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Fish Physiology, Toxicology, and Water Quality

Proceedings of the Seventh International Symposium, Tallinn, Estonia May 12-15, 2003

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FISH PHYSIOLOGY, TOXICOLOGY, AND WATER QUALITY

Proceedings of the Seventh International Symposium, Tallinn, Estonia May 12-15, 2003

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FOREWORD

Joint ecological research involving scientists and environmental managers from every country in the world is essential if global environmental problems are to be solved. Recognition of this international aspect of environmental protection is reflected in the joint activities undertaken under Annex 3, Item 4 of the United States of America-People's Republic of China Protocol for Environmental Protection. This component of the protocol provides for cooperative research on the effects of pollution on freshwater organisms, environmental processes, soils, surface water and groundwater, and on the application of pollutant transport and transformation models. Activities include seminars, workshops, joint symposia, training programs, joint research, and publications exchange.

This Symposium focuses on the result of international cooperation in environmental research dealing with such complex issues as: land use and euthrophication; euthrophication and hypoxia/anoxia; effect of hypoxia/ anoxia on fish physiology, behavior, and toxicology; and modeling biogeochemical processes to predict water quality.

This is the seventh international symposium to bring together researchers from the U.S., China, Russia, and other countries to report on and exchange information in the area of fish physiology, toxicology, and water quality. The seventh symposium was held in Tallinn, Estonia, May 12-15, 2003. Scientists from 16 countries presented 23 papers at the symposium sponsored by the U.S. Environmental Protection Agency, the Estonian Agricultural University, and Montana State University. The six previous symposia were held in Guangzhou, China, September 14-16, 1988; in Sacramento, California, USA, September 18-19, 1990; in Nanjing, China, November 3-5, 1992; in Bozeman, Montana USA, September 19-21, 1995; in Hong Kong, China, November 10-13, 1998 and in La Paz, B.C.S. Mexico, January 22-26, 2001.

Symposia are effective means of fostering cooperation among scientists from different countries as environmental organizations seek to gain the information necessary to predict the effects of pollutants on ecosystems and apply the results on a global scale. The symposia provide a forum through which distinguished scientists from laboratories and institutions from several countries can exchange scientific knowledge on environmental problems of concern to the U.S. Environmental Protection Agency and the international environmental community.

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ABSTRACT

Sixty-five scientists from sixteen countries convened in Tallinn, Estonia, May 12-15, 2003, for the Seventh International Symposium on Fish Physiology, Toxicology, and Water Quality. These Proceedings include 23 papers presented in sessions that took place over three days. Relative to their ecological threat, papers addressed the global spread of the phenomenon of hypoxia and anoxia, trends in specific large water bodies, and the land use practices that drive the phenomenon. Toxicological papers focused on possible computational approaches, the effects of hypoxia and metals on fish survival, and effects of ammonia toxicity on fish organ systems and biochemistry. Papers that addressed fish physiology and behavior spanned a very wide range of subjects, ranging from the oceanic distribution of cod as a function of temperature, through behavior of reef fishes during diurnal cycles of oxygen depletion, to blood hemoglobin regulation in hypoxic fish and the inhibition of fish reproduction as a function of hypoxia and other factors. Papers dealing with biogeochemistry and water quality covered the water quality conditions of the Gulf of Finland and Lakes Peipsi and Võrtsjärv in Estonia, the modeling of metal binding on humic substances, and a large-scale ecological modeling approach for predicting estuarine water quality conditions.

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ACKNOWLEDGMENTS

The Seventh Symposium is largely the legacy of Dr. Robert Vance Thurston of Montana State University, who died in February 2002. Beginning with its origin in 1988, the symposium series relied on his energy and broad scientific knowledge to bring together investigators from many nations to address all aspects of hypoxia, eutrophication and fish health. This particular meeting could not have come together without the work of the steering committee: Dr. George Bailey of the U.S. Environmental Protection Agency, Dr. Arvo Tuvikene of the Estonian Agricultural University, Dr. John Steffensen of Copenhagen University, and Dr. Dave Randall of the University of British Columbia and the City University of Hong Kong. Funding for the symposium was provided by these sponsoring organizations: the U.S. Environmental Protection Agency, Montana State University and the Estonian Agricultural University. Dr. Arvo Tuvikene, Dr. Lea Tuvikene, Dr. Ain Järvalt, Kai Piirsoo and their colleagues from the Võrtsjärv Limnoloogiajaam were exemplary hosts in Tallinn. The meeting, the poster session and the field trip were all efficient and enjoyable because of their hard work. The symposium chairs are also grateful to Dr. Mike Newman of the Virginia Institute of Marine Sciences and to Dr. Dave Randall, for serving as session chairs. Assistance in preparing this document was provided by Suzanne Faber, Rose Adams, and Sheila Walker. Finally, we express our profound thanks to all the symposium participants, who persevered in spite of considerable global challenges to make this a successful scientific meeting.

Gretchen Rupp and Michelle D. White
Symposium Co-Chairs

A GLOBAL PERSPECTIVE ON THE EFFECTS OF EUTROPHICATION AND HYPOXIA ON AQUATIC BIOTA

Robert J. Diaz[1], Janet Nestlerode[1], Minnie L. Diaz[2]

ABSTRACT

Development associated with human populations has led to the globalization of many environmental problems. In marine systems, the most serious of these problems are directly related to the process of eutrophication. The increased production of organic matter in these marine systems associated with eutrophication is the primary factor impacting species abundance and composition and dissolved oxygen budgets. Oxygen, which is essential to maintaining balance in ecosystem processes through its role in mediating microbial and metazoan activities, has declined to critically low levels in many systems, which has led to the development of hypoxia (<2 ml O_2/l) and anoxia (0 ml O_2/l). Currently, most oxygen depletion events are seasonal, but trends toward longer periods that could eventually lead to persistent hypoxic or anoxic conditions are emerging. Over the last 50 years, there has been an increase in the number of systems reporting problems associated with low dissolved oxygen. Currently there are over 100 hypoxic/anoxic areas around the globe, ranging in size from <1 km^2 to 70000 km^2, that exhibit a graded series of responses to oxygen depletion, ranging from no obvious change to mass mortality of bottom fauna. Ecosystems currently severely stressed by eutrophication-induced hypoxia continue to be threatened with the loss of fisheries, loss of biodiversity, alteration of food webs, and simplification of energy flows.

INTRODUCTION

Cloern (2001) succinctly summarized current understanding of coastal eutrophication, indicating that the long-term records of nutrient discharges over the past 50 years provide compelling evidence of a rapid increase in the fertility of many temperate coastal ecosystems (for example, Baltic and adjoining seas – Karlson *et al.* 2002; Northwest Black Sea – Mee 1992; Northern Adriatic Sea – Solic *et al.* 1997; North Sea rivers – Howarth *et al.* 2002; United States bays and estuaries - Jaworski *et al.* 1997, Howarth *et al.* 1996; Northern Gulf of Mexico – Rabalais *et al*. 1996, Rabalais and Turner 2001; Japan – Suzuki 2001). In each of these systems, the fertilization is directly related to an expanding human population, which recently passed 6 billion and will likely exceed 8 to 10 billion by the year 2050 (Wilson 2002). Seitzinger *et al.* (2002) found that at scales of regions and continents, human population was a good predictor of

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dissolved inorganic nitrogen (DIN) exported to coastal systems. By 2050, projections indicate that a 2.4 to 2.7-fold increase in nitrogen and phosphorus driven eutrophication will result from this population expansion (Tilman *et al.* 2001), with serious consequences for coastal ecosystems.

Fertilization of marine systems, mainly from excess nitrogen, has been linked in a complicated way to many ecosystem-level changes associated with eutrophication, or more precisely, cultural eutrophication. Cultural eutrophication is specific to impacts related to human populations on the environment and separates the conditions in these coastal systems from natural processes that can also lead to eutrophic-like conditions, such as those associated with coastal upwelling zones and oxygen minimum zones (OMZ) where oxygen consumption exceeds resupply. Oxygen depletion associated with upwelling events tends to be episodic, severe, short-lived (less than a year), and associated with the western boundaries of continental landmasses (Brongersma-Sanders 1957, Rosenberg *et al.* 1983). OMZs are unusual oxygen-depleted areas that are widespread and stable oceanic features occurring at intermediate depths (typically 400 to 1000 m), persisting for long periods of time (at greater than decadal scales), and are completely controlled by natural processes and cycles (Wyrtki 1966, Kamykowski and Zentara 1990, Olson *et al.* 1993, Childress and Seibel 1998).

While eutrophication can be defined simply as the production of organic matter in excess of what an ecosystem is normally adapted to processing (Nixon 1995), it is actually only part of a complex web of stressors that interact to shape and direct ecosystem-level processes (Breitburg *et al.* 1998, Cloern 2001) (Figure 1). From Figure 1, the most visible ecosystem response to this set of multiple stressors is the greening of the water column as primary production increases in direct response to nutrient enrichment. However, the unseen is most dangerous. For nutrient enrichment, which leads to increased organic matter production (eutrophication), the unseen decrease in dissolved oxygen in bottom waters created by the increased flux of particulate organic matter to the bottom is most threatening. The degree to which an ecosystem responds to any of the multiple stressors is dependent upon physical, chemical, and biological characteristics that act to filter and modulate the response (Cloern 2001).

Human impacts are accelerating the rate and magnitude of change within an ecosystem as more and more ecosystem level processes are affected (Jackson *et al.* 2001). The history and pattern of human disturbance in terrestrial, aquatic, coastal, and oceanic ecosystems have brought us to a point at which oxygen depletion is likely to become the keystone impact for the 21st century, replacing the 20th century keystone of overfishing (Jackson *et al.* 2001). A mounting volume of literature documenting change in marine ecosystems indicates oxygen depletion as a major phenomenon that is a tertiary manifestation of the severe levels of stress experienced by many ecosystems. The primary stress is nutrient enrichment, which regulates the secondary response of eutrophication. See reviews and summaries by Gray (1992), Nixon (1995), Diaz and Rosenberg (1995), Cloern (2001), Turner (2001), and Karlson *et al.* (2002) for examples of ecosystem responses. The correlation between human activities and declining dissolved oxygen is strong, with the oxygen budgets of many marine ecosystems around the world adversely affected by eutrophication.

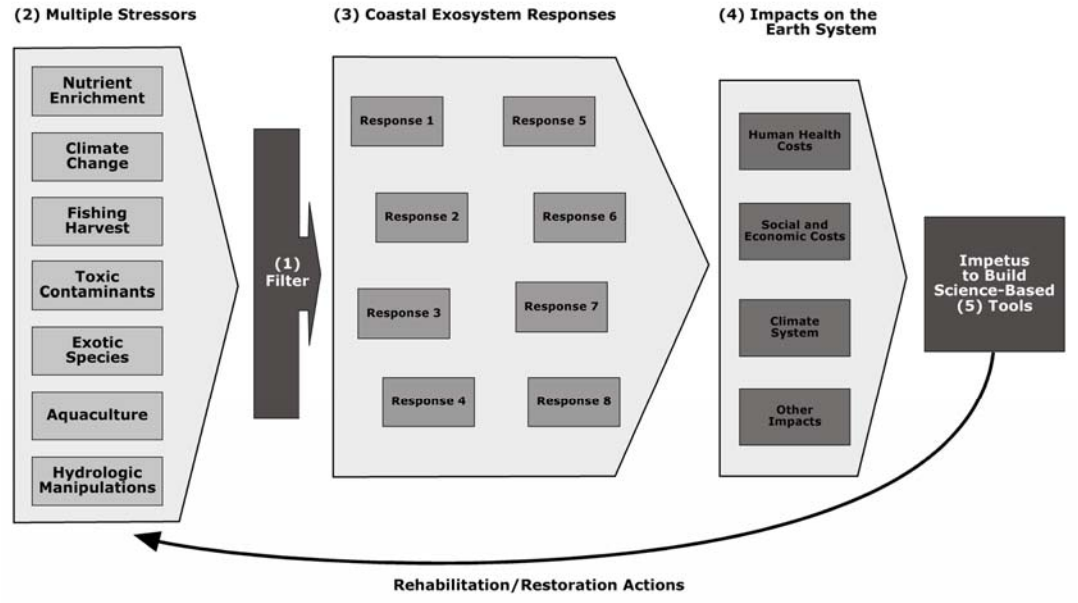

Figure 1. Conceptual model of coastal eutrophication modified from Cloern (2001). (1) system attributes that determine responses to nutrient enrichment; (2) nutrient enrichment as one of many stressors; (3) complex linkage between responses to multiple stressors; (4) change in coastal ecosystems; (5) application of scientific understanding of eutrophication with the goal of building rational management strategies for ecosystem rehabilitation/restoration.

The emphasis of this paper is ecosystem response to oxygen depletion resulting directly from eutrophication. The emphasis on dissolved oxygen is warranted given the importance of oxygen for sustaining life for all fishes and invertebrates. Metaphorically speaking, the American Lung Association motto could be adopted for this situation. "When you can't breathe, nothing else matters." When the supply of dissolved oxygen in aquatic environments is cut off or the consumption rate exceeds resupply, oxygen concentrations quickly decline beyond the point that sustains most animal life. Two factors are required for the development of hypoxia, and at times anoxia; one is water column stratification that isolates the bottom water from oxygen-rich surface water, and the second is decomposition of organic matter that reduces oxygen levels in the isolated bottom water. The first factor is generated primarily by salinity stratification and the second by microbial metabolism. Both factors must be at work for hypoxia to develop and persist. In fact, the reaction of microbial populations to eutrophication has been explosive, particularly in systems with the greatest oxygen depletion problems (Jackson *et al.* 2001).

The terms used to describe low dissolved oxygen or oxygen depletion are hypoxia and anoxia. Hypoxia is defined by dissolved oxygen concentrations <2 ml O_2/l or <2.8 mg O_2/l; for seawater this is about 18% of air saturation (Tyson and Pearson 1991). Anoxia is the complete absence of dissolved oxygen (0 ml O_2/l). The point at which various animals suffocate varies, but effects generally appear when oxygen drops below 2 ml O_2/l (Diaz and Rosenberg 1995,

Breitburg *et al.* 2001, Karlson *et al.* 2002). The relationship between declining oxygen and animal response are graded and follow a predictable path, good examples of which are given by Diaz and Rosenberg (1995) and Rabalais *et al.* (2001b). This paper presents a brief overview and update of hypoxic conditions in estuarine and marine systems around the world.

OXYGEN DEPLETION AROUND THE WORLD

On a geological time scale, low-dissolved-oxygen environments (hypoxia and anoxia) have been major factors in shaping the evolution of life (Caplan and Bustin 1999). Today, the vestiges of naturally occurring oxygen depletion are the oceanic OMZs, the largest pools of hypoxic water in world oceans, particularly in the Pacific and Indian Oceans and the Arabian Sea (Olsen *et al.* 1993). The largest pool of naturally occurring anoxic water is the Black Sea (Kideys 2002). The Black Sea anoxic zone does not support any eukaryotic life, which is typical of all areas experiencing extended periods of anoxia, whether natural or anthropogenic. However, the temporal and spatial stability of OMZs has allowed the development of species aerobically adapted to dissolved oxygen concentrations from 0.5 ml O_2/l to about 0.1 ml O_2/l (Levin *et al.*1991, Childress and Seibel 1998). This is in stark contrast to the faunal response to cultural-eutrophication-induced hypoxia in shallow coastal and estuarine areas, where oxygen concentrations of <0.5 ml O_2/l lead to mass mortality of individuals and major change in community structure.

The worldwide distribution of coastal oxygen depletion is either centered on major population concentrations, or closely associated with developed watersheds that deliver large quantities of nutrients (Figure 2, Table 1). The historical perspective indicates that many of these currently hypoxic systems were not so when they were first studied. Since at least the 1950s and 1960s, dissolved oxygen concentrations of many major coastal ecosystems around the world have been adversely affected by eutrophication. Most of these coastal systems have documented declines in dissolved oxygen through time, starting in most cases from their initial oxygen measurements (Rosenberg 1990). The declining trend in dissolved oxygen seems to have lagged about 10 to 20 years behind the increased use of chemical fertilizer that began in the 1940s (Howarth *et al*.2002). For systems with historical data from the first half of the 20th century, declines in oxygen concentrations started in the 1950s and 1960s for the northern Adriatic Sea (Justic 1987), between the 1940s and 1960s for the northwest continental shelf of the Black Sea (Mee 1992, Kideys 2002), and in the 1970s for the Kattegat (Baden *et al*.1990a). Declining dissolved oxygen levels were noted in the Baltic Sea as early as the 1930s (Fonselius 1969), but it was in the 1950s that hypoxia became widespread (Karlson *et al.* 2002). Other systems have experienced hypoxia since the beginning of oxygen data collection, for example, in the 1930s for the Chesapeake Bay (Officer *et al.* 1984), and the 1970s for the northern Gulf of Mexico (Rabalais and Turner 2001) and many Scandinavian fjord systems (Karlson *et al.* 2002). However, the longer-term geochronological records indicate that hypoxia was not always present in these particular ecosystems (Sen Gupta *et al.* 1996; Karlson *et al.* 2002; Zimmerman and Canuel 2002). Not all nutrient-enriched systems have developed eutrophic conditions and related oxygen depletion problems. San Francisco Bay receives higher levels of nutrients than the Chesapeake Bay, but has lower primary production and oxygen depletion due to strong tidal mixing and turbid water (Cloern 2001).

Table 1. Eutrophication-associated hypoxic areas around the world with an emphasis on benthic and fisheries responses. Several of these systems also experience anoxia. Hypoxia is characterized as Episodic: events occurring at irregular intervals greater than one year; Periodic: events occurring at regular intervals shorter than one year; Annual: yearly events related to summer or autumnal stratification; Persistent: year-round hypoxia. Benthic faunal response is categorized as None: communities appear similar before and after hypoxic event; Mortality: moderate reductions of populations, many species survive; Mass Mortality: drastic reduction or elimination of the benthos. Benthic recovery is described by No Change: dynamics appear unrelated to hypoxia; Reduced: recolonization occurs but community does not return to prehypoxic structure; Multi-year: gradual return of community structure; Annual: return of similar community structure in a year. First observed is usually first documentation in literature and in most cases not the first occurrence of oxygen depletion.

System	Country/State	First Observations	Recent	Area (km^2)	Fisheries Response	Benthic Response	Benthic Recovery	Reference
Annual Oxygen Depletion								
Åland archipelago	Finland	1990		10		Mortality	Reduced	Norkko and Bonsdorff 1996
Aquaculture	Taiwan	2000			Mortality of prawns	Mortality		Cheng *et al*. 2002
Århus Bay	Denmark	1980		1300		Mass Mortality	Multi-year	Fallesen and Jorgensen 1991
Barnegatt Inlet	New Jersey	1990				Mass Mortality	Annual	Moser 1998
Belt Sea	Denmark	1970		2150				Karlson *et al*. 2002
Bilbao Estuary	Spain	1990				Mortality		Gonzales-Oreja and Saiz-Salinas 1998; Saiz-Salinas and Frances-Zubillaga 1997
Bornholm Basin	Baltic Sea	1950		2500		Mass Mortality	Multi-year	Tulkki 1965; Leppakoski 1969
Cabbage Tree Basin	Australia	1980				Mortality	Annual	Rainer and Fitzhardinge 1981
Chesapeake Bay Mainstem	Maryland	1930		2750	Avoidance, some mortality	Mortality	Annual	Holland *et al.* 1987; Boesch *et al*. 2001; Seliger and Boggs 1988
Corpus Christi Bay	Texas	1980		15		Reduced	Reduced	Ritter and Montagna 1999
Dead-end canals	Maryland, Delaware	1990		5				Maxted *et al*. 1997
Delaware River, Lower	Delaware	1910	Improved		Recovery American shad/striped bass fishery	Mass Mortality	Increasing	Weisberg *et al.* 1996
Delaware River, Upper	Delaware	1910	Improved					Patrick 1988
Eckernforde Bay	Germany	1990				Mortality	Annual	D'Andrea *et al.* 1996
Elbe Estuary	Germany	1980	Improved		Stressed			Thiel *et al.* 1995
Elefsis Bay, Aegean Sea	Greece	1980				Mass Mortality	Annual	Friligos and Zenetos 1988; Theodorou 1996
Fjords of Skagerrak coast	Sweden	1950						Rosenberg 1990
Flushing Bay	New York	1990				Mortality		Diaz, unpublished data
German Bight	Germany	1980			Mortality	Mortality	Reduced	Brockmann *et al.* 1988; Niermann *et al.* 1990
Gialova Lagoon	Ionian Sea, Greece	1990		2500		Mortality	Annual	Arvanitidis *et al.* 1999
Goro Lagoon	Italy	1990				Mortality	Annual	Reizopoulou *et al.* 1996
Great Egg Harbor River	New Jersey	1990						Glenn *et al.* 1996
Guanabara Bay	Brazil	1990			Tropical	Mortality		Valentin *et al.* 1999
Gulf of Mexico	Louisiana	1970		17000	Avoidance	Mortality/avoidance	Reduced	Rabalais and Turner 2001
Gulf of Trieste	Adriatic Sea	1960			Stressed	Mass Mortality	Multi-year	Stachowitsch 1984, 1991; Simunovic *et al.* 1999; Justic *et al.* 1987
Gullmarsfjord	Sweden	1980			Stressed	Mass Mortality	Reduced	Nilsson and Rosenberg 2000; Josefson and Widbom 1988
Hakata Bay	Japan	1970		120		Mortality	Annual	Karim *et al*. 2002
Havstens Fjord	Sweden	1990						Gustafsson and Nordberg 2000
Herring River	Massachusetts	1980			Fish kills, decline of alewife fishery			Portnoy 1991
Hillsborough Bay	Florida	1980				Mass Mortality	Annual	Santos and Simon 1980

System	Country/State	Observations		(km^2)	Fisheries Response	Response	Recovery	Reference
Hiuchi Sound	Japan	1970				Mass Mortality		Sanukida *et al.* 1984
Hood Canal	Washington	1980						Paulson *et al.* 1993
Horseshoe Lagoon	Australia	1990						Donnelly *et al*. 1999
Hudson River	New York	1960	Improved					Bronsnan and O'Shea 1996
Inre Verkviken	Finland	1970		0.5				Lindholm 1996
Ise Bay	Japan	1990			Stressed	Mortality		Nakata *et al.* 1997
Kattegat	Sweden, Denmark	1980		3850	Collapse of Norway lobster	Mass Mortality	Multi-year	Baden *et al.* 1990a; Josefson and Jensen 1992; Rosenberg *et al.* 1992
Kiel Bay	Germany	1960		890	Stressed	Mass Mortality	Annual	Arntz 1981; Rumohr 1986; Weigelt 1990, 1991; Oeschger and Storey 1990
La Coruna Bay	Spain	1990						Lopez-Jamar *et al*. 1995
		First	Recent	Area		Benthic	Benthic	
Laholm Bay	Sweden	1980			Mortality	Mortality	Annual	Baden *et al.* 1990b; Rosenberg and Loo 1988
Lake Nakaumi	Japan	1990			Mortality/avoidance			Ishitobi *et al*. 2000
Lake Shinji	Japan	1990				Mass Mortality		Yamamuro *et al.* 1998
Limfjord	Denmark	1980		440	Demersal fisheries gone	Mass Mortality	Annual	Jorgensen 1980; Hylleberg 1993
Loire Estrary	France	1990			Mortality of migratory species			Thouvenin *et al*. 1994
Long Island Sound	New York	1980		232	Avoidance, some mortality	Mortality		Howell and Simpson 1994; Welsh *et al.* 1994; Schimmel *et al.* 1999; NOAA 1997
Los Angeles Harbor	California	1950	Improved			Mass Mortality	Reduced	Reish 1955, 2000
Lough Ine	Ireland	1970				Mass Mortality	Annual	Kitching *et al.* 1976
Mecklenburg Bay	Germany	1980		1860				Weigelt and Rumohr 1986
Mikawa & Ise Bays	Japan	1980				Mortality/avoidance		Suzuki and Matsukawa 1987
Mobile Bay	Alabama	1880		1060	Mortality	Mass Mortality		May 1973; Engle and Summers 1999; Pennock *et al.* 1994
Mullica River Estuary	New Jersey	1990						Glenn *et al*. 1996
Neuse River Estuary	North Carolina	1990			Fish kills, mortality of oyster	Mortality/avoidance	Annual	Paerl *et al.* 1995, 1998; Lenihan and Peterson 1998; Lenihan 1999
New York City Harbor	New York	1990				Mass Mortality	Annual	Diaz, unpublished data
Nichupti Lagoon	Mexico	1980						Diaz, unpublished data
Northern Adriatic Sea	Italy	1970		3750				Barmawidjaja *et al*. 1995; Justic *et al.* 1987, 1993
NW Gulf of Mexico	Louisiana	1980				Mortality	Annual	Gaston 1985
NW Shelf Black Sea	Ukraine, Romania	1960		40000	Reduced	Mass Mortality	Annual	Zaitsev 1993; Bakan and Buyukgungor 2000
Oder Lagoon	Germany	1990						Pohl *et al.* 1998
Omura Bay	Japan	1980						Iizuka and Min 1989
Osaka Bay	Japan	1980						Tanimoto and Hoshika 1997
Oslofjord	Norway	1910		150	Reduced	Mortality	Annual	Petersen 1915; Mirza and Gray 1981; Rosenberg *et al*. 1987
Palude della Rosa	Italy	1990				Mortality	Annual	Tagliapietra *et al.* 1998
Pamlico River	North Carolina	1960			Mortality	Mass Mortality	Annual	Tenore 1972; Hobbie *et al.* 1975; Stanley and Nixon, 1992
Patuxent Piver	Maryland	1990			Avoidance, low egg hatching/larval mortality	Avoidance/mortality	Annual	Keister *et al*. 2000, Breitburg *et al*. 1997
Perdido Bay	Florida	1990						Flemer *et al.* 1999
Pomeranian Bay	Germany	1990		170		Mass Mortality	Reduced	Powilleit and Kube 1999
Potomac River	Maryland	1990		264		Mortality	Annual	NOAA 1997
Raritan Bay	New York, New Jersey	1970						Christensen and Packard 1976
SE Kattegat	Sweden	1980			Reduced demersal fishes	Avoidance/mortality	Multi-year	Peterson and Pihl 1995
Seine Estuary	France	1990						Michel *et al.* 2000
Seto Inland Sea	Japan	1980				Mortality	Annual	Imabayashi 1986
St. Johns River	Florida	1990				Mortality	Annual	Mason 1998
Swedish West Coast Fjords	Sweden	1980			Stressed	Mortality	Reduced	Josefsen and Rosenberg 1988

System	Country/State	Observations	(km^2)	Fisheries Response	Response	Recovery	Reference
Thau	France	1990		Mortality/Reduced shellfish production	Mass Mortality		Souchu *et al*. 1998; Mazouni *et al.* 1996; Chapelle *et al.* 2000
Tolo Harbor	Hong Kong	1980			Mass Mortality	Annual	Wu 1982
Tome Cove	Japan	1980			Mortality	Annual	Tsutsumi 1987
Townsend-Hereford Inlet	New Jersey	1990					Glenn *et al.* 1996
Western Gulf of St. Lawrence	Canada	1990			No response		Comeau *et al* 2002
Episodic Oxygen Depletion							
Baie de Somme	France	1990		Collapse of cockle industry	Mass Mortality		Rybarczyk *et al*. 1996
Beacon Key, Biscane Bay	Florida	1990					Leverone 1995
Bude Bay	SW England	1990	12		Mortality		Gibbs *et al.* 1999
Buzzard Bay	Massachusetts	1990	2				NOAA 1997
Cape Fear River	North Carolina	1990		Fish kills	Reduced	Annual	Mallin *et al.* 1999; Posey *et al.* 1999
Chester River	Maryland	1990	24				NOAA 1997
Choptank River	Maryland	1990	48				NOAA 1997
		First Recent	Area		Benthic	Benthic	
Connecticut River	Connecticut	1990	9				NOAA 1997
East Frisian, Wadden Sea	Netherlands	1990					Kaiser and Lutter 1998
Finnish Archipelago	Finland	1970					Karlson *et a*l. 2002
German Bight	Germany	1980	15000		Mass Mortality	Annual	Dethlefsen and Westernhagen 1983 Brockmann *et al.* 1988
Gulf of Mexico, off Freeport	Texas	1970	50	Mortality	Avoidance/mortality	2 years	Harper and Rabalais 1995
Krka, Adriatic Sea	Yugoslavia	1980			Mortality	Annual	Legovic *et al*. 1991
Lake Pontchartrain	Louisiana	1990		Loss of large clams	Reduced		Abadie and Poirrier 2000
Loch Ailort	Scotland	1990		Salmon farms in the system	Reduced		Gillibrand *et al.* 1996
New York Bight	New York, New Jersey	1970	987	Surf clam/finfish mortality, Avoidance	Mass Mortality	Multi-year	Garlo *et al.* 1979; Sindermann and Swanson 1980
North Sea coast	Germany	1980	25		Mortality	Reduced	Koenig 1996
Off Cape Rodney	New Zealand	1980		Mortality			Taylor *et al.* 1985
Pamlico Sound	North Carolina	1990		Mortality	Mortality		Paerl *et al.* 2000
Salts Hole	United Kingdom	1990	6		Mortality		McArthur 1998
SE North Sea	Denmark	1980		Stressed	Mortality	Annual	Dyer *et al.* 1983; Westernhangen and Dethlefson 1983
Sommone Bay	France	1980	3	Collapse of cockle fishery	Mass Mortality	Multi-year	Desprez *et al.* 1992
Texas Shelf, Deep	Texas	1980		Stressed	Mortality	Annual	Harper *et al.* 1981, 1991
Texas Shelf, Shallow	Texas	1980		Stressed	Mass Mortality	Multi-year	Harper *et al.* 1981, 1991
Wadden Sea	Wadden Sea	1990	3000	Stressed	Mortality		deJonge *et a*l. 1994
Wismar Bay	Baltic Sea	1980		Stressed	Mortality	Reduced	Prena 1995a, 1995b
Vestfjord		1970					Karlson *et a*l. 2002
Periodic Oxygen Depletion (>1 event per year)							
Bon Secour Bay	Alabama	2000		Loss of oyster	Mortality		Rikard *et al*. 2000
Florida Keys	Florida	1990			Mortality		Lapointe and Matzie 1996
Gironde Estuary	France	1990					Abril *et al.* 1999
Great South Bay	New York	1990	15				NOAA 1997
Gullmarsfjord, Alsback Deep	Sweden	1990			Mortality		Gustafsson and Nordberg 2001
Jamaica Bay	New York	1990	26				NOAA 1997
James Island Creek	South Carolina	1990		Avoidance	Avoidance		Cochran and Burnett 1995
Koljo Fjord	Sweden	1990			Mortality	Annual	Gustafsson and Nordberg 1999; Rosenberg *et al*. 2001
Narragansett Bay	Rhode Island	1990	11				NOAA 1997
Prevost Lagoon	France	1990		Reduced aquaculture production	Mass Mortality	Annual	Guyoneaud *et al*. 1998

System	Country/State	Observations		(km^2)	Fisheries Response	Response	Recovery	Reference
Rappahannock River	Virginia	1990		55	Avoidance	Mortality	Annual	Llanso 1992; NOAA 1997
St. Joseph Bay	Florida	1990			Avoidance			Leonard and McClintock 1999
St. Lucie River	Florida	1990						Chamberlain and Hayward 1996
York River	Virginia	1980		30	Avoidance	No response	No Change	Pihl *et al.* 1991; Diaz *et a*l. 1992; Sagasti *et al*. 2001
Persistent Oxygen Depletion								
Arkona Basin	Sweden	1980		1000				Karlson *et al* 2002
Baltic proper	Baltic Sea	1960		70000	Avoidance, mortality/low hatch cod eggs	Mortality/avoidance		deJonge *et al.* 1994
Big Glory Bay	New Zealand	2000			Caused by salmon farming	Mass Mortality		Morrisey 2000
Byfjord	Sweden	1970			Pelagic only	Mortality	Reduced	Rosenberg 1990, Rosenberg *et al.* 1977
Caspian Sea	Caspian Sea	1990				Mortality	Some?	Dumont 1998
Gdansk Basin	Poland	1960		1200				Karlson *et a*l. 2002
Gotland Basin	Sweden	1960			Avoidance, mortality/low hatch cod eggs	Mortality	Reduced	Laine *et al.* 1997
Gulf of Finland, Deep	Gulf of Finland, Deep	1960	Improved	2330		Reduced	Increasing	Laine *et al.* 1997; Andersin and Sandler 1991
Himmerfjord	Sweden	1970	Improved	11				Karlson *et al.* 2002
Idefjord	Sweden, Norway	1960	Improved	80		Mortality	Reduced	Rosenberg 1980
Loch Carron	Scotland	1970				Mass Mortality	No Change	
Scheldt Estuary	Belgium	1990						Verlaan *et al.* 1998
Sea of Azov	Russia-Ukraine	1990			Lower production	Mortality	Reduced	Balkas *et al.* 1991; Chechum 1998
		First	Recent	Area		Benthic	Benthic	
Skagerrak Coast Fjords	Sweden, Norway	1920		54	Stressed	Mortality		AnnualJohannessen and Dahl 1996a,b
St. Anna Archipelago	Sweden	1970		25				Karlson *et al.* 2002
Stockholm Inner Archipelago	Sweden	1970		60		No Benthos	No Change	Rosenberg and Diaz 1993
Sullom Voe	Shetland	1980				Mass Mortality	No Change	Pearson and Eleftheriou 1981
Tan Shui Estuary	Taiwan	1990						Jeng and Han 1996
Unknown Oxygen Depletion Cause								
Etang de Berre	France	1970		132				Stora and Arnoux 1983
Kilviken Fjord	Sweden	1970				Reduced		Hendelberg and Jensen 1993
Marmara Sea	Marmara Sea	1990			Mass Mortality	Mass Mortality		Orhon and Yuksek, unpublished data
Mauritius Island	Mauritius Island	1990			Coral reef affected			Thomassin *et al*. 1998
Mondego River	Portugal	1990						Kamp-Nielsen *et al.* 1997
Pettaquamscutt River	Rhode Island	1990						Wilkin and Barnes 1997
Roskilde Fjord	Denmark	1990						Kamp-Nielsen *et al.* 1998
Waquoit Bay	Massachusetts	1990						Fritz *et al*. 1996

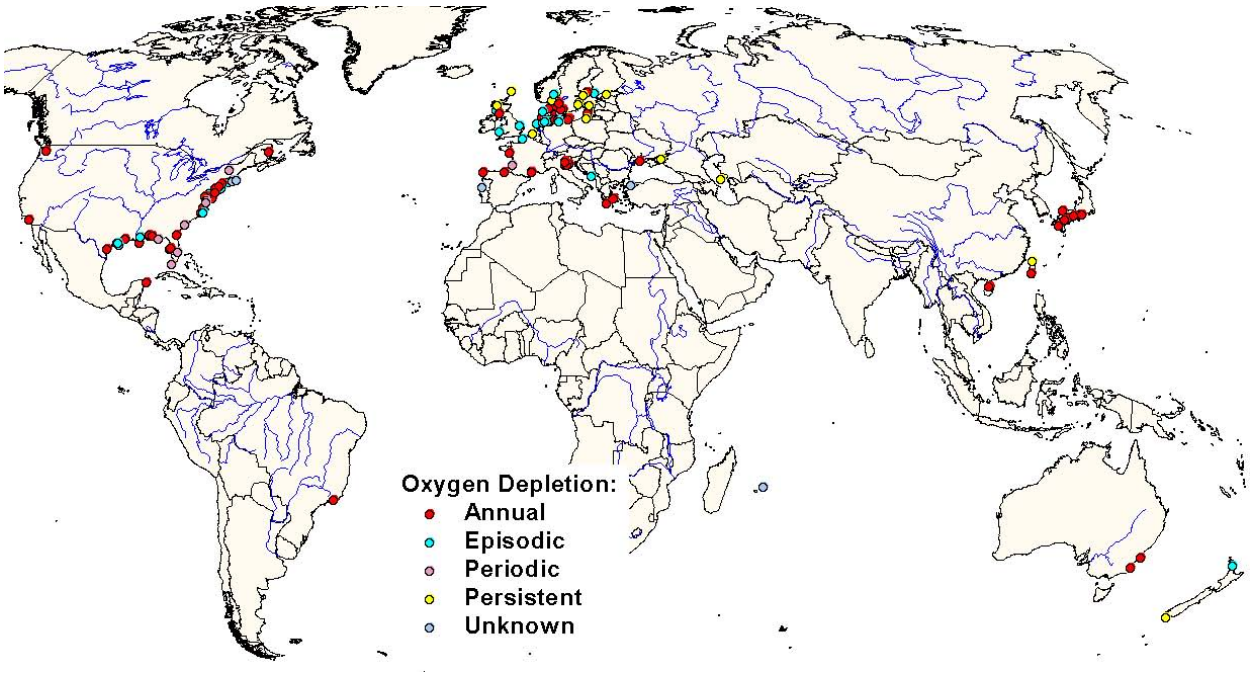

Figure 2. Global distribution of the 146 oxygen depletion zones related to cultural eutrophication listed in Table 1. Systems are categorized by type of hypoxia (see Table 1 for details).

The most common form of hypoxia is one annual event, occurring at 54% of the 146 oxygen-depleted systems. The most common response to annual oxygen depletion was mortality of benthos followed by some level of recolonization with the return of normal oxygen conditions (Table 1). In essence, annual hypoxia forces an annual pulsing of energy over the shortened interval of normal dissolved oxygen conditions (Diaz and Rosenberg 1995). The second most common form of oxygen depletion is episodic, occurring 18% of the time in the 146 systems. It appeared that episodic oxygen depletion was the first signal that a system had reached a critical point. Many systems, such as the Kattegat, first experienced episodic events that initially caused mass mortality of benthic organisms, but now experience annual oxygen depletion (Karlson *et al.* 2002).

Since the 1960s, the number of oxygen-depleted ecosystems has doubled every ten years (Figure 3). Prior to 1960, we found nine systems with cultural eutrophication-related oxygen depletion. During the 1960s, another ten systems were added, but by the 1970s estuarine and coastal ecosystems around the world were becoming over enriched with organic matter (Nixon 1995) and the number of oxygen-depleted ecosystems had doubled (Figure 3). After this point, hypoxia quickly became an annual event and a prominent feature affecting energy flow processes in marine ecosystems (Elmgren 1989, Pearson and Rosenberg 1992). During the 1980s, 37 systems were added, and in the 1990s 68 more were added (Table 1). By the end of the 20th century, oxygen depletion had become a major, worldwide environmental problem with only a small fraction of systems (6%) showing signs of improvement.

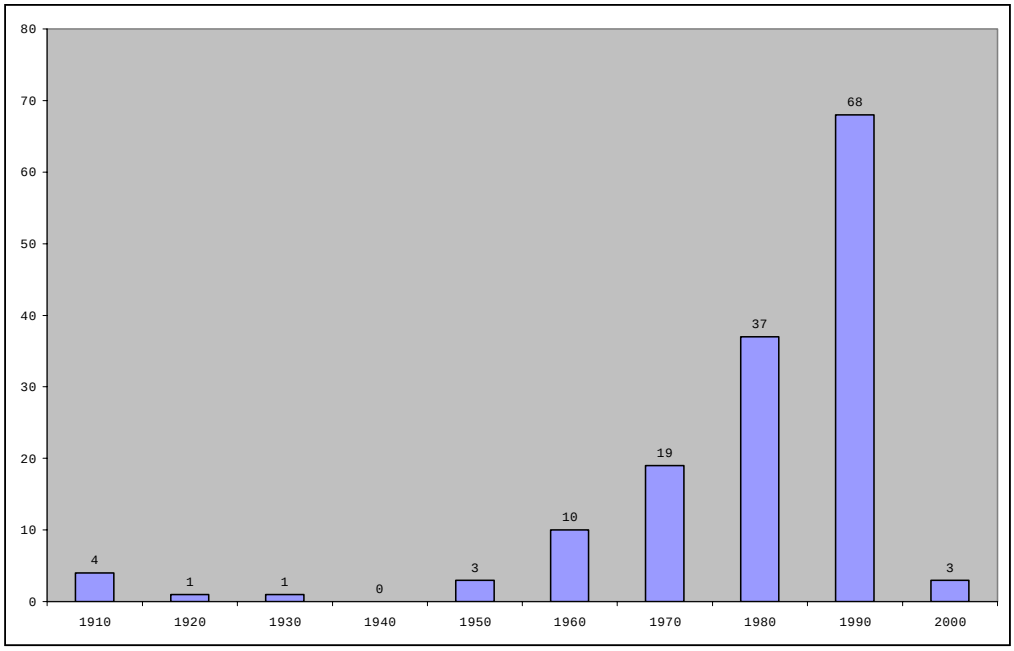

Figure 3. Histogram of the number of ecosystems reporting oxygen depletion by decade. The decade was determined either from the first time hypoxia was seen in historical data or the first year a published account appeared in the literature. Data from Table 1.

The largest systems with improved dissolved oxygen conditions were the northwest Black Sea and the Gulf of Finland. In the Black Sea, a reduction in nutrients and possibly a balancing of exotic species led to an improvement in ecosystem function and reduction in hypoxia (Kidey 2002). In the Gulf of Finland, a decrease in water column stratification led to improved dissolved-oxygen conditions and the return of benthic fauna (Karlson *et al.* 2002). Conditions have also improved in some systems that have experienced intensive regulation of nutrient or carbon inputs, such as the Hudson River, New York, and the Delaware River in Pennsylvania and New Jersey. In others, such as the Chesapeake Bay, improvements in dissolved oxygen await the 'burn off' of nitrogen that has accumulated within the system's sediments. Many examples of small-scale reversals in hypoxia associated with improvements in treatment of sewage and pulp mill effluents as early as the 1970s (Rosenberg 1972, 1976) also exist. In the northern Gulf of Mexico, the hypoxic zone is very tightly coupled with runoff from the Mississippi River. During low flow years, the area of hypoxia is greatly reduced, only to increase when river flow increases (Rabalais *et al.* 2001a, unpublished data). Similarly, the Baltic Sea can experience temporary dissolved oxygen increases associated with episodic water exchanges across the belt seas. Even though the exchange of deep water in the Baltic is episodic, there is convincing evidence that eutrophication accelerates oxygen consumption in its bottom waters (Karlson *et al.* 2002).

In general, coastal hypoxia is not a natural condition. Only hypoxia and anoxia associated with naturally-occurring events have a historical context dating back 100 to 150 years.

This includes: areas of natural upwelling, such as those off Peru and Central America (Tarazona *et al.* 1988) or West Africa's Namibian shelf (Hamukuaya *et al.* 1998); oceanic OMZ, such as in the Arabian Sea (Gooday *et al.* 2000); and stagnant basins, such as Santa Barbara Basin off California (Bernhard and Reimers 1991). Methodologies for measuring dissolved oxygen were not developed until the 1880s (Winkler 1888). Accounts in the historical literature do imply the occurrence of oxygen depletion prior to development of Winkler's method, and were generally in water bodies associated with human development. For example, in 1884 the Mobile Register (Alabama, USA) described what was very likely a hypoxic/anoxic event in Mobile Bay, with fishes congregating in shallow water where they were easily caught by hand (J. Pennock, University of Alabama, personal communication). Mobile Bay has a well-documented history of oxygen depletion that extends back to at least the 1960s, with descriptions of similar attempts by fish to escape oxygen-depleted waters (May 1973). A detailed review of the historical literature will likely find hundreds of such accounts, and we predict that most of them can be associated with some sort of human development.

The most serious effects of the combined problems associated with eutrophication and hypoxia are seen in the Black Sea and Baltic Sea, where demersal trawl fisheries have either been eliminated or severely stressed (Elmgren 1989, Mee 1992). Karlson *et al.* (2002) provide a detailed summary of the development of oxygen depletion in the Baltic and surrounding seas. The Black Sea, in particular, provides an excellent example of how multiple stressors conspired to alter an entire system. In the 1980s and early 1990s, the northwest coastal shelf of the Black Sea was in a severe state of deterioration from stress exerted by multiple factors, including over-fishing, exotic species introduction (the ctenophore *Mnemiopsis* spp.), pollution, altered hydrology and nutrient enrichment that led to eutrophication-induced hypoxia (Mee 1992, Kideys 1994, 2002). Historical data show that in the 1940s, the northwest Black Sea was considered to be oligotrophic. However, by the 1970s nutrient enrichment had led to a highly eutrophic condition that, in turn, led to alterations in the composition and quality of phytoplankton production, including harmful algal blooms (HAB). In the 1970s prior to the introduction of the ctenophore, and in the 1980s before ctenophore populations exploded, eutrophication resulted in increased anchovy (*Engraulis encrasicolus*) production and widespread hypoxia. Through the 1970s and 1980s hypoxia and anoxia became more prevalent and were the primary cause of mass mortality of the benthos, including demersal fish. Other complex changes that occurred and were likely a response to the multiple stressors included increased turbidity, decrease in nongelatinous zooplankton, decline in biodiversity, and replacement of highly-valued demersal fish species with less desirable planktonic omnivores (Mee 1992, Kideys 2002). Of the 26 commercial species fished in the 1960s, only six still supported a fishery in the early 1990s (Mee 1992). In 1989, the ctenophore populations exploded and caused a crash in the pelagic anchovy and nongelatinous zooplankton communities that was not oxygen-related. This event indicates that the combination of stressors affecting the Black Sea needs to be examined in order to understand ecosystem response. The resilience of the Black Sea ecosystem was observed in the 1990s when nutrient loads declined between 1991 and 1997. Primary production declined, there was a species shift back to diatoms, harmful algal blooms decreased, nongelatinous zooplankton increased, and pelagic fish reappeared (Kideys 2002). The introduction of the ctenophore *Beroe* spp., a predator of *Mnemiopsis* spp., further improved the Black Sea ecosystem.

The eutrophication-related oxygen depletion zone in the northwest Black Sea is not

related to the central Black Sea anoxic zone, which over the last 5000 years has evolved an oxic/anoxic ecosystem in a precise redox balance (Konovalov and Murray 2001). However, there is now evidence that even the central Black Sea anoxic zone is showing the signs of eutrophication due to an increased flux of organic matter. This, in turn, has increased the rate of sulfate reduction and created an imbalance in the sulfide budget. As a result, sulfide concentrations have increased in the anoxic zone over the past 20 to 25 years (Konovalov and Murray 2001).

As early as the 1980s, the occurrence of hypoxia in coastal systems was closely linked to eutrophication. In the German Bight, van Pagee *et al*. (1983) found that from 1930 to 1983 an increase in nutrients corresponded with an increase in the duration and severity of hypoxia. In all recent cases, (listed in Table 1), hypoxia appears to be a result of general ecosystem eutrophication, with other stressors acting to complicate ecosystem response. It is difficult or impossible to separate the response of an ecosystem to eutrophication-induced hypoxia from the other multiple stressors on ecosystem functioning; the Black Sea provides a good example. However, some level of eutrophication appears to be a positive force in enhancing a system's secondary productivity (Nixon and Buckley 2002), and to a point enhances fisheries yield (Caddy 1993, 2001). The critical point in the ecosystem response trajectory to eutrophication is the appearance of severe hypoxia or anoxia, either of which can potentially cause mass mortality of both benthic and pelagic species. The general effect of eutrophication and hypoxia to favor benthic macrofaunal communities and species with opportunistic life histories, shorter life spans, and smaller body size is well characterized by the Pearson and Rosenberg (1978) organic gradient response model. However, eutrophication has a preconditioning effect on benthic fauna by eliminating sensitive species, which tends to lessen the acute response of the system to hypoxia when it does occur. This is the reason some systems that experience mild hypoxia show no acute effect, such as the York River, in Virginia (Neubauer 1993, Sagastie *et al.* 2001).

Climate change, whether from global warming or from microclimate variation, will have direct consequences for eutrophication-related oxygen depletion. The form of climate change effect will depend primarily upon how the strength of water column stratification is affected, and secondarily on factors that affect organic matter production such as nutrient supplies. At a global scale, general circulation models predict large changes in rainfall patterns under a CO_2 doubling scenario. If these changes in rainfall lead to increased discharges of freshwater to coastal ecosystems, stratification is likely to increase and oxygen depletion will expand in those systems already affected, and may begin in other systems. Conversely, if stratification decreases, oxygen depletion or the chances for depletion will decrease. For that part of the Mississippi River basin associated with the northern Gulf of Mexico annual oxygen depletion, a doubling of CO_2 would increase river discharge by 20% and temperature by $2^{o}C$ to $4^{o}C$ (Miller and Russell 1992). Justic *et al.* (1996) predicted that these changes would lead to a 50% increase in primary production, a 30% to 60% decrease in subpycnocline dissolved oxygen, and expansion of the oxygen-depleted area. Smaller-scale climate variation, such as the North Atlantic Oscillation (NAO) index, may have similar effects on dissolved oxygen budgets. Nordberg *et al.* (2000, 2001) found that the NAO index was correlated to hydrographic conditions in Swedish west coast fords, and may in part be responsible for changes in dissolved oxygen budgets, particularly in fjords not subjected to significant human pollution.

SUMMARY

Hypoxia related to anthropogenic activities appears to develop within a system as a result of the cumulative effects of eutrophication in combination with other stressors. Many times hypoxia is not noticed until higher-level ecosystem effects are manifested. For example, hypoxia did not become a prominent environmental issue in the Kattegat until the collapse of a Norway lobster fishery several years after hypoxic bottom waters were first reported (Baden *et al.* 1990b). The northern Gulf of Mexico is representative of severely stressed coastal ecosystems that currently experience seasonal hypoxia, but have not experienced hypoxia-related loss of fisheries. Although hypoxia in the northern Gulf of Mexico has affected benthic invertebrate communities over the last several decades, there is no clear signal of hypoxia in fisheries landings statistics (Rabalais *et al.* 2001a, Chesney and Baltz 2001). However, ecosystem level change is rarely the result of a single factor, and several forms of stress typically act in concert to cause change within an ecosystem. The critical point for fisheries losses in the northern Gulf of Mexico may be potential effects from global warming. The shallow, northwest continental shelf of the Black Sea (not part of the deep central basin anoxia) is another example of a system that was stressed by eutrophication-driven hypoxia in combination with other stressors that led to drastic reductions in bottom fisheries (Mee 1992, Kideys 1994, 2002).

Until the 1950s, reports of mass mortality of marine animals caused by lack of oxygen were limited to small systems that had histories of oxygen stress (Brongersma-Sanders 1957). In the 1960s, the number of systems with reports of hypoxia-related problems started to increase, but it was in the 1970s and 1980s when most initial reports of hypoxia occurred. By the 1990s, most estuarine and marine systems in close proximity to population centers had reports of hypoxia or anoxia. It does not appear that reports of oxygen depletion have leveled off, and the number of systems affected by hypoxia/anoxia continues to rise. There is encouraging news since 2000 that some large systems such as the Black Sea and Gulf of Finland have responded positively to a decrease in stressors.

Coastal and estuarine hypoxia does not appear to be a natural condition, except in areas influenced by OMZs, upwelling, or some enclosed fjord systems. The main factor in development of hypoxia in coastal and estuarine systems has been the input of excess nutrients leading to eutrophication. The determination of population or ecosystem level effects from hypoxia is complicated by many factors, including inadequate data on historic trends of species populations and dissolved oxygen concentrations and the interaction and synergistic effects of multiple stressors such as fishing pressure, habitat loss, etc. (Figure 1). Hypoxia and anoxia are among the most widespread deleterious anthropogenic effects in estuarine and marine environments. The effects of hypoxia may be reversed by the reduction of nutrient or organic inputs to a system that lead to a reduction or elimination of the hypoxia.

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REFERENCES

Abadie, S.W. and M.A. Poirrier. 2000. Increased density of large *Rangia* clams in Lake Pontchartrain after the cessation of shell dredging. Journal of Shellfish Research 19:481-485.

Abril, G., H. Etcheber, P.L. Hir, P. Bassoullet, B. Boutier, and M. Frankignoulle. 1999. Oxic/anoxic oscillations and organic carbon mineralization in an estuarine maximum turbidity zone (The Gironde, France). Limnology and Oceanography 44:1304-1315.

Andersin, A.B., J. Lassig, L. Parkkonen, and H. Sandler. 1978. The decline of macrofauna in the deeper parts of the Baltic proper and the Gulf of Finland. Kieler Meeresforschungen, Sonderheft 4:23-52.

Andersin, A.-B. and H. Sandler. 1991. Macrobenthic fauna and oxygen deficiency in the Gulf of Finland. Memoranda Soc Fauna Flora Fennica 67:3-10.

Arntz, W. 1981. Zonation and dynamics of macrobenthos biomass in an area stressed by oxygen deficiency. *In:* Stress effects on natural ecosystems, G.W. Barrett and R. Rosenberg (eds.), John Wiley and Sons, Chichester. pp. 215-225.

Arvanitidis C., D. Koutsoubas, C. Dounas, and A. Eleftheriou. 1999. Annelid fauna of a Mediterranean Lagoon (Gialova Lagoon, south-west Greece): community structure in a severely fluctuating environment. Journal of Marine Biology Association UK 1999: 849-856.

Baden, S.P., L.O. Loo, L. Pihl, and R. Rosenberg. 1990a. Effects of Eutrophication on benthic communities including fish - Swedish west coast. Ambio 19:113-122.

Baden, S.P., L. Pihl, and R. Rosenberg. 1990b. Effects of oxygen depletion on the ecology, blood physiology and fishery of the Norway lobster *Nephrops norvegicus*. Marine Ecology Progress Series 67:141-155.

Bakan, G. and H. Buyukgungor. 2000. The Black Sea. Marine Pollution Bulletin 41:24-43.

Barmawidjaja, D. M., G. J. Van Der Zwaan, F.J.Jorissen, and S. Puskaric. 1995. 150 years of eutrophication in the northern Adriatic Sea: Evidence from a benthic formaminiferal record. Marine Geology. 122: 367–384.

Bentley, S.J. and C.A. Nittrouer. 1999. Physical and Biological Influences on the Formation of Sedimentary Fabric in an Oxygen-restricted Depositional Environment: Eckernfoerde Bay, Southwestern Baltic Sea. Palaios 14.

Bernhard, J.M. and C.E. Reimers. 1991. Benthic foraminiferal population fluctuations related to anoxia: Santa Barbara basin. Biogeochemistry 15:127-149.

Boesch, D.F., R.B. Brinsfield, and R.E. Magnien. 2001. Chesapeake Bay Eutrophication: Scientific understanding, ecosystem restoration, and challenges for agriculture. Journal of Environmental Quality 30:303-320.

Breitburg, D.L, T. Loher, C.A. Pacey, and A. Gerstein. 1997. Varying effects of low dissolved oxygen on trophic interactions in an estuarine food web. Ecological Monographs 67:489-507.

Breitburg, D.L., J. Baxter, C. Hatfield, R.W. Howarth, C.G. Jones, G.M. Lovett, and C. Wigand. 1998. Understanding effects of multiple stresses: ideas and challenges. *In*: Successes, Limitations and Frontiers in Ecosystem Science, M. Pace and P. Groffman (eds.), Springer, New York, pp. 416-431.

Breitburg, D.L., L. Pihl, and S.E. Kolesar. 2001. Effects of low dissolved oxygen on the behavior, ecology and harvest of fishes: a comparison of the Chesapeake and Baltic systems. *In:* Coastal Hypoxia Consequences for Living Resources and Ecosystems, N.N. Rabalais and R.E. Turner (eds.), American Geophysical Union, Washington, D.C. pp. 241-267.

Brockmann, U., G. Billen, and W. Gieskes. 1988. North Sea Nutrients and Eutrophication. *In:* Pollution of the North Sea: An Assessment, W. Salomons, B. Bayne, E. Duursma, and U. Förstner (eds.), Springer-Verlag, New York, pp. 348-389.

Brongersma-Sanders, M. 1957. Mass mortality in the sea. *In*: Treatise on Marine Ecology and Paleoecology, Volume 1, J.W. Hedgpeth (ed.), Waverly Press, Baltimore, pp. 941-1010.

Brosnan, T.M. and M.L. O'Shea. 1996. Long-term improvements in water quality due to sewage abatement in the lower Hudson River. Estuaries 19:890-900.

Caddy, J. 1993. Toward a comparative evaluation of human impacts on fishery ecosystems of enclosed and semi-enclosed seas. Review of Fishery Science 1:57-96.

Caddy, J. 2001. A brief overview of catchment basin effects on marine fisheries. *In:* Coastal Hypoxia Consequences for Living Resources and Ecosystems, N.N. Rabalais and R.E. Turner (eds.), American Geophysical Union, Washington, D.C. pp. 355-370.

Caplan, M.L. and R.M. Bustin. 1999. Devonian-Carboniferous Hangenberg mass extinction event, widespread organic-rich mudrock and anoxia: causes and consequences. Palaeogeography, Palaeoclimatology, Palaeoecology 148:187-208.

Chamberlain, R. and D. Hayward. 1996. Evaluation of water quality and monitoring in the St. Lucie Estuary, Florida. Water Resources Bulletin 32:681-696.

Chapelle, A., A. Menesguen, J.-M. Deslous-Paoli., P. Souchu, N. Mazouni, A. Vaquer, and B. Millet. 2000. Modelling nitrogen, primary production and oxygen in a Mediterranean lagoon. Impact of oysters farming and inputs from the watershed. Ecological Modelling 127: 161–181.

Chechun, T.Y. 1998. Nutrition of the Acipenseridae of the Sea of Azov under present conditions. Journal of Ichthyology 38:147-154.

Cheng, W., C.-H. Liu, J.-P. Hsu, and J.-C. Chen. 2002. Effect of hypoxia on the immune response of giant freshwater prawn *Macrobrachium rosenbergii* and its susceptibility to pathogen Enterococcus. Fish and Shellfish Immunology 13:351-365.

Chesney, E.J. and D.M. Baltz. 2001. The effects of hypoxia on the northern Gulf. *In:* Coastal Hypoxia Consequences for Living Resources and Ecosystems, N.N. Rabalais and R.E. Turner (eds.), American Geophysical Union, Washington, D.C. pp. 321-354.

Childress, J.J. and B.A. Seibel. 1998. Live at stable low oxygen levels: adaptations of animals to oceanic oxygen minimum layers. Journal of Experimental Biology 201:1223-1232.

Christensen, J. and T. Packard. 1976. Oxygen utilization and plankton metabolism in a Washington fjord. Estuarine and Costal Marine Science 4:339-347.

Cloern, J.E. 2001. Our evolving conceptual model of the coastal eutrophication problem. Marine Ecology and Progress Series 210:223-253.

Cochran, R.E. and L.E. Burnett. 1995. Respiratory responses of the salt marsh animals *Fundulus heteroclitus*, *Leiostomus xanthurus*, and *Palaeominetes pugio* to environmental hypoxia and hypercapnia, and to the organophosphate pesticide, azinphosmethyl. Journal of Experimental Marine Biology and Ecology 195:125-144.

Comeau, L.A., S.E. Campana, and G.A. Chouinard. 2002. Timing of cod (*Gadus morhua* L.) seasonal migrations in the southern Gulf of St. Lawrence: interannual variability and proximate control. ICES Journal of Marine Science 59: 333-351.

D'Andrea, A.F., N.I. Craig, and G.R. Lopez. 1996. Benthic macrofauna and depth of bioturbation in Eckernfoerde Bay, southwestern Baltic Sea. Geo-Marine 16:155-159.

deJonge, V.N., W. Boynton, C.F. D'Elia, R. Elmgren, and B.L. Welsh. 1994. Responses to developments in eutrophication in four different North Atlantic estuarine systems. *In*: Changes in Fluxes in Estuaries: Implications from Science to Management, K. Dyer and R. Orth (eds.), Olsen and Olsen, Fredensborg, Denmark, pp. 179-196.

Desprez, M., H. Rybarczyk, J.G. Wilson, J.P Ducrotoy, F. Sueur, R. Olivesi, and B. Elkaim. 1992. Biological impact of eutrophication in the Bay of Somme and the induction and impact of anoxia. Netherlands Journal of Sea Research 30:149-159.

Dethlefsen, V. and H.V. Westernhagen. 1983. Oxygen deficiency and effects on bottom fauna in the eastern German Bight 1982. Meeresforschung 60:42-53.

Diaz, R.J. and R. Rosenberg. 1995. Marine benthic hypoxia - review of ecological effects and behavioural responses on macrofauna. Oceanography and Marine Biology: an Annual Review 33:245-303.

Diaz, R.J. and R. Rosenberg. 2001. Overview of anthropogenically-induced hypoxia effects on marine benthic fauna. *In:* Coastal Hypoxia Consequences for Living Resources and Ecosystems, N.N. Rabalais and R.E. Turner (eds.), American Geophysical Union, Washington, D.C. pp. 129-146.

Diaz, R.J., R.J. Neubauer, L.C. Schaffner, L. Pihl, and S.P. Baden. 1992. Continuous monitoring of dissolved oxygen in an estuary experiencing periodic hypoxia and the effect of hypoxia on macrobenthos and fish. Science of the Total Environment, Supplement 1992, pp. 1055-1068.

Donnelly, T.H., P.W. Ford, D. McGregor, and D. Allen. 1999. Anthropogenic changes to a billabong in New South Wales. 1. Lagoon evolution and phosphorus dynamics. Marine and Freshwater Research 50:689-698.

Dumont, H.J. 1998. The Caspian Lake: History, biota, structure, and function. Limnology and Oceanography 43:44-52.

Dyer, M.F., J.G. Pope, P.D. Fry, R.J. Law, and J.E. Portmann. 1983. Changes in fish and benthos catches off the Danish coast in September 1981. Journal of the Marine Biological Association of the United Kingdom 63:767-775.

Elmgren, R. 1989. Man's impact on the ecosystem of the Baltic Sea: Energy flows today and at the turn of the Century. Ambio 18:326-332.

Engle, V.D., J.K. Summers, and J.M. Macauley. 1999. Dissolved oxygen conditions in northern Gulf of Mexico estuaries. Environmental Monitoring and Assessment 57:1-20.

Engle, V.D. and J.K. Summers. 1999. Refinement, validation, and application of a benthic condition index for northern Gulf of Mexico estuaries. Estuaries 22(3A):624-635.

Fallesen, G. and H.M. Jørgensen. 1991. Distribution of *Nephtys hombergii* and *N. ciliata* (Polychaeta: Nephtyidae) in Århus Bay, Dennmark, with emphasis on the effect of sewage oxygen deficiency. Ophelia Supplement 5:443-450.

Flemer, D.A., W.L. Kruczynski, B.F. Ruth, and C.M. Bundrick. 1999. The relative influence of hypoxia, anoxia, and associated environmental factors as determinants of macrobenthic community structure in a northern Gulf of Mexico estuary. Journal of Aquatic Ecosystem Stress and Recovery 6:311-328.

Fonselius, S.H. 1969. Hydrography of the Baltic deep basins III. Fishery Board of Sweden, Series Hydrography report No.23. pp. 1-97.

Friedrich, J., C. Dinkel, G. Friedl, N. Pimenov, J. Wijsman, M.-T. Gomoiu, A. Cociasu, L. Popa, and B. Wehrli. 2002. Benthic nutrient cycling and diagenetic pathways in the north-western Black Sea. Estuarine, Coastal and Shelf Science 54: 369–383.

Friligos, N. and A. Zenetos. 1988. Elefsis Bay anoxia: nutrient conditions and benthic community structure. Marine Ecology 9:273-290.

Fritz, C., E. LaBrecque, J. Tober, P.J. Behr, and I. Valiela. 1996. Shrimp in Waquoit Bay: Effects of nitrogen loading on size and abundance. General Scientific Meetings of the Marine Biological Laboratory, Woods Hole, MA. 191:326-327.

Garlo, E.V., C.B. Milstein, and A.E. Jahn. 1979. Impact of hypoxic conditions in the vicinity of Little Egg Inlet, New Jersey in summer 1976. Estuarine and Coastal Marine Science 8:421-432.

Gaston, G. 1985. Effects of hypoxia on macrobenthos of the inner shelf off Cameron, Louisiana. Estuarine, Coastal and Shelf Science 20: 603-613.

Gibbs, P.E., J.C. Green, and P.L. Pascoe. 1999. A massive summer-kill of the dog whelk, Nucella lapillus, on the north Cornwall coast in 1995: freak or forerunner? Journal of Marine Biology Association UK 1999: 103-109.

Gillibrand, P.A., W.R. Turrell, D.C. Moore, and R.D. Adams. 1996. Bottom water stagnation and oxygen depletion in a Scottish sea loch. Estuarine, Coastal and Shelf Science 43:217-235.

Glenn, S., M. Crowley, D. Haidvogel, and Y. Song. 1996. Underwater observatory captures coastal upwelling event off New Jersey. EOS Transaction, American Geophysical Union, 77:223-236.

Gonzales-Oreja, J., and J. Saiz-Salinas. 1998. Loss of herterotrophic biomass structure in an extreme estuarine environment. Estuarine, Coastal and Shelf Science 48:391-399.

Gooday, A.J., J.M. Bernhard, L.A. Levin, and S.B. Suhr. 2000. Foraminifera in the Arabian Sea oxygen minimum zone and other oxygen-deficient settings: taxonomic composition, diversity, and relation to metazoan faunas. Deep-Sea Research Part II 47:25-54.

Gray, J.S. 1992. Eutrophication in the sea. *In*: Marine Eutrophication and Population Dynamics, G. Colombo, I. Ferrari, V.U. Ceccherelli and R. Rossi (eds.), Proceedings 25th European Marine Biology Symposium. Olsen and Olsen, Fredensporg, pp. 3-15.

Gustafsson, M. and K. Nordberg. 1999. Benthic foraminifera and their response to hydrography, periodic hypoxic conditions and primary production in the Koljo fjord on the Swedish west coast. Journal of Sea Research 41: 163-178.

Gustafsson, M., and K. Nordberg. 2000. Living (stained) benthic foraminifera and their response to the seasonal hydrographic cycle, periodic hypoxia and to primary production in Havstens Fjord on the Swedish west coast. Estuarine, Coastal and Shelf Science 51: 743-761.

Gustafsson, M. and K. Nordberg. 2001. Living (stained) benthic foraminiferal response to primary production and hydrography in the deepest part of the Gullmar Fjord, Swedish west coast, with comparisons to Hoglund's 1927 material. Journal of Foraminiferal Research 31: 2-11.

Guyoneaud, R., R. De Wit, R. Matheron, and P. Caumette. 1998. Impact of macroalgal dredging on dystrophic crises and phototrophic bacterial blooms (red waters) in a brackish coastal lagoon. Oceanologica Acta 21:551-561.

Hagerman, L., A. B. Josefson and J.N. Jensen. 1996. Benthic macrofauna and demersal fish. *In*: Eutrophication in Coastal Marine Ecosystems, B.B. Jørgensen and K. Richardson (eds.), Coastal and Estuarine Studies, Volume 52. American Geophysical Union, Washington, D.C. pp. 155-178.

Hamukuaya, H., M.J. O'Toole, and P.M.J. Woodhead. 1998. Observations of severe hypoxia and offshore displacement of Cape hake over the Namibian shelf in 1994. South African Journal of Marine Science 19:57-59.

Harper, D.E., Jr., and N.N. Rabalais. 1995. Responses of Benthonic and Nektonic Organisms, and communities, to severe hypoxia on the inner continental shelf of Louisiana and Texas. *In*: Proceedings of the First Gulf of Mexico Hypoxia Management Conference, Dec 5-6, 1995, Kenner, Louisiana, USEPA-55-R-97-002, pp. 41-56.

Harper, D.E., L.D. McKinney, J.M. Nance, and R.B. Salzer. 1991. Recovery responses of two benthic assemblages following an acute hypoxic event on the Texas continental shelf, northwestern Gulf of Mexico. *In*: Modern and Ancient Continental Shelf Anoxia, R.V. Tyson and T.H. Pearson (eds.), Geological Society Special Publication No. 58, London, pp. 49-64.

Harper, D.E., L.D. McKinney, R.B. Salzer, and R.J. Case. 1981. The occurrence of hypoxic bottom water off the upper Texas coast and its effects on the benthic biota. Contributions in Marine Science 24:53-79.

Hendelberg, M. and P. Jensen. 1993. Vertical distribution of the nematode fauna in a coastal sediment influenced by seasonal hypoxia in the bottom water. OPHELIA 37: 83-94.

Hobbie, J. E., B. J. Copeland, and W. G. Harrison. 1975. Sources and fates of nutrients in the Pamlico River estuary of North Carolina. *In:* Estuarine Research. Vol.1, L. E. Cronin (ed.), Academic Press, New York, pp. 287-302.

Holland, A.F., A.T. Shaughnessy, and M.H. Hiegel. 1987. Long-term variation in Mesohaline Chesapeake Bay macrobenthos: spatial and temporal patterns. Estuaries 10:370-378.

Howell, P. and D. Simpson. 1994. Abundance of marine resources in relation to dissolved oxygen in Long Island Sound. Estuaries 17:394-402.

Howarth, R.W., G. Billen, D. Swaney, A. Townsend, N. Jaworski, K. Lajtha, J.A. Downing, R. Elmgren, N Caraco, T. Jordan, F. Berendse, J. Freney, V. Kudeyarov, P. Murdoch, and Z. Zhao-Liang. 1996. Regional nitrogen budgets and riverine N & P fluxes for the drainage to the North Atlantic Ocean: natural and human influences. Biogeochemistry 35:75-139.

Howarth, R.W., A. Sharpley, and D. Walker. 2002. Sources of nutrient pollution to coastal waters in the United States: implications for achieving coastal water quality goals. Estuaries 40:656-676.

Hylleberg, J. 1993. Extinction and immigration of benthic fauna, the value of historical data from Limfjorden, Denmark. *In:* Symposium Mediterranean Seas 2000, Santa Margherita Ligure, September 23-27, 1991, Instituto di Scienze Ambientali Marine, Santa Margherita Ligure, Italy, pp. 43-70, N. Della Croce (ed.).

Iizuka, S. and S. Min. 1989. Formation of anoxic bottom waters in Omura Bay. Engan Kaiyo Kenkyu. 2:75-86.

Imabayashi, H. 1986. Effect of oxygen-deficient water on the settled abundance and size composition of the bivalve *Theora lubrica*. Bulletin of the Japanese Society of Scientific Fisheries. 5:391-397.

Ishitobi, Y., J. Hiratsuka, H. Kuwabara, and M. Yamamuro. 2000. Comparison of fish fauna in three areas of adjacent eutrophic estuarine lagoons with different salinities. Journal of Marine Systems 26:171–181.

Jackson, J.B.C., M.X. Kirby, W.H. Berger, K.A. Bjorndal, L.W. Botsford, B.J. Bourque, R.H. Bradbury, R. Cooke, J. Erlandson, J.A. Estes, T.P. Hughes, S. Kidwell, C.B. Lange, H.S. Lenihan, J.M. Pandolfi, C.H. Peterson, R.S. Steneck, M.J. Tegner, and R.R. Warner.

2001. Historical overfishing and the recent collapse of coastal ecosystems. Science 293:629–638.

Jaworski, N.A., R.W. Howarth, and L. J. Hetling. 1997. Atmospheric deposition of nitrogen oxides onto landscape contributes to coastal eutrophication in the northeast United States. Environmental Science and Technology 31:1995-2004.

Jeng, W-L. and B-C. Han. 1996. Coprostanol in a sediment core from the anoxic Tan-Shui Estuary, Taiwan. Estuarine, Coastal and Shelf Science 42:727-735.

Johannessen, T. and E. Dahl. 1996a. Declines in oxygen concentrations along the Norwegian Skagerrak coast, 1927-1993: A signal of ecosystem changes due to eutrophication? Limnology and Oceanography 41: 766-778.

Johannessen, T. and E. Dahl. 1996b. Historical changes in oxygen concentraions along the Norwegion Skagerrak coast: Reply to the comment by Gray and Abdullah. Limnology and Oceanography 41: 1847-1852.

Jørgensen, B.B. 1980. Seasonal oxygen depletion in the bottom waters of a Danish fjord and its effect on the benthic community. Oikos 34:68-76.

Jørgensen, B.B. and K. Richardson. 1996. Eutrophication in Coastal Marine Ecosystems. Coastal and Estuarine Studies, Volume 52. American Geophysical Union, Washington, D.C., pp. 273.

Josefson, A.B. and J. N. Jensen. 1992. Effects of hypoxia on soft-sediment macrobenthos in southern Kattegat. *In*: Marine Eutrophication and Population Dynamics, G. Colombo, I. Ferrari, V. U. Ceccherelli and R. Rossi (eds.), Olsen and Olsen, Fredensborg, pp. 21-28.

Josefson, A.B. and R. Rosenberg. 1988. Long-term soft-bottom faunal changes in three shallow fjords, west Sweden. Netherlands Journal of Sea Research 22, 149-159.

Josefson, A.B. and B. Widbom. 1988. Differential response of benthic macrofauna and meiofauna to hypoxia in the Gullmar Fjord basin. Marine Biology 100:31-40.

Justic, D. 1987. Long-term eutrophication of the northern Adriatic Sea. Marine Pollution Bulletin. 18:281-284.

Justic, D., T. Legovic, and L. Rottini-Sandrini. 1987. Trends in oxygen content 1911-1984 and occurrence of benthic mortality in the northern Adriatic Sea. Estuarine Coastal Shelf Science 25:435-445.

Justic, D., N.N. Rabalais, and R.E. Turner. 1996. Effects of climate change on hypoxia in coastal waters: a doubled CO_2 scenario for the northern Gulf of Mexico. Limnology and Oceanography 41: 992 - 1003.

Justic, D., N.N. Rabalais, R.E. Turner, and W.J. Wiseman, Jr. 1993. Seasonal coupling between riverborne nutrients, net productivity and hypoxia. Marine Pollution Bulletin 26: 184-189.

Kaiser, J. and S. Lutter. 1998. Do we have the right strategies to combat eutrophication in the Wadden Sea? A critical review of current policies. Ecosystem Research in the Wadden Sea Area. 9. International Scientific Wadden Sea Symposium, Norderney, Germany, 5-8 Nov 1996. Senckenbergiana maritima 29:17-24.

Kamykowski, D. and S-J. Zentara. 1990. Hypoxia in the world oceans as recorded in the historical data set. Deep Sea Research 37:1861-1874.

Karim, M.R., S. Masahiko, and U. Masao. 2002. Simulation of eutrophication and associated occurrence of hypoxic and anoxic condition in a coastal bay in Japan. Marine Pollution Bulletin 45: 280–285.

Karlson, K., R. Rosenberg, and E. Bonsdorff. 2002. Temporal and spatial large-scale effects of eutrophication and oxygen deficiency on benthic fauna in Scandinavian and Baltic waters – a review. Oceanography and Marine Biology: an Annual Review 40:427-489.

Keister, J.E., E.D. Houde, and D.L. Breitburg. 2000. Effects of bottom-layer hypoxia on abundances and depth distributions of organisms in Patuxent River, Chesapeake Bay. Marine Ecology Progress Series 205:43-59.

Kideys, A.E. 1994. Recent dramatic changes in the Black Sea ecosystem: the reason for the sharp decline in Turkish anchovy fisheries. Journal of Marine Systems 5:171-181.

Kideys, A.E. 2002. Fall and rise of the Black Sea ecosystem. Science 297:1482-1484.

Kitching, J.A., F.J. Ebling, J.C. Gable, R. Hoare, A.A.Q.R. McLeod, and T.A. Norton. 1976. The ecology of Lough Ine. XIX. Seasonal changes in the western trough. Journal of Animal Ecology 45:731-758.

Koenig, R. 1996. Black spots blot German coastal flats. Science 273: 25.

Konovalov, S.K. and J.W. Murray. 2001. Variations in the basic chemical properties of the Black Sea on a scale of decades (1960-1995). Journal of Marine Systems 31: 217-243.

Laine, A.O., H. Sandler, A-B. Andersin, and J. Stigzelius. 1997. Long-term changes of macrozoobenthos in the eastern Gotland Basin and the Gulf of Finland (Baltic Sea) in relation to the hydrographical regime. Netherlands Journal of Sea Research 38:135-159.

Lapointe, B.E. and W.R. Matzie. 1996. Effects of stormwater nutrient discharges on eutrophication processes in nearshore waters of the Florida Keys. Estuaries 19:422-435.

Legović, T., D. Petricioli, and V. Zutic. 1991. Hypoxia in a pristine stratified estuary (Krka, Adriatic Sea). Marine Chemistry 32: 347-359.

Lenihan, H.S. 1999. Physical-biological coupling on oyster reefs: how habitat structure influences individual performance. Ecological Monographs 69: 251-275.

Lenihan, H.S. and C.H. Peterson. 1998. How habitat degradation through fishery disturbance enhances impacts of hypoxia on oyster reefs. Ecological Applications 8:128-140.

Leonard, C.L. and J.B. McClintock. 1999. The population dynamics of the brittlestar *Ophioderma brevispinum* in near- and farshore seagrass habitats of Port Saint Joseph Bay, Florida. Gulf of Mexico Science 17:87-94.

Leppäkoski, E. 1969. Transitory return of the benthic fauna of the Bornholm Basin, after extermination by oxygen insufficiency. Cahiers de Biologie Marine 10:163-172.

Leverone, J.R. 1995. Diurnal Dissolved Oxygen in two Tampa Bay seagrass meadows: Ramifications for the survival of adult bay scallops (*Argopectin irradians concentricus*). Florida Scientist 58: 141-152.

Levin, L.A., C.L. Huggett, and K.F. Wishner. 1991. Control of deep-sea benthic community structure by oxygen and organic-matter gradients in the eastern Pacific Ocean. Journal of Marine Research 49:763-800.

Lindholm, T. 1996. Periodic anoxia in an emerging coastline lake basin in SW Finland. Hydrobiologia 325: 223-230.

Llansó, R.J. 1992. Effects of hypoxia on estuarine benthos: the lower Rappahannock River (Chesapeake Bay), a case study. Estuarine, Coastal and Shelf Science 35:491-515.

Lopez-Jamar, E., O. Francesch, A.V. Dorrio, and S. Parra. 1995. Long-term variation of the infaunal benthos of La Coruna Bay (NW Spain): results from a 12-year study (1982-1993). Scientia marina 59:49-61.

Mallin, M.A., M.H. Posey, G.C. Shank, M.R. McIver, S.H. Ensign, and T.D. Alphin. 1999. Hurricane effects on water quality and benthos in the Cape Fear watershed: Natural and anthropogenic impacts. Ecological Applications 9:350-362.

Mason, W.T., Jr. 1998. Macrobenthic monitoring in the Lower St. Johns River, Florida. Environmental Monitoring and Assessment 50:101-130.

Maxted, J.R., R.A. Eskin, S.B. Weisberg, J.C. Chaillou, and F.W. Kutz. 1997. The ecological condition of dead-end canals of the Delaware and Maryland coastal bays. Estuaries 20: 319-327.

May, E. 1973. Extensive oxygen depletion in Mobile Bay, Alabama. Limnology and Oceanography 18:353-366.

Mazouni, N., J.-C. Gaertner, J.M. Deslous-Paoli, S. Landrein, and M. Geringer d'Oedenberg. 1996. Nutrient and oxygen exchanges at the water–sediment interface in a shellfish farming lagoon (Thau, France). Journal of Experimental Marine Biology and Ecology 205: 91–113.

McArthur, V.E. 1998. Predation and the survival of juvenile *Cerastoderma glaucum* Bruguiere (Mollusca: Bivalvia) in a coastal lagoon. Journal of Experimental Marine Biology And Ecology 225: 79-97.

Mee, L.D. 1992. The Black Sea in crisis: A need for concerted international action. Ambio 21:278-286.

Michel, M., B. Averty, J.-F. Chiffoleau, and L.-A. Roman. 2000. Biogeochemical Behavior of Arsenic Species in the Seine Estuary in Relation to Successive High-Amplitude Primary Production, Anoxia, Turbidity, and Salinity Events. Estuaries 24: 1066–1073.

Mirza, F.B. and J.S. Gray. 1981. The fauna of benthic sediments from the organically enriched Oslofjord, Norway. Journal of Experimental Marine Biology and Ecology 54:181-207.

Morrisey, D.J. 2000. Predicting impacts and recovery of marine-farm sites in Stewart Island, New Zealand, from the Findlay-Watling model. Aquaculture 185:257-271.

Moser, F.C. 1998. Sources and Sinks of Nitrogen and Trace Metals, and Benthic Macrofauna Assemblages in Barnegat Bay, New Jersey. Dissertation Abstracts International Part B: Science and Engineering, University Microfilms International 58:5849.

Nakata, K., S-I. Sugioka, and T. Hosaka. 1997. Hindcast of a Japan Sea Oil Spill. Spill Science and Technology Bulletin 4:219-229.

National Oceanographic and Atmospheric Administration (NOAA) 1997. NOAA's Estuarine Eutrophication Survey. Volume 2: Mid-Atlantic Region. Silver Spring, MD: Office of Ocean Resources Conservation and Assessment. p. 51.

Neubauer, R.J. 1993. The relationship between dominant macrobenthos and cyclical hypoxia in the lower York River. Masters Thesis, College of William and Mary, Williamsburg, Virginia.

Niermann, U., E. Bauerfeind, W. Hickel, and H .V. Westernhagen. 1990. The recovery of benthos following the impact of low oxygen content in the German Bight. Netherlands Journal of Sea Research 25:215-226.

Nilsson, H. and R. Rosenberg. 2000. Succession in marine benthic habitats and fauna in response to oxygen deficiency: analysed by sediment profile-imaging and by grab samples. Marine Ecology Progress Series 197: 139-149.

Nixon, S.W. 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. Ophelia 41:199-219.

Nixon, S.W. and B.A. Buckley. 2002. "A strikingly rich zone" - nutrient enrichment and secondary production in coastal marine ecosystems. Estuaries 25:782-296.

Norkko, A. and E. Bonsdorff. 1996. Rapid zoobenthic community responses to accumulations of drifting algae. Marine Ecology Progress Series 131:143-157.

Nordberg, K., M. Gustafsson, and A-L. Krantz. 2000. Decreasing oxygen concentration in the Gullmar Fjord, Sweden, as confirmed by benthic foraminifera, and the possible association with NAO. Journal of Marine Systems 23: 303-316.

Nordberg, K., H. L. Filipsson, M. Gustafsson, R. Harland, and P. Roos. 2001. Climate, hydrographic variations and marine benthic hypoxia in Koljö Fjord, Sweden. Journal of Sea Research 46: 187-200.

Oeschger, A. and K.B. Storey. 1990. Regulation of glycolytic enzymes in the marine invertebrate *Halicryptus spinulosus* (Priapulida) during environmental anoxia and exposure to hydrogen sulfide. Marine Biology 106: 261-266.

Officer, C.B., Biggs, R.B., Taft, J.L., Cronin, L.E., Tyler, M.A, and W.R. Boynton. 1984. Chesapeake Bay anoxia: origin, development, and significance. Science 223:22-27.

Olsen, D.B., G.L. Hitchcock, R.A. Fine and B.A. Warren. 1993. Maintenance of the low-oxygen layer in the central Arabian Sea. Deep-Sea Research II 40:673-685.

Paerl, H.W., J.L. Pinkney, and S.A. Kucera. 1995. Clarification of the structural and functional roles of heterocysts and anoxic microzones in the control of pelagic nitrogen fixation. Limnology and Oceanography 40:634-638.

Paerl, H.W., J.L. Pinckney, J.M. Fear, and B.L. Peierls. 1998. Ecosystem responses to internal and watershed organic matter loading: Consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA. Marine Ecology Progress Series 166:17-25.

Paerl, H.W., J.D. Bales, L.W. Ausley, C.P. Buzzelli, L.B. Crowder, L.A. Eby, M. Go, B.L. Peierls, T.L. Richardson, and J.S. Ramus. 2000. Hurricanes' hydrological and ecological effects linger in major US estuary. EOS Transaction, American Geophysical Union, 81: 457-462.

Patrick, R. 1988. Changes in the chemical and biological characteristics of the Upper Delaware River estuary in response to environmental laws. *In:* Majumdar, E., W. Miller and L.E. Sage (eds.), Unnamed publication of the Pennsylvania Academy of Science, Philadelphia, PA, pp. 332-359.

Paulson, A.J., H.C. Curl, Jr., and R.A. Freely. 1992. The biogeochemistry of nutrients and trace metals in Hood Canal, a Puget Sound fjord. Marine Chemistry 43: 157-173.

Pearson, T.H. and A. Eleftheriou. 1981. The benthic ecology of Sollom Voe. Proceedings of the Royal Society of Edinburgh 80B:241-269.

Pearson, T.H. and R. Rosenberg. 1992. Energy flow through the SE Kattegat: a comparative examination of the eutrophication of a coastal marine ecosystem. Netherlands Journal of Sea Research 28:317-334.

Pennock, J.R., J.H. Sharp, and W.W. Schroeder. 1994. What Controls the Expression of Estuarine Eutrophication? Case Studies of Nutrient Enrichment in the Delaware Bay and Mobile Bay Estuaries, USA. *In:* Changes in Fluxes in Estuaries: Implications from Science to Management, K.R. Dyer and R.J. Orth (eds.), Olsen and Olsen. Fredensborg. pp. 139-146.

Petersen, C.G.J. 1915. On the animal communities of the sea bottom in the Skagerak, the Christiania Fjord and the Danish waters. Report from the Danish Biological Station 23:1-28.

Petersen, J.K. and L. Pihl. 1995. Responses to hypoxia of plaice, *Pleuronectes platessa* and dab, *Limanda limanda*, in the south-east Kattegat: distribution and growth. Environmental Biology of Fishes. 43: 311-321.

Pihl, L., S.P. Baden, and R.J. Diaz. 1991. Effects of periodic hypoxia on distribution of demersal fish and crustaceans. Marine Biology 108:349-360.

Pohl, C., U. Hennings, I. Petersohn, and H. Siegel. 1998. Trace metal budget, transport, modification and sinks in the transition area between the Oder and Peene rivers and the southern Pomeranian Bight. Marine Pollution Bulletin 36:598-616.

Portnoy, J.W. 1991. Summer Oxygen Depletion in a Diked New England Estuary. Estuaries 14:122-129.

Posey, M.H., T.D. Alphin, L. Cahoon, D. Lindquist, and M.E. Becker. 1999. Interactive effects of nutrient additions and predation on infaunal communities. Estuaries 22: 785-792

Powilleit, M. and J. Kube. 1999. Effects of severe oxygen depletion on macrobenthos in the Pomeranian Bay (southern Baltic Sea): a case study in a shallow, sublittoral habitat characterised by low species richness. Netherlands Journal of Sea Research 42:221-234.

Prena, J. 1995a. Effects of eutrophication on macrobenthos zonation in Wismar Bay (western Baltic Sea). Archiv fur Hydrobiologie. Stuttgart 133:245-257

Prena, J. 1995b. Temporal irregularities in the macrobenthic community and deep-water advection in Wismar Bay (western Baltic Sea). Estuarine, Coastal and Shelf Science 41:705-717.

Rabalais, N.N. and R.E. Turner. 2001. Hypoxia in the northern Gulf of Mexico: description, causes and change. *In:* Coastal Hypoxia Consequences for Living Resources and Ecosystems. N.N. Rabalais and R.E. Turner (eds.). American Geophysical Union, Washington, D.C. pp. 1- 36.

Rabalais, N.N., R.E. Turner, and W.J. Wiseman, Jr. 1996. Nutrient changes in the Mississippi River and system responses on the adjacent continental shelf. Estuaries 19:386-407.

Rabalais, N.R., L.E. Smith, and D.E. Harper, Jr. 2001a. Effects of seasonal hypoxia on continental shelf benthos. *In:* Coastal hypoxia consequences for living resources and ecosystems. N.N. Rabalais and R.E. Turner (eds.). American Geophysical Union, Washington, D.C. pp. 185-210.

Rabalais, N.N., R.E. Turner, and W.J. Wiseman, Jr. 2001b. Hypoxia in the Gulf of Mexico. Journal of Environmental Quality 30: 320-329.

Rainer, S.F. and R.C. Fitzhardinge. 1981. Benthic communities in an estuary with periodic deoxygenation. Australian Journal of Marine and Freshwater Research 32:227-243.

Reish, D.J. 1955. The relation of polychaetous annelids to harbor pollution. Public Health Report 70:1168-1174.

Reish, D.J. 2000. The seasonal settlement of polychaete larvae before and after pollution abatement in Los Angeles-Long Beach Harbors, California. Bulletin of Marine Science 67:672.

Reizopoulou, S, Thessalou-Legaki, M, and A. Nicolaidou. 1996. Assessment of disturbance in Mediterranean lagoons: An evaluation of methods. Marine Biology. Berlin, Heidelberg 125:189-197.

Richardson, K. and B.B. Jørgensen. 1996. Eutrophication: Definition, history, and effects. *In*: Eutrophication in Coastal Marine Ecosystems, B.B. Jørgensen and K. Richardson (eds.), Coastal and Estuarine Studies, Volume 52. American Geophysical Union, Washington, D.C. pp. 1-19.

Rikard, F.S., R.K. Wallace, D. Rouse, and I. Saoud. 2000. The effect of low oxygen on oyster survival during reef restoration efforts in Bon Secour Bay, Alabama. Journal of Shellfish Research 19:640.

Ritter, C. and P.A. Montagna. 1999. Seasonal hypoxia and models of benthic response in a Texas bay. Estuaries 22:7-20.

Rosenberg, R. 1980. Effects of oxygen deficiency on benthic macrofauna in fjords. *In*: Fjord Oceanography, H.J. Freeland, D.M. Farmer, and C.D. Levings (eds.), Plenum Publishing Corp., New York. pp. 499-514.

Rosenberg, R. 1972. Benthic faunal recovery in a Swedish fjord following the closure of a sulphite pulp mill. Oikos 23:92-108.

Rosenberg, R. 1976. Benthic faunal dynamics during succession following pollution abatement in a Swedish estuary. Oikos 27:414-427.

Rosenberg, R. 1990. Negative oxygen trends in Swedish coastal bottom waters. Marine Pollution Bulletin 21: 335-339.

Rosenberg, R. and R.J. Diaz. 1993. Sulphur bacteria (*Beggiatoa spp.)* mats indicate hypoxic conditions in the Inner Stockholm Archipelago. Ambio 22:32-36.

Rosenberg, R. and L.O. Loo. 1988. Marine eutrophication induced oxygen deficiency: effects on soft bottom fauna, western Sweden. Ophelia 29:213-225.

Rosenberg, R., I. Olsson, and E. Olundh. 1977. Energy flow model of an oxygen-deficient estuary on the Swedish west coast. Marine Biology 42: 99-107.

Rosenberg, R., W.E. Arntz, E. Chumán de Flores, L.A. Flores, G. Carbajal, I. Finger, and J. Tarazona. 1983. Benthos biomass and oxygen deficiency in the upwelling system off Peru. Journal of Marine Research 41: 263-279.

Rosenberg, R., L.O. Loo, and P. Möller. 1992. Hypoxia, salinity and temperature as structuring factors for marine bethic communities in a eutrophic area. Netherlands Journal of Sea Research 30:121-129.

Rosenberg, R., H.C. Nilsson, and R.J. Diaz. 2001. Response of benthic fauna and changing sediment redox profiles over a hypoxic gradient. Estuarine, Coastal and Shelf Science 53: 343-350.

Rosenberg, R., J.S. Gray, A.B. Josefson, and T.H. Pearson. 1987. Petersen's benthic stations revisited. II. Is the Oslofjord and eastern Skagerrak enriched? Journal of Experimental Marine Biology and Ecology 105:219-251.

Rumohr, H. 1986. Historical evidence for eutrophication processes in the Kieler Bucht (western Baltic). Meeresforschung 31:115-123.

Rybarczyk, H., B. Elkaim, J.G. Wilson, and N. Loquet. 1996. Eutrophication in the Baie de Somme: Consequences and impacts on the benthic population. Oceanologica Acta 19:131-140.

Sagasti, A., L.C. Schaffner, and J.E. Duffy. 2001. Effects of periodic hypoxia on mortality, feeding and predation in an estuarine epifaunal community. Journal of Experimental Marine Biology and Ecology 258: 257-283.

Saiz-Salinas, J.I. and G. Frances-Zubillaga. 1997. Tidal zonation on mud flats in a polluted estuary caused by oxygen-depleted water. Journal of Experimental Marine Biology and Ecology 209: 157-170.

Santos, S.L. and J.L. Simon. 1980. Marine soft-bottom community establishment following annual defaunation: larval or adult recruitment. Marine Ecology Progress Series 2:235-241.

Sanukida, S., H. Okamato, and M. Hitomi. 1984. Alteration of pollution indicator species of macrobenthos during stagnant periods in eastern Hiuchi Sound. Bulletin Japanese Society of Scientific Fisheries 50:727.

Schimmel, S.C., S.J. Benyi, and C.J. Strobel. 1999. An assessment of the ecological condition of Long Island Sound, 1990-1993. Environmental Monitoring and Assessment 56:27-49.

Seitzinger, S.P., C. Kroeze, A.F. Bouwman, N. Caraco, F. Dentener, and R.V. Styles. 2002. Global patterns of dissolved inorganic and particulate nitrogen inputs to coastal systems: recent conditions and future projections. Estuaries 25:640-655.

Seliger, H.H. and J.A. Boggs. 1988. Long term pattern of anoxia in the Chesapeake Bay. *In:* Understanding the estuary: Advances in Chesapeake Bay research: Proceedings of conference. 29-31 March 1988, Baltimore, MD. Chesapeake Research Consortium Publication 129. CBP/TRS 24/88, pp. 570-583.

Sen Gupta, B.K., R.E. Turner, and N.N. Rabalais. 1996. Seasonal oxygen depletion in the continental shelf waters of Louisiana: Historical record of benthic foraminifers. Geology 24:227-230

Sindermann, C. and R. Swanson. 1980. Chapter 1. Historical and Regional Perspective. *In:* Oxygen Depletion and Associated Benthic Mortalities in New York Bight, 1976. R.L. Swanson and C.J. Sindermann (eds.), Rockville. U.S. Department of Commerce National Oceanic and Atmospheric Administration. pp. 1-16.

Simunovic, A., C. Piccinetti, and M. Zore-Armanda. 1999. Kill of benthic organisms as a response to an anoxic state in the northern Adriatic (a critical review). Acta Adriatica 40:37-64.

Solic, M., N. Krstulovic, L. Marasovic, A. Baranovic, T. Pucher-Petkovic, and T. Vucetic. 1997. Analysis of time series of planktonic communities in the Adriatic Sea: distinguishing between natural and man-induced changes. Oceanologica Acta 20:131-143.

Souchu, P., A. Gasc, Y. Collos, A. Vaquer, H. Tournier, B. Bibent, and J-M. eslous-Paoli. 1998. Biogeochemical aspects of bottom anoxia in a Mediterranean lagoon (Thau, France). Marine Ecology Progress Series 164:135-146.

Stachowitsch, M. 1984. Mass mortality in the Gulf of Trieste: The course of community destruction. Marine Ecology 5: 243-264.

Stachowitsch, M. 1991. Anoxia in the Northern Adriatic Sea: rapid death, slow recovery. *In*: Modern and ancient continental shelf anoxia R.V. Tyson and T.H. Pearson (eds.), London: Geological Society Special Publication No. 58. pp. 119-129.

Stanley, D.W. and S.W. Nixon. 1992. Stratification and bottom-water hypoxia in the Pamlico River Estuary. Estuaries 15:270-281.

Stora, G. and A. Arnoux. 1983. Effects of large freshwater diversions on benthos of a Mediterranean lagoon. Estuaries 6: 115-125.

Suzuki, T. 2001. Oxygen-deficient waters along the Japanese coast and their effects upon the estuarine ecosystem. Journal of Environmental Quality 30:291-302.

Suzuki, T. and Y. Matsukawa. 1987. Hydrography and budget of dissolved total nitrogen and dissolved oxygen in the stratified Mikawa Bay, Japan. Journal of the Oceanographic Society of Japan 43:37-48.

Tagliapietra, D., M. Pavan, and C. Wagner. 1998. Macrobenthic community changes related to eutrophication in Palude della Rosa (Venetian Lagoon, Italy). Estuarine, Coastal and Shelf Science 47:217-226.

Tanimoto, T. and A. Hoshika. 1997. Transport of total suspended matter, paticulate organic carbon, organic nitrogen and phosphorus in the inner part of Osaka Bay. Journal of Oceanography., 53, 365-371.

Tarazona, J., H. Salzwedel, and W. Arntz. 1988. Positive effect of "El Nino" on macrozoobenthos inhabiting hypoxic areas of the Peruvian upwelling system. Oecologia 76:184-190.

Taylor, F.J., N.J. Taylor, and J.R. Walsby. 1985. A bloom of the planktonic diatom, *Cerataulina pelagica*, off the coast of northeastern New Zealand in 1983, and its contribution to an associated mortality of fish and benthic fauna. Internationl Revue ges Hydrobiol. 70: 773-795.

Tenore, K.R. 1972. Macrobenthos of the Pamlico River estuary, North Carolina. Ecological Monographs 42:51-69.

Thiel, R., A. Sepulveda, R. Kafemann, and W. Nellen. 1995. Environmental factors structuring the fish community of the Elbe estuary. Journal of Fisheries Biology 46:47-69.

Theodorou, A.J. 1996. Long-term environmental effects of raw sewage sea disposal in Elefsis Bay (Saronikos Gulf, Greece). Partnership in coastal zone management, Samara Publishing ltd., Cardigan, UK. pp. 547-556.

Thomassin, B.A., P. Gourbesville, B. Gout, and A. Arnoux. 1998. Impact of an industrial and urban sewage off a coral fringing reef at Mauritius (Indian Ocean): modeling of plumes, distribution of trace metals in sediments and effects of the eutrophication on coral reef communities. Oceans 98 - Engineering for sustainable use of the oceans: conference proceedings, Nice, IEEE/OES, Piscataway, New Jersey. 1:301-305

Thouvenin, B., P. Le Hir, and L.A. Romana. 1994. Dissolved oxygen model in the Loire Estuary. Changes in fluxes in estuaries: implications from science to management. Olsen and Olsen, Fredensborg, Denmark. pp. 169-178.

Tilman, D., J. Fargione, B. Wolff, C. D'Antonio, A. Dobson, R. Howarth, D. Schindler, W.H. Schlesinger, D. Simberloff and D. Swackhamer. 2001. Forecasting agriculturally driven global environmental change. Science 292:281-284.

Tsutsumi, H. 1987. Population dynamics of *Capitella capitata* (Polychaeta, Capitellidae) in an organically polluted cove. Marine Ecology Progress Series 36:139-149.

Tulkki, P. 1965. Disappearance of the benthic fauna from the basin of the Bornholm (southern Baltic) due to oxygen deficiency. Cahiers de Biologie Marine 6:455-463.

Turner, R.E. 2001. Some effects of eutrophication on pelagic and demersal marine food webs. *In:* Coastal Hypoxia Consequences for Living Resources and Ecosystems. N. N. Rabalais and R.E. Turner (eds.), American Geophysical Union, Washington, D.C. pp. 371-398.

Tyson, R.V. and T.H. Pearson (eds.) 1991. Modern and ancient continental shelf anoxia: an overview. London: Geological Society Special Publication No. 58, pp. 1-24.

Valentin, J., D. Tenenbaum, A. Bonecker, S. Bonecker, C. Nogueira, R. Paranhos, and M.C. Villac. 1999. Hydrobiological characteristics of the Guanabara Bay (Brazil). Journal de recherche Oceanographique 24:33-41.

van Pagee, J.A., P.C.G. Glas, A.A. Markus, and L. Postma. 1988. Mathematical modeling as a tool for assessment of North Sea pollution. *In:* Pollution of the North Sea: An Assessment, W. Salomons, B. Bayne, E. Duursma, and U. Förstner (eds.), Springer-Verlag, New York. pp. 400-422.

Verlaan, P.A.J., M. Donze, and P. Kuik. 1998. Marine vs. fluvial suspended matter in the Scheldt Estuary. Estuarine, Coastal and Shelf Science 46:873-883.

Weigelt, M. 1990. Oxygen conditions in the deep water of Keil Bay and the impact of inflowing salt-rich water from the Kattegatt. Meeresforschung 33:1-22.

Weigelt, M. 1991. Short- and long-term changes in the benthic community of the deeper parts of Kiel Bay (Western Baltic) due to oxygen depletion and eutrophication. Meeresforsch 33: 197-224.

Weigelt, M. and H. Rumohr. 1986. Effects of wide-range oxygen depletion on benthic fauna and demersal fish in Kiel Bay 1981-1983. Meeresforsch 31: 124-136.

Weisberg, S.B., H.T. Wilson, P. Himchak, T. Baum, and R. Allen. 1996. Temporal trends in abundance of fish in the tidal Delaware River. Estuaries 19:723-729.

Welsh, B., R. Welsh, and M. DiGiacomo-Cohen. 1994. Quantifying hypoxia and anoxia in Long Island Sound. *In:* Changes in fluxes in estuaries: Implications from science to management, K. Dyer and R. Orth (eds.), Fredensborg: Olsen and Olsen. pp. 131-137.

Westernhagen, H. V. and V. Dethlefsen. 1983. North Sea oxygen deficiency 1982 and its effects on the bottom fauna. Ambio 12:264-266.

Wilkin, R. and H.L. Barnes. 1997. Pyrite formation in an anoxic estuarine basin. American Journal of Science 297:620-650.

Wilson, E.O. 2002. The future of life. Alfred A. Knopf, New York. p. 229.

Winkler, L. 1888. The determination of dissolved oxygen in water. Berichte der Deutschen chemicer Gesellschaft 21:28-43.

Wu, R.S.S. 1982. Periodic defaunation and recovery in a subtropical epibenthic community, in relation to organic pollution. Journal of Experimental Marine Biology and Ecology 64:253-269.

Wyrtki, K. 1966. Oceanography of the eastern equatorial Pacific Ocean. Oceanography and Marine Biology: an Annual Review 4:33-68.

Yamamuro, M., N. Oka, and J. Hiratsuka. 1998. Predation by diving ducks on the biofouling mussel *Musculista senhousia* in a eutrophic estuarine lagoon. Marine Ecology Progress Series 174:101-106.

Zaitsev, Y.P. 1993. Impacts of eutrophication on the Black Sea fauna. *In:* Fisheries and Environment Studies in the Black Sea System, Food and Agricultural Organization of the United Nations, Rome, Italy. pp. 64-86.

Zimmerman, A.R. and E.A. Canuel. 2000. A geochemical record of eutrophication and anoxia in Chesapeake Bay sediments: anthropogenic influence on organic matter composition. Marine Chemistry 69: 117-137.

ESTUARINE EUTROPHICATION, HYPOXIA AND ANOXIA DYNAMICS: CAUSES, CONSEQUENCES AND CONTROLS

Hans W. Paerl[1]

ABSTRACT

Estuaries are among the most productive, resourceful, and dynamic aquatic ecosystems on Earth. Their productive nature is linked to the fact that they process much of the world's riverine and coastal watershed discharges. Coastal watersheds support more than 75% of the world's human population and, as such, are sites of large increases in nutrient loading associated with urban and agricultural expansion. Increased nutrient loading translates into accelerated primary production, or eutrophication, the symptoms of which include increased algal bloom activity, and accumulation of organic matter. Excess organic matter serves as the "fuel" for microbial decomposition that consumes large amounts of oxygen (O_2), leading to oxygen depletion. Hypoxia (<4 mg O_2 L^{-1}) and anoxia (<1 mg O_2 L^{-1}), respectively, are stressful and fatal to finfish, shellfish and higher plant life, and are prime indicators of declining water quality and habitat conditions. While nutrient-enhanced eutrophication is a "driver" of hypoxia and anoxia, specific physical characteristics, including low flushing rates, strong vertical stratification, stagnant conditions, and elevated temperatures are also involved. Therefore, anthropogenic factors (*i.e.*, excessive nutrient loading), geomorphological and climatic factors (*i.e.*, storm-related freshwater and nutrient discharge) are jointly implicated in determining eutrophication potential. The complex interactions of these factors determine the magnitude, duration and aerial extent of hypoxia and anoxia (jointly termed hypoxia). Using the eutrophic Neuse River Estuary (NRE), North Carolina, USA as a case study, we will examine the physical-chemical mechanisms underlying and controlling hypoxia dynamics. Because primary production in the NRE and many other estuaries is nitrogen (N) limited, emphasis is placed on reducing N inputs to reduce hypoxia. Both the amounts and chemical forms of N play roles in determining the composition and extent of phytoplankton blooms that supply the bulk of the organic carbon fueling hypoxia. Bloom organisms that are readily grazed represent minimal C flux to the sediments, while toxic or inedible blooms lead to the greatest amount of sedimentary C flux and hence hypoxia potential. Nutrient input reductions are the main options for reducing hypoxia. Being able to distinguish physical, chemical and biotic forcing features of hypoxia is key to understanding, predicting, and ultimately managing hypoxia potentials. Long-term monitoring, experimental manipulations and modeling of causative agents of hypoxia over appropriate spatial and temporal scales will be invaluable for developing realistic, ecologically sound, and cost-effective nutrient input reduction strategies aimed at minimizing this undesirable process.

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THE PROBLEM: NUTRIENT-DRIVEN EUTROPHICATION, ORGANIC MATTER ACCUMULATION, HYPOXIA AND ANOXIA IN HYDROLOGICALLY-VARIABLE ESTUARIES

Estuaries are among the most productive, ecologically-diverse, economically-important, and hydrologically-variable ecosystems on Earth. The bulk of the worlds' commercial and recreational fish stocks depend on estuaries as nurseries, refuges and feeding grounds. Estuaries also receive and process a large share of land-based nutrients and other pollutants via surface runoff, atmospheric deposition and groundwater discharge, much of it delivered via rivers draining urban and rural watersheds (Howarth *et al.* 1996, Paerl 1997, Jaworski *et al*. 1997, Paerl *et al.* 2002). Coastal watersheds support approximately 75% of the world's human population and the number of coastal residents continues to rise (Vitousek *et al.* 1997). The productive nature and resourcefulness of estuaries depends to a large extent on externally-supplied, or "new" nutrient inputs; however, anthropogenic nutrient inputs have increased dramatically in recent years, far exceeding what is needed to sustain desirable production (Vollenweider *et al*. 1992, Jørgensen and Richardson 1996, Boesch *et al*. 2001). Many estuaries are now facing nutrient-over-enrichment, or "too much of a good thing" (D'Elia 1997, NRC 2000), which greatly stimulates primary production, the process by which inorganic carbon (CO_2) is converted to organic matter (OM). Accelerated production of organic matter at the base of the food web is commonly referred to as eutrophication (Nixon 1995). High rates of eutrophication can lead to the rapid accumulation of organic matter (*e.g.* algal blooms) due to the inability of consumers to assimilate it. Eventually, the unused organic matter settles to the sediment, where it serves as "fuel" for microbial decomposition that converts organic matter back to CO_2 and inorganic nutrients.

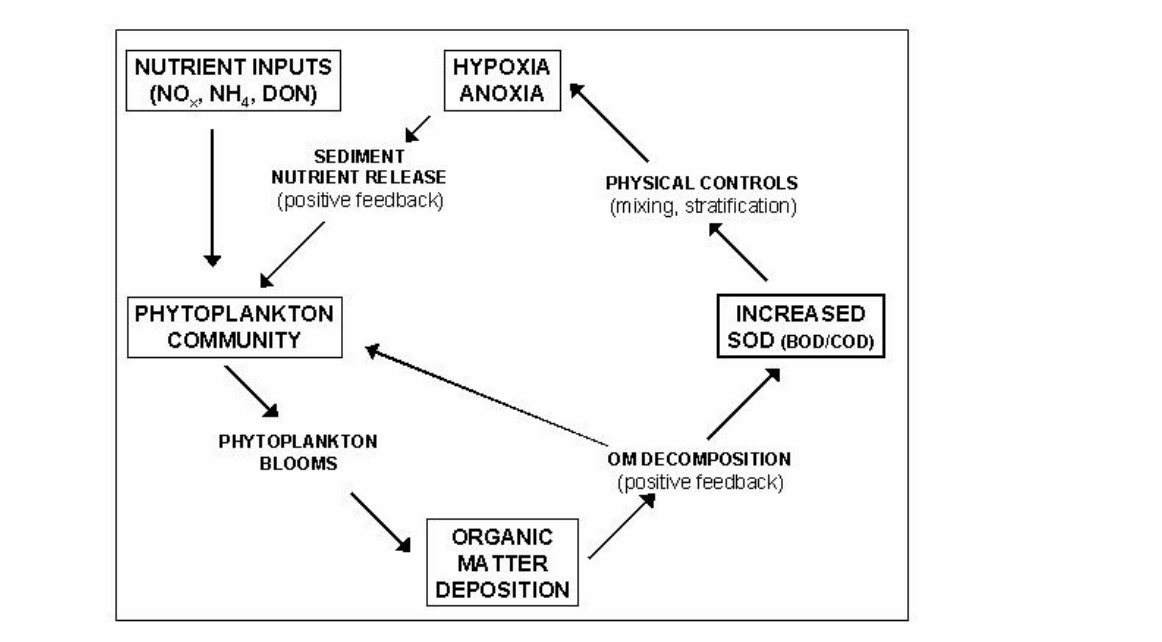

Figure 1. Functional linkages between nutrient inputs, eutrophication (phytoplankton blooms) and hypoxia/anoxia in aquatic ecosystems.

This aerobic decomposition requires large amounts of O_2 (as an oxidant). Therefore, waters enriched with decomposable organic matter tend to consume large amounts of O_2. If the affected waters are stratified, slowly flushed, or stagnant, consumption of O_2 can exceed its re-supply from either atmospheric or photosynthetic (*i.e.*, O_2 evolution) sources. The imbalance between relatively high rates of O_2 consumption and low rates of O_2 re-supply causes dissolved oxygen (DO) content to drop to levels that are low enough to adversely affect oxygen-requiring animal and plant life. Dissolved O_2 concentrations of less than 4 mg O_2 L^{-1} are stressful and termed hypoxic, while O_2 concentrations less than 1 mg O_2 L^{-1} are termed anoxic and may be fatal to many fish, shellfish and invertebrate species (Renaud 1986, Pihl *et al*. 1991, Diaz and Rosenberg 1995). Both conditions are generally considered unhealthy and undesirable from water quality perspectives (Rabalais and Turner 2001).

Hypoxia and anoxia are naturally-occurring processes resulting from a combination of physical-chemical conditions, including: persistent water column stratification; long residence times and stagnation; elevated temperatures that favor high rates of microbial decomposition and hence O_2 consumption; and adequate organic matter supplies. These conditions can vary dramatically in time and space. Hypoxia is exacerbated by excessive organic matter input, hence the link to eutrophication (Rabalais and Turner 2001). There are numerous examples of hypoxia and anoxia in estuarine and coastal waters experiencing nutrient-enhanced eutrophication, the symptoms of which range from barely perceptible increases in primary production and plant biomass (*e.g.*, chlorophyll *a*), to massive accumulations of nuisance (odoriferous, noxious and toxic) algae, or "blooms" (Figure 2) (Paerl 1988, Richardson 1997, Rabalais and Turner 2001). Examples include: 1) summer stratification in the main stem of the Chesapeake Bay (Officer *et al.* 1984, Boynton and Kemp 2001); 2) the Baltic Sea and its embayments (Elmgren and Larsson 2001); 3) The Northern Adriatic Sea downstream of the Po River discharge plume (Justic 1991); 4) Hong Kong Harbor and the adjacent Pearl River Delta that have experienced accelerating nutrient loads from rapid urban and agricultural development in its watershed (Yin *et al*. in press); 5) the Mississippi River discharge plume entering the Northern Gulf of Mexico (Rabalais *et al*. 2001); and 6) Scandinavian fjords under the influence of agricultural runoff and intensive aquaculture operations (Rosenberg 1980, 1990). There are additional examples worldwide (c.f., Turner *et al.* 1987, Van Dolah and Anderson 1991, Welsh and Eller 1991, Diaz and Rosenberg 1995, Rabalais and Turner 2001).

Primary production is controlled or limited by nitrogen (N) availability in most of the world's estuaries (Ryther and Dunstan 1971, Nielsen and Cronin 1974, Nixon 1986, 1995). Growing and more diversified inorganic and organic N inputs from agricultural, urban and industrial expansion in coastal watersheds have been identified as key "drivers" of eutrophication in these estuaries (D'Elia *et al.* 1986, Nixon 1995, 1996, Paerl 1997; Seitzinger and Sanders 1997, Peierls and Paerl 1997). In many N-sensitive estuarine and coastal systems, N loading rates are good predictors of primary production response (Nixon 1995) (Figure 3). It follows that N reduction is a broadly prescribed nutrient management step for reducing estuarine eutrophication, hypoxia and anoxia. There are cases, however, where estuarine and coastal primary production can be co-limited by N and P (*e.g.*, Chesapeake Bay, North Sea, Mediterranean, Northern Gulf of Mexico in Mississippi plume). Here, both N and P reductions must be considered for effective, long-term control of eutrophication (Vollenweider *et al*. 1992, Jonge *et al.* 1996).

Figure 2. Representative algal blooms that provide the organic matter "fuel" for bottom-water hypoxia in vertically-stratified estuarine and coastal waters. Upper left; cyanobacterial (*Nodularia* spp.*, Aphanizomenon flos aquae*) bloom in the Baltic Sea, near the Gulf of Finland. Upper right; Red tide dinoflagellate bloom in the coastal Sea of Japan. Lower left; cyanobacterial (*Anabaena* spp., *Microcystis aeruginosa*) bloom on the St Johns River, a tidal estuary in Florida, USA. Lower right; Algal bloom dominated by cyanobacteria and chlorphytes in a coastal embayment, North Island, New Zealand.

The linkage between nutrient loading, eutrophication and hypoxia/anoxia dynamics is often non-linear and complex in estuarine and coastal systems (Cloern 2001). This is because these systems are hydrodynamically and biogeochemically distinct and dynamic. Climatic and physiographic differences between these systems profoundly affect physical-chemical and biological processes mediating organic matter production and accumulation, oxygen dynamics, and nutrient cycling. The complex interplay between hydrologic discharge (*i.e.*, flushing, residence time), vertical and horizontal thermal and salinity stratification, wind and tidal mixing, and frontal (*e.g.*, "nor-easters") passages and even larger storm events (*i.e.* hurricanes) determines the frequency, spatial and temporal extent of hypoxia events in estuaries. Here, I will explore the interplay of anthropogenic (nutrient) and natural (climatic) forcing features in the Neuse River Estuary (NRE), North Carolina, a system whose watershed has experienced rapid population growth and agricultural and urban development, accompanied by substantial increases in nutrient (N and P) loading.

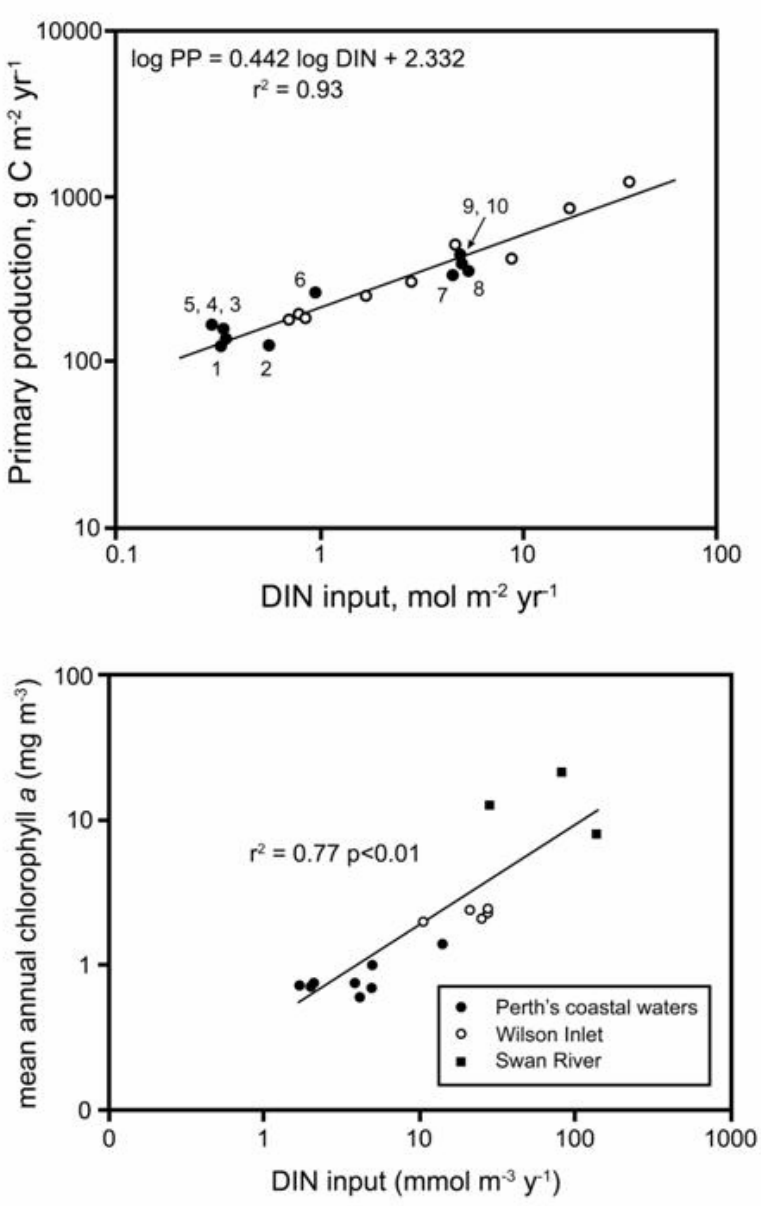

Figure 3. Upper frame. Direct relationship between dissolved inorganic N input and primary production in various North American and European estuarine and coastal ecosystems*. Figure adapted from Nixon *et al.* (1996). Lower frame. Direct relationship between dissolved inorganic N input and phytoplankton biomass, as mean annual chlorophyll *a* content, in several Western Australian estuarine systems. Figure adapted from Twomey and Thompson (2001).

*Details of systems: The open circles are for large (13 m^3, 5 m deep), well-mixed mesocosm tanks at the Marine Ecosystems Research Laboratory (MERL) during a multi-year fertilization experiment (Nixon *et al.* 1986, Nixon 1992). Natural systems (solid circles) include (1) Scotian shelf – DIN from Houghton *et al.* (1978), production from (Mills & Fournier 1979), (2) Sargasso Sea – DIN from (Jenkins 1988), production from (Lohrenz *et al.* 1992) mean of 1989 and 1990 values of 110

and 144 g C m^{-2} yr^{-1}, respectively, (3) North Sea – DIN from (Laane *et al.* 1993) assuming that the ratio of DIN/TN in the input from the Atlantic equals that in the Channel, production from Seitzinger & Giblin (this volume), (4) the Baltic Sea – DIN and production from (Ronner 1985), including DIN flux across the halocline, (5) North Central Pacific – DIN from 0 and 185 g C m^{-2} yr^{-1}, (6) Tomales Bay, CA – DIN and production from (Smith 1991), (7) Continental shelf off New York – DIN and production from (Walsh *et al.* 1987), (8) Outer continental shelf off southeastern U.S. – DIN and production from (Verity *et al.* 1993), (9) Peru upwelling – DIN calculated from annual mean upwelling rate of 0.77 m d^{-1} (Guillen & Calienes 1981) and an initial 20 μM concentation of NO_3 in upwelled water (Walsh *et al.* 1980), production off Chimbote from (Guillen & Calienes 1981), (10) Georges Bank – DIN from (Walsh *et al.* 1987), production from (O'Reilly *et al.* 1987). The equation is a functional regression.

Human and natural disturbances in the NRE and other coastal watersheds have altered both the amounts and ratios of nutrients discharged to coastal waters. Timing, modality, and composition of N (and other nutrient) inputs play roles in determining phytoplankton growth responses (Dugdale and Goering 1967, Harrison *et al.* 1987, Collos 1989, Antia *et al*. 1991), with possible ramifications for hypoxia. Natural phytoplankton communities are exposed to a range of inorganic and organic N compounds at varying ratios and supply rates. Uptake rates of N and other nutrient compounds vary spatially and temporally among phytoplankton communities in the NRE and adjacent coastal waters, suggesting differential responses (Boyer *et al.* 1994, Peierls *et al.* 1997, Paerl *et al.* 2002). These taxa-specific responses play a key role in structuring natural phytoplankton communities, and determining bloom dynamics (Turpin and Harrison 1979, Collos 1989, Stolte *et al.* 1994). Specific phytoplankton taxa can affect hypoxia and anoxia dynamics; especially if they are differentially utilized by zooplankton, larval fish, and benthic grazers. For example, preferentially grazed phytoplankton groups such as diatoms and flagellates, are often effectively utilized and metabolized in the water column, while relatively poorly grazed taxa, such as filamentous cyanobacteria and toxic dinoflagellates, will form a larger fraction of the sedimented organic matter and hence a larger hypoxia burden to the system (Figure 4). Previous studies in the NRE indicate that cyanobacterial and dinoflagellate blooms tend to precede large hypoxia and anoxia events (Pinckney *et al.* 1998, 2001, Paerl *et al*. 2002), suggesting links between phytoplankton community composition and hypoxic and anoxic conditions. There are also strong positive relationships between the extent of vertical stratification (*i.e.* stratification index) and the frequency and aerial extent of bottom water hypoxia (Figure 5) (Buzzelli *et al.* 2002). To some extent, there are strong interactions among all these abiotic parameters and trophic state, since organic matter, much of it originating from phytoplankton production, is the fuel supporting hypoxia in this estuary (Paerl *et al.* 1998, Buzzelli *et al.* 2002).

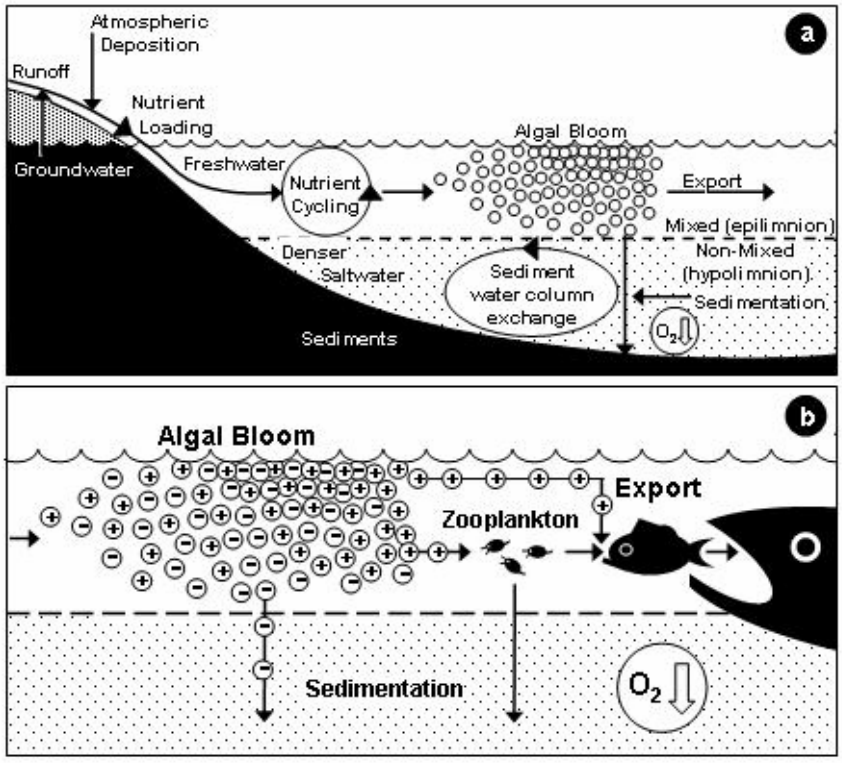

Figure 4. Figure 4a: Conceptual figure showing the linkage between external nutrient loading, internal nutrient cycling, nutrient enhanced algal bloom formation and hypoxia under salinity-stratified estuarine conditions. Figure 4b: Differential impact on hypoxia of phytoplankton species that are readily consumed (labeled +) vs. species that are not (-). Species that are not consumed form a larger share of sedimented organic matter and, therefore, represent a larger burden on the hypoxia potential in the estuary.

Human development of coastal watersheds also accelerates "external" organic matter loading to estuaries in the form of soil erosion, agricultural waste and urban discharge. Enhanced organic matter loading exacerbates hypoxia potential in estuaries (Stanley and Nixon 1992, Paerl *et al.* 1998). Recent research has pointed to the role of dissolved organic nitrogen (DON) in algal nutrition, competitive interactions and determination of community structure (Neilson and Lewin 1974, Antia *et al*. 1991, Seitzinger and Sanders 1997, Peierls and Paerl 1997). In particular, certain harmful (*i.e.*, toxic, hypoxia/anoxia-inducing, food web-altering) algal bloom groups, including dinoflagellates and blue-green algae (cyanobacteria), are known to contain species capable of growing in either autotrophic or heterotrophic modes (Antia *et al.* 1991), enabling them to exploit both inorganic and organic nutrient enrichment. There is concern that substantial quantities of organically-bound nutrients emanating from the watershed can overshadow potentially-beneficial effects of inorganic nutrient reduction strategies in eutrophying waters. Therefore, in order to understand and manage the nutrient "drivers" of eutrophication and hypoxia dynamics, *both* inorganic and organic nutrient enrichment must be considered.

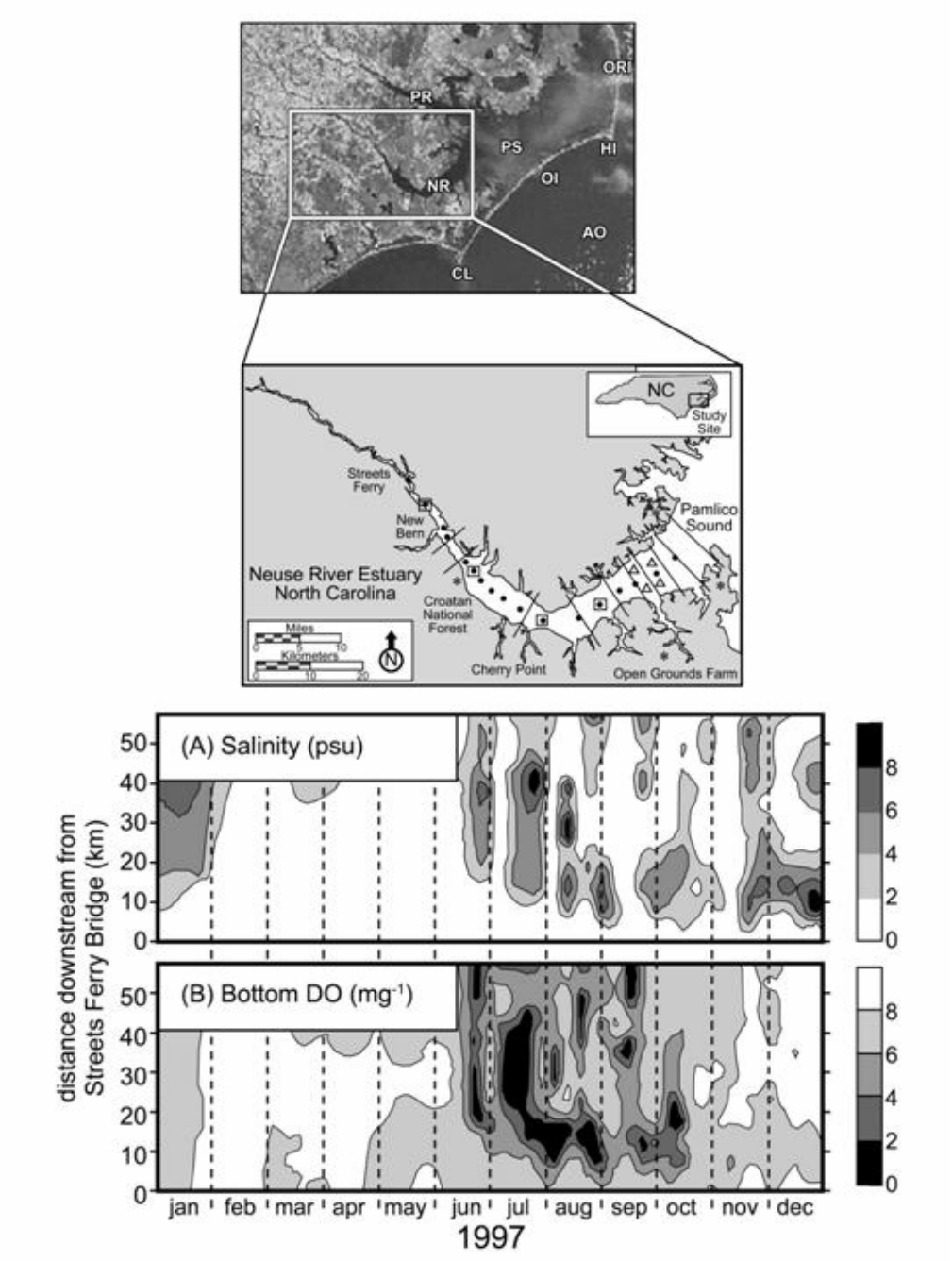

Figure 5. Upper Frame: Location of the Neuse River Estuary and Pamlico Sound, North Carolina. Shown are the Atlantic Ocean (AO), Oregon, Hatteras, and Ocracoke Inlets (ORI, HI, OI, respectively), Cape Lookout (CL), Pamlico Sound (PS), and the Pamlico and Neuse Rivers (PR and NR). The NRE sampling sites for mid-river water quality (19 filled circles) and continuous in-stream monitoring (4 open boxes) are shown. Triangles indicate sites for diel and other periodic studies during which additional samples are collected. Lower Frames: Spatio-temporal

contour plots of salinity and dissolved oxygen characteristics of the Neuse River Estuary during an annual cycle. The sampling locations from which the data were derived are shown. Near surface and near bottom samples were collected biweekly as part of the Neuse River Modeling and Monitoring Program (www.marine.unc.edu/neuse/modmon). The data are plotted along a transect spanning upstream freshwater (Streets Ferry Bridge (SFB), designated 0 km) to a downstream mesohaline location above the entrance to Pamlico Sound (50km downstream of Streets Ferry Bridge). Data were plotted using Surfer Plot software.

HYPOXIA DYNAMICS IN THE NEUSE RIVER ESTUARY, NORTH CAROLINA, USA

The NRE is a tributary of North Carolina's Albemarle-Pamlico Estuarine System (APES), the US's 2nd largest estuarine complex. It drains a rapidly growing urban, industrial, and agricultural watershed, and illustrates the plight of many coastal river systems. This estuary is approximately 100 km long from its fresh headwaters to the mesohaline (15-25 psu) waters of Pamlico Sound (Figure 5). Its physical, chemical and biological characteristics have been intensively monitored, and are the focus of modeling studies described at www.marine.unc.edu/neuse/modmon, www.ferrymon.org (Luettich *et al.* 2000).

Primary production in the NRE is controlled by N inputs (Paerl 1987, Rudek *et al.* 1991, Boyer *et al.* 1994) that have nearly doubled in the past 3 decades (Stanley 1988, Dodd *et al.* 1993). Within this time frame, the NRE has experienced trophic, biogeochemical and water quality decline, exemplified by proliferating nuisance (*i.e.*, toxic and food web disrupting) dinoflagellate and cyanobacterial blooms (Christian *et al.* 1986, Paerl 1987, Paerl *et al.* 1995). Non-point sources contribute ca. 75% of the external or "new" N loading, much of it attributable to agricultural activities (NC Dept. of Environment and Natural Resources 2002). Agricultural expansion, including creation of new farm and forest land, widespread use of nitrogen fertilizers, proliferating livestock (swine, cattle) and poultry (chicken, turkey) operations, coastal urbanization and increasing inputs of groundwater and atmospheric deposition have led to unprecedented increases in total N loading (Paerl *et al.* 2002). Industrial-style farms have increased the region's hog population from approximately 1 million to over 12 million between 1989-1999. As a result, land-applied and atmospherically-emitted N are input into this estuary in increasingly-large amounts (Whitall and Paerl 2001).

Eutrophication and algal bloom formation have been linked to enhanced deposition of organic matter (Clesceri et al. in press), leading to growing frequencies, magnitudes, and aerial coverage of large-scale, bottom-water hypoxia and anoxia (Paerl *et al.* 1998) (Figure 6). Relatively long water residence times (>52 d), low flushing rates, and persistent stratification exacerbate low DO conditions during summer that can cover at least half the bottom of the estuary (Luettich *et al.* 2000, Buzzelli *et al.* 2002) (Figure 7). Finfish and shellfish kills have been linked to this chain of events (Lenihan and Peterson 1998, Paerl *et al.* 1998) (Figure 7).

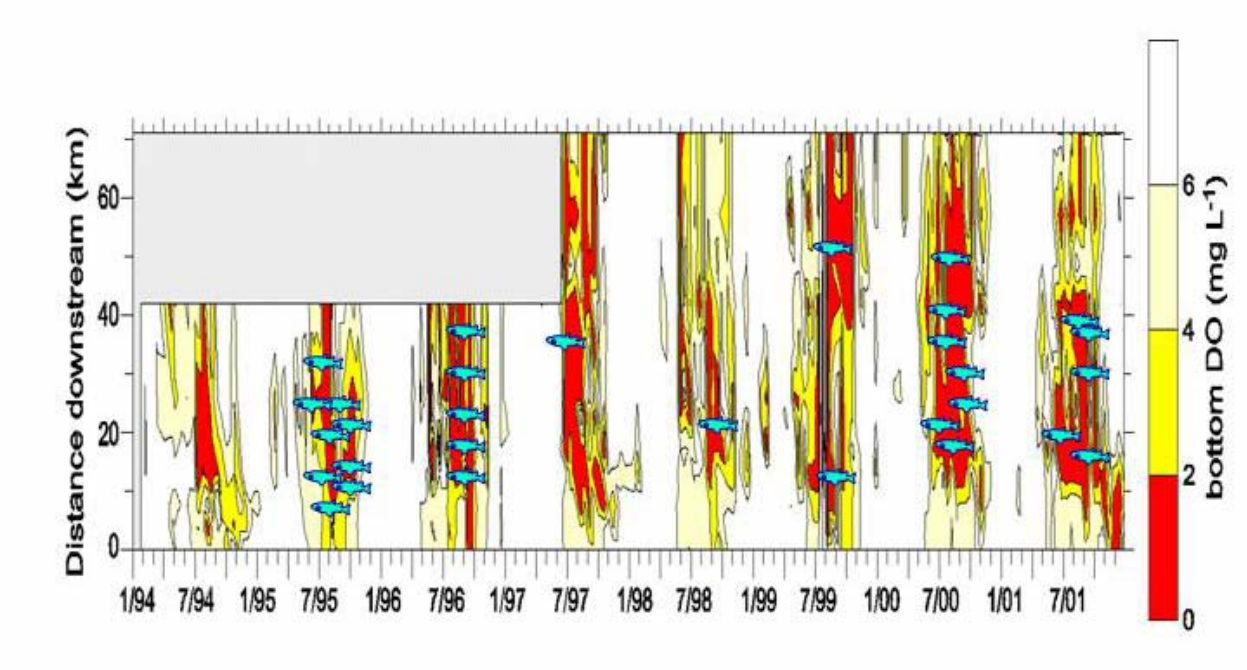

Figure 6. Spatio-temporal relationship between bottom water hypoxia and fish kills, plotted for 1994-2001 in a mesohaline segment of the Neuse River Estuary between New Bern and a location midway between Minnesott Beach and the entrance to Pamlico Solund (see Figure 5). Dissolved oxygen data were obtained by the Neuse River Modeling and Monitoring Program (ModMon; www.marine.unc.edu/neuse/modmon). The fish kill events (each representing at least 500 dead fish) were recorded by the North Carolina Department of Environment and Natural Resources, Division of Water Quality.

The NRE has also recently been under the influence of elevated tropical storm and hurricane activity, possibly reflecting a larger-scale Atlantic basin trend predicted to last 10-40 years (Goldenberg *et al*. 2001). At least 6 major hurricanes have impacted the NRE watershed in the past 6 years alone. During the fall of 1999, 3 sequential hurricanes, Dennis, Floyd and Irene, inundated the NRE watershed with up to a meter of rain during a 6 week period. This caused a 200 year flood in the NRE watershed. Floodwaters turned the NRE and other tributaries of the Pamlico Sound completely fresh, and accounted for more than half the annual N load to this N-sensitive system (Paerl *et al.* 2001). Biogeochemical and ecological effects included hypoxic bottom waters, altered nutrient (N, P, C) cycling, a 3-fold increase in algal biomass, shifts in microbial community structure and function, altered fish distributions and catches, and an increase in fish disease (Paerl *et al*. 2001, Eby and Crowder unpublished data).

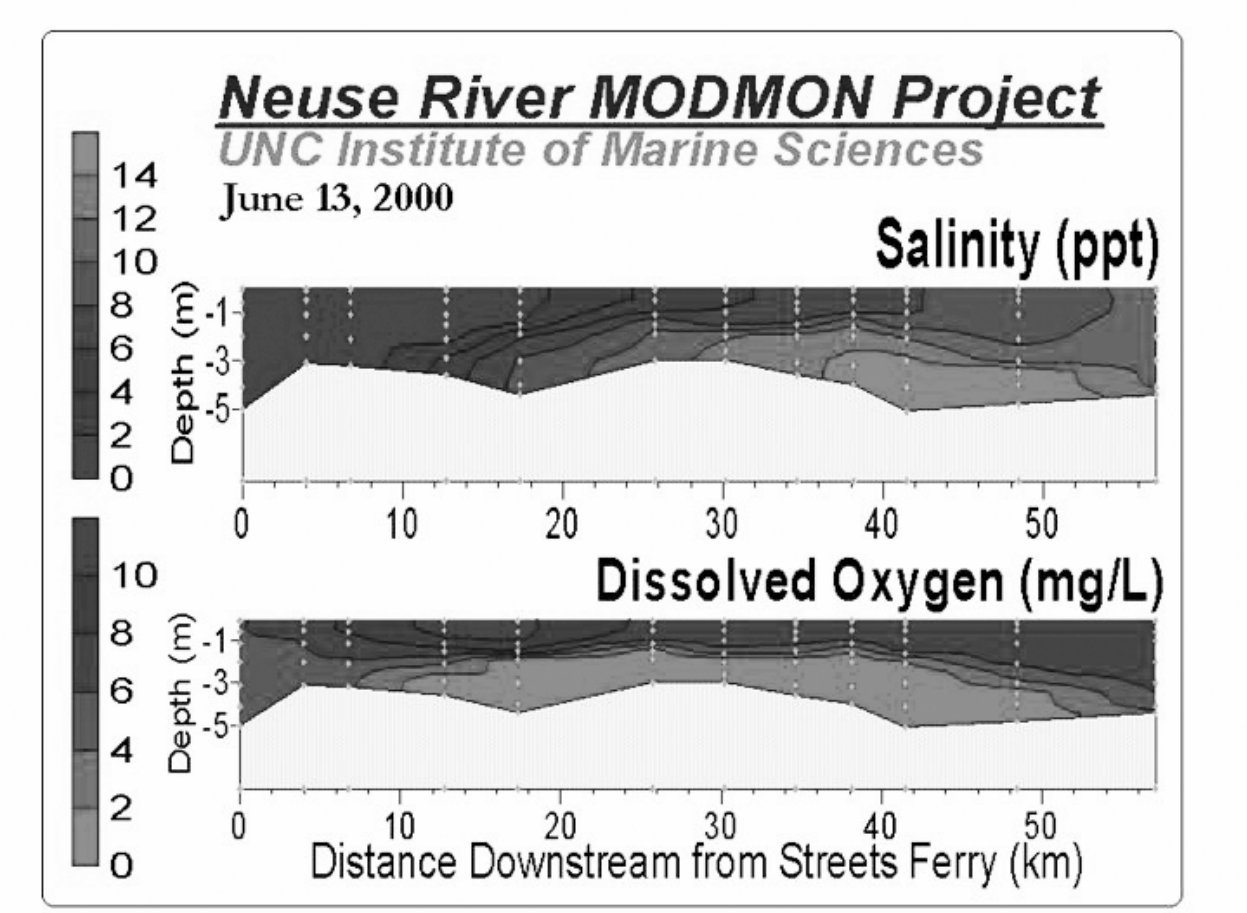

Figure 7. Vertical section of the eutrophic Neuse River Estuary, NC, ranging from the freshwater head of the estuary (left hand side) to the mesohaline entrance to Pamlico Sound. Data plotted were obtained from the biweekly Neuse River Modeling and Monitoring Program, ModMon database (www.marine.unc.edu/neuse/modmon). The upper frame illustrates the strong, vertical salinity stratification that results from light, freshwater inflow floating over denser, salt water entering from Pamlico Sound. Stratification persists during the summer months. The lower frame shows hypoxia that characterizes the bottom water as a result of the persistent vertical stratification.

Numerous estuarine studies throughout the world clearly demonstrate that interactions between the sediment and the water column play an important role in regulating phytoplankton production and the extent of bottom water hypoxia/anoxia (*e.g.*, Billen 1978, Matson *et al.* 1983, Nixon and Pilson 1983, Kemp and Boynton 1992, Jørgensen 1996). Estuarine sediments are rich in organic matter (typically 3-10 % organic carbon) and represent vast storage reservoirs for nutrients and oxygen demand. For example, the upper 10 cm of sediment in the NRE contains 500 times more N than the entire water column (Alperin *et al.* 2000). Likewise, the benthic oxygen flux in the Neuse (20-40 mmol m^{-2} d^{-1}) is capable of depleting the bottom water of oxygen in just 10 days (Alperin *et al.* 2002).

Sediment biogeochemical processes are driven by the flux of organic detritus from the water column. The quantity of organic matter deposited at the sediment surface depends on productivity in the overlying water (internal loading), the flux of organic matter from the watershed (external loading), and the efficiency with which the organic matter is exported from the estuary by either physical processes or consumption by grazers. Since, on an annual basis, external organic matter constitutes less than a third of the total load to the NRE (Paerl *et al.* 1998), productivity and export are the major controls on the flux of organic matter reaching the sediment surface. While productivity is largely controlled by the supply of fixed N, the export of organic matter from the estuary depends on phytoplankton community structure. Taxonomic groups that are poorly grazed (*e.g.*, cyanobacteria, dinoflagellates) or that flocculate and settle as their blooms "crash" have a greater tendency to be retained by the sediment.

Pinckney *et al.* (1998) have shown that phytoplankton bloom dynamics and community structure in the NRE are variable in time and space. Typically, total productivity is highest in the spring, coincident with a peak in N loading associated with maximal river discharge. The spring bloom is dominated by dinoflagellates, which grazers find less palatable than diatoms and chlorophytes, allowing a greater portion of the production to escape export and settle to the sediment. This sedimentation process is an important mechanism for retaining C and N fixed during the spring since high flow during this period would tend to flush the suspended organic matter from the estuary in < 1.5 months (Christian *et al.* 1991). A secondary bloom -- composed of diatoms, cyanobacteria, cryptomonads, and chlorophytes -- often occurs during the summer. This bloom coincides with the period of minimal external N loading (Paerl *et al.* 1998), implying that recycled N released from the sediment plays a significant role in fueling this productivity. The oxygen demand associated with organic matter from the summertime bloom combined with warm temperatures (up to 30^{o} C in the summer), low flow rates, fewer mixing events, and intense water column stratification, result in pervasive hypoxia and anoxia throughout the upper estuary.

During summer, long residence times, low flushing rates, persistent vertical stratification, and elevated temperatures exacerbate hypoxic conditions that can persist for weeks and cover large areas (Luettich *et al.* 2000). Non-motile (sessile) fauna are unable to escape these conditions and, therefore, large areas of the benthos are subjected to periodic defaunation events (Lenihan and Peterson 1998). In addition, these conditions overlap with fish nursery/refuge habitats (Luettich *et al.* 2000). The ecosystem-level trophic implications are that a large component of the estuarine food web can be negatively impacted, or even removed, altering both the structure and function of the system until benthic and nektonic communities are reestablished. Hypoxia and anoxia also influence biogeochemical cycling processes in affected habitats. Benthic nutrient release, especially of NH_4^+ and PO_4^{-3}, is enhanced at low DO concentrations (Rizzo and Christian 1996). These nutrients, which are critical for supporting phytoplankton production and standing stock, are present in high concentrations during and following anoxic events. Periodic pulse nutrient loading from storm events throughout the watershed, as well as point and non-point source discharges near the estuary, help sustain phytoplankton blooms and perpetuate hypoxic conditions (Figure 8). As the frequency, duration, and aerial coverage of these perturbations increase, both the structure and function of the estuarine ecosystem can experience long-term change.

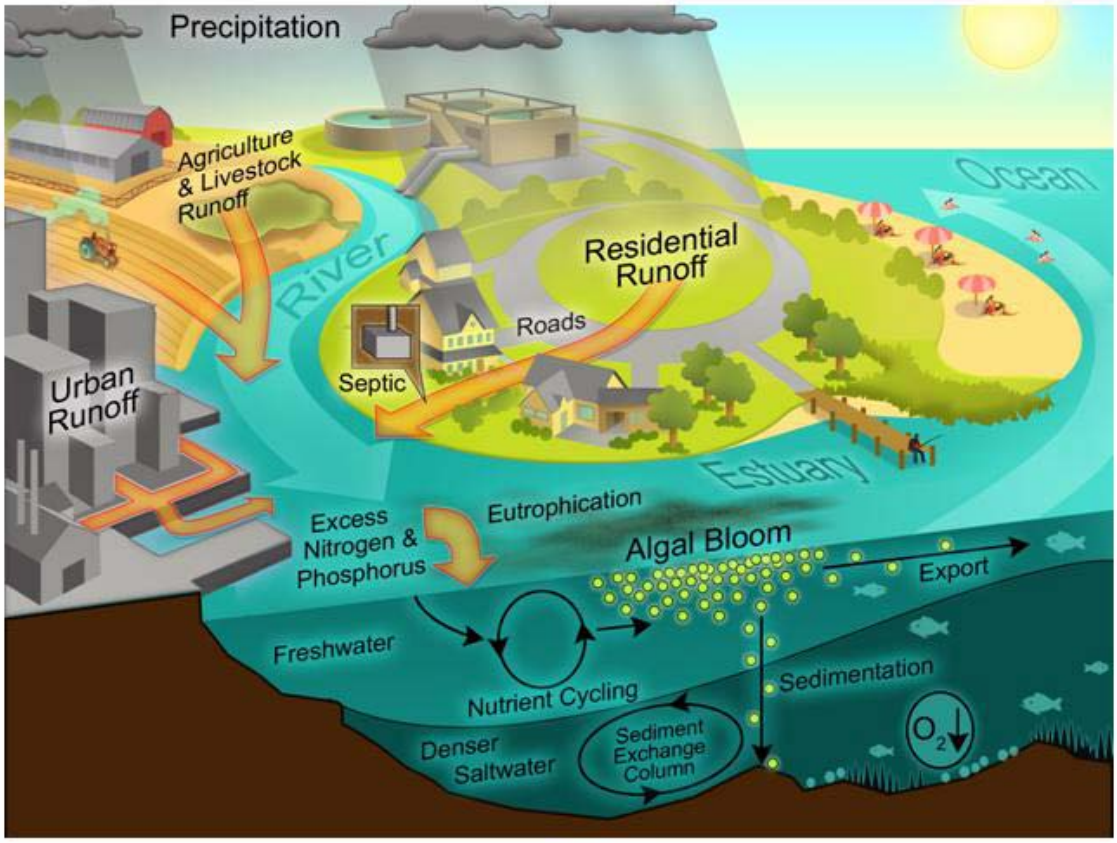

Figure 8. Conceptual illustration showing the linkage between specific nutrient inputs, freshwater discharge, eutrophication, algal bloom formation, and hypoxia dynamics in a stratified estuarine ecosystem.

MANAGING HYPOXIA IN THE NEUSE AND OTHER ESTUARIES: WHAT ARE THE OPTIONS AND APPROACHES?

Bottom-water hypoxia in the NRE and other estuaries result from the interaction of several non-biological variables, including freshwater discharge, vertical stratification, mixing associated with atmospheric forcing, and mixing associated with the tides, with sufficient oxidizable organic matter to lead to net O_2 consumption (Paerl *et al*. 1998, Buzzelli *et al.* 2002). There are key physical prerequisites for hypoxia. These include vertical salinity and/or temperature stratification, long residence time (*i.e.* restricted flushing) and a lack of or minimal wind or tidal mixing. As a rule, estuaries with stable water columns that exhibit periods of vertical stratification have a natural propensity for hypoxia to develop. Conversely, well-mixed, non-stratified estuarine or coastal waters rarely exhibit water column hypoxia. From a management perspective, these physical forcing features are seldom controllable, the exception

being small systems such as aquaculture ponds that can lend themselves to artificial destratification. The introduction of organic matter, the "fuel" of hypoxia, is, therefore, the key manipulative factor. Organic matter (OM) arises from two main sources, externally-supplied OM derived from the watershed, and internally-supplied OM, derived from primary production within the system. A vast amount of watershed-based OM input is attributable to natural processes, including leaching of decomposing plant and animal OM materials from soils, forests, wetlands and swamps. Leaching is often enhanced in agricultural soils due to tilling and the application of organic fertilizers (*i.e.*, animal wastes, manures). Therefore, land management practices that minimize OM losses from these sources can help reduce OM losses to downstream estuarine and coastal waters.

As mentioned previously, estuarine and coastal OM inputs are dominated by *in situ* primary production, originating from phytoplankton and macroalgae or rooted higher aquatic plants (Nixon 1986, Valiela 1995, Paerl *et al*. 1998, Hobbie 2000). Therefore, controlling the rate of primary production is a direct approach to minimizing OM loading, and potentially reducing hypoxia. Because estuarine and coastal primary production is, at least, in part controlled by nutrient (*i.e.*, N and P) supply, nutrient input constraints are the prescribed approaches for minimizing the hypoxia potential of sensitive waters (Nixon 1995, Paerl *et al.* 1998, Boynton and Kemp 2000). As to what, how much, when and where to reduce nutrient inputs are site-specific questions that require answers intimately tied to the interactive effects of physical, chemical and biological forcing features. Demonstrating a linkage between phytoplankton and O_2 dynamics within the framework of annual variability requires a process-based understanding of the combined effect of individual factors. Synoptic measurements of the major variables at relevant time scales are a critical first step in developing data useful for formulating, validating, and testing realistic conceptual/mathematical models describing environmental controls of the primary production process on an ecosystem scale.

The linkages between nutrients, phytoplankton and hypoxia/anoxia are usually viewed in a simple conceptual manner that translates biomass into carbon deposition and decomposition (c.f. Figure 1). However, this approach needs to incorporate the complex interactions of changing nutrient sources and chemical forms (*e.g.*, nitrate vs. ammonium vs. organic N) associated with human and climatic perturbations in coastal watersheds that impact phytoplankton community structural and functional responses. We now know that these responses impact trophic transfer and C deposition rates, both of which affect hypoxia potentials (Figure 8). Amounts and forms of watershed-discharged nutrients should be the primary targets of management strategies aimed at reducing estuarine hypoxia potentials. In most instances, the focus should be on N controls, since productivity of the receiving waters is largely N limited (Nixon 1995). However, N and P co-limitation occurs in some estuaries, in which case both nutrients should be included in source/input reduction strategies (Jonge 1990, Elmgren and Larsson 2001). Modeling efforts should be focused on determining naturally-occurring "background" hypoxia potentials, *i.e.*, the tendency of a system to develop hypoxia independent of man-made nutrient inputs. Models able to predict the difference in hypoxia potentials between "natural" and anthropogenically-enriched nutrient conditions will help management devise practical approaches to control estuarine hypoxia using quantifiable, ecologically sound and cost-effective nutrient input reduction strategies. Modeling efforts under way in the NRE to

distinguish the relative importance and roles of physical and chemical drivers of hypoxia (Borsuk *et al.* 2001, Buzzelli *et al.* 2002) may prove useful for other, hydrologically and biogeochemically-variable estuaries.

In any case, it should be recognized and kept in perspective that hypoxia is a natural phenomenon that frequently takes place in strongly stratified, productive waters. Therefore, it is unlikely that management efforts will entirely eliminate the tendency of these waters to periodically exhibit localized oxygen depletion. However, there is evidence that excessive organic matter loading, most commonly as nutrient-enhanced eutrophication, can increase hypoxia potentials even in these waters (c.f., Rabalais and Turner 2001). As such, steps taken to reduce the unwanted symptoms of eutrophication will also help mitigate, but not necessarily eliminate, hypoxia potentials. Management strategies and steps will most likely need to be site and ecosystem specific. Formulating effective nutrient management strategies for specific ecosystems and regions is a rational, scientifically-sound approach to minimizing negative ecological and economic impacts of excessive hypoxia.

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REFERENCES

Alperin, M., E. Clesceri, J. Wells, D. Albert, J. McNinch, and C. Martens. 2000. Sedimentary processes and benthic-pelagic coupling. *In:* Neuse River Modeling and Monitoring Project Stage 1: Hydrography and Circulation, Water Column Nutrients and Productivity, Sedimentary Processes and Benthic-Pelagic Coupling, and Benthic Ecology. UNC-Water Resources Research Institute. Raleigh, NC.

Antia N., P. Harrison, and L. Oliveira. 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia 30:1-89.

Billen, G. and C. Lancelot. 1988. Modelling benthic nitrogen cycling in temperate coastal ecosystems. *In:* Nitrogen Cycling in Coastal Marine Environments. T.H. Blackburn and J. Sorensen, (eds.). Wiley. pp. 341-378.

Borsuk, M.E., C.A. Stow, R.A. Luettich, H.W. Paerl, and J.L. Pinckney. 2001. Modeling oxygen dynamics in an intermittently stratified estuary. Estimation of process rates using field data. Estuarian Coastal Shelf Science 52:33-49.

Boesch, D.F., E. Burreson, W. Dennison, E. Houde, M. Kemp, V. Kennedy, R. Newell, K. Paynter, R. Orth, and R. Ulanowicz. 2001. Factors in the decline of coastal ecosystems. Science 293:629-638.

Boyer, J.N., D.W. Stanley, and R.R. Christian. 1994. Dynamics of NH_4^+ and NO_3^- uptake in the water column of the Neuse River estuary, North Carolina. Estuaries 17:361-371.

Boynton, W.R. and W.M. Kemp. 2000. Influences of river flow and nutrient loads on selected ecosystem processes. A synthesis of Chesapeake Bay data. *In:* Estuarine Science: A Synthesis Approach to Research and Practice, J.E. Hobbie (ed.). Island Press, Washington, DC. pp. 269-298

Buzzelli, C.P., S P. Powers, R.A. Luettich Jr., J.E McNinch, C.H. Peterson, J.L. Pinckney, and H.W. Paerl. 2002. Estimating the spatial extent of bottom water hypoxia and benthic fishery habitat degradation in the Neuse River Estuary, NC. Marine Ecology Progress Series 230:103-112.

Christian, R.R., W. Bryant, and D.W. Stanley. 1986. The relationship between river flow and *Microcystis aeruginosa* blooms. *In:* The Neuse River, NC. UNC Water Resources Research Institute Report No. 223. Raleigh, NC.

Christian R.R., J.N. Boyer, and D.W. Stanley. 1991. Multi-year distribution patterns of nutrients within the Neuse River Estuary. Marine Ecology Progress Series 71:259-274.

Clesceri, E.J., M.H. Cotte-Krief, M.J. Alperin, C.S. Martens, and H.W. Paerl. 2003. Stable Isotope Evidence for Changes in the Source and Depositional Patterns of Particulate Organic Matter in the Neuse River Estuary, North Carolina (USA): Impact of Three Consecutive Hurricanes. Estuaries (in press).

Cloern, J.E. 2001. Our evolving conceptual model of the coastal eutrophication problem. Marine Ecology Progress Series 210:23-253.

Collos Y. 1989. A linear model of external interactions during uptake of different forms of inorganic nitrogen by microalgae. Journal of Plankton Research 11:521-533.

D'Elia, C.F. 1987. Nutrient enrichment of the Chesapeake Bay: too much of a good thing. Environment 29:2.

D'Elia C.F., J.G. Sanders, and W.R Boynton. 1986. Nutrient enrichment studies in a coastal plain estuary: phytoplankton growth in large scale, continuous cultures. Canadian Journal of Fisheries and Aquatic Sciences 43:397-406.

Diaz, R.J. and R. Rosenberg. 1995. Marine benthic hypoxia: A review of its ecological effects and the behavioural responses of benthic macrofauna. Oceanography and Marine Biology: an Annual Review 33:245-303.

Dodd R.C., P.A. Cunningham, R.J. Curry, and S.J. Stichter. 1993. Watershed planning in the Albemarle-Pamlico Estuarine System, Report No. 93-01, Research Triangle Institute, Research Triangle Park, N.C. Department of Environment, Health & Natural Resources.

Dugdale, R. and J. Goering. 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. Limnology and Oceanography 12:196-206.

Elmgren, R. and U. Larsson. 2001. Eutrophication of the Baltic Sea area. Integrated coastal management issues. *In:* Science and Integrated Coastal Management, B. von Bodungen and R.K. Turner (eds.). Dahlem Press, Berlin. pp. 15-35.

Goldenberg, S.B., C.W. Landsea, A.M. Mestas-Nuñez, and W.M. Gray. 2001. The recent increase in Atlantic hurricane activity: Causes and implications. Science 293: 747-718.

Harrison, P and D. Turpin. 1982. The manipulation of physical, chemical, and biological factors to select species from natural phytoplankton populations. *In:* Marine Mesocosms: Biological and Chemical Research in Experimental Ecosystems, G Grice & M. Reeve (eds.). Springer-Verlag, New York. pp. 275-287.

Harrison, W., T. Platt, and M. Lewis. 1987. F-ratio and its relationship to ambient nitrate concentration in coastal waters. Journal of Plankton Research 9:235-245.

Hobbie, J.E. (ed.). 2000. Estuarine Science: A Synthetic Approach to Research and Practice. Island Press, Washington D.C.

Howarth, R.W., G. Billen, D. Swaney, A. Townsend, N. Jaworski, K. Lajtha, J.A. Downing, R. Elmgren, N. Caraco, T. Jordan, F. Berendse, J. Freney, V. Kudeyarov, P. Murdoch, and Z. Zhao-Liang. 1996. Regional nitrogen budgets and riverine N and P fluxes for the drainages to the North Atlantic Ocean: Natural and human influences. Biogeochemistry 35:75-79.

Jaworski, N., R. Howarth, and L. Hetling. 1997. Atmospheric deposition of nitrogen oxides onto the landscape contributes to coastal eutrophication in the Northeast United States. Environmental Science and Technology 31:1995-2004.

Jonge, V.N. de. 1990. Response of the Dutch Wadden Sea ecosystem to phosphorus discharges from the Rhine River. Hydrobiologia 195:49-62.

Jørgensen, B.B. 1996. Material flux in the sediment. *In:* Eutrophication in Coastal Marine Ecosystems, B.B. Jørgensen and K. Richardson, (eds.). American Geophysical Union. pp. 115-135.

Jørgensen, B.B and K. Richardson (eds.). 1996. Eutrophication of Coastal Marine Ecosystems, Coastal and Estuarine Studies 52. American Geophysical Union, Washington, DC. pp. 267.

Justic, D. 1991. Hypoxic conditions in the northern Adriatic Sea: historical development and ecological significance. *In:* Modern and Ancient Continental Shelf Anoxia, R.V. Tyson and T.H. Pearson (eds.). Geological Society Special Publication No. 58:95-105. The Geological Society, London. pp. 470.

Kemp, W.M. and W.R. Boynton. 1992. Benthic-pelagic interactions: nutrient and oxygen dynamics. *In:* Oxygen Dynamics in the Chesapeake Bay, D. E. Smith, M. Leffler, and G. Mackiernan, (eds.). Maryland Sea Grant. pp. 149-221.

Lenihan, H and C.H. Peterson. 1998. How habitat degradation through fishery disturbance enhances impacts of hypoxia on oyster reefs. Ecological Applications 8:128-140.

Luettich, R.A., J.E., McNinch, H.W. Paerl, C.H. Peterson, J.T. Wells, M. Alperin, C.S. Martens and, J.L. Pinckney. 2000. Neuse River Modeling and Monitoring Study (ModMon); Phase 1, 1997-1999. Water Resources Research Institute Report 325B, UNC Water Resources Research Institute. Raleigh, NC. pp. 172.

Matson E.A., M.M. Brinson, D.D. Cahoon, and G.J. Davis. 1983. Biogeochemistry of the sediments of the Pamlico and Neuse River Estuaries, NC. Water Resources Research Institute Report No. 191. UNC Water Resources Research Institute. Raleigh, NC.

National Research Council (NRC). 2000. Clean Coastal Waters: Understanding and Reducing the Effects of Nutrient Pollution. National Academy Press. Washington, DC.

Neilsen, A. and R. Lewin. 1974. The uptake and utilization of organic carbon by algae: An essay in comparative biochemistry. Phycologia 13:246-264.

Neilson, B. and L. Cronin (eds.). 1981. Estuaries and nutrients. Humana Press. Clifton, NY.

Nixon, S.W. 1986. Nutrient dynamics and the productivity of marine coastal waters. *In:* Coastal Eutrophication, Halwagy, R.D., Clayton, B. and M. Behbehani (eds.). The Alden Press. Oxford. pp.97-115.

Nixon, S.W. 1995. Coastal marine eutrophication: A definition, social causes, and future concerns. Ophelia 41:199-220.

Nixon, S.W. and M.E.Q. Pilson. 1983. Nitrogen in estuarine and coastal marine ecosystems. *In:* Nitrogen in the Marine Environment, E. J. Carpenter and D. G. Capone, (eds.). Academic Press. pp. 565-648.

North Carolina Department of Environment and Natural Resources (DENR). 2002. Neuse River Water Quality Program, Division of Water Quality, Raleigh, NC.

Officer, C.B., R.B. Biggs, J.L. Taft, L.E. Cronin, M.A. Tyler, and W.R. Boynton. 1984. Chesapeake Bay anoxia, origin, development and significance. Science 223:22-27.

Paerl, H.W. 1987. Dynamics of blue-green algal (*Microcystis aeruginosa*) blooms in the lower Neuse River, NC: causative factors and potential controls. UNC Water Resources Research Institute. Raleigh, NC. Report 229:164.

Paerl, H.W. 1988. Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. Limnology and Oceanography 33:823-847.

Paerl, H.W. 1997. Coastal eutrophication and harmful algal blooms: Importance of atmospheric deposition and groundwater as "new" nitrogen and other nutrient sources. Limnology and Oceanography 42:1154-1165.

Paerl, H.W., J. Rudek, and M.A. Mallin. 1990. Stimulation of phytoplankton production in coastal waters by natural rainfall inputs: nutritional and trophic implications. Marine Biology 107:247-254.

Paerl, H.W., M.A. Mallin, C. Donahue, M. Go, and B.L. Peierls. 1995. Nitrogen loading sources and eutrophication of the Neuse River Estuary, NC: Direct and indirect roles of atmospheric deposition. UNC Water Resources Research Institute Report 291. Raleigh, NC.

Paerl, H.W., J.L. Pinckney, J.M. Fear, and B.L. Peierls. 1998. Ecosystem responses to internal and watershed organic matter loading: Consequences for hypoxia in the eutrophying Neuse River Estuary, NC, USA. Marine Ecology Progress Series 166:17-25.

Paerl, H.W., J.D. Bales, L.W. Ausley, C.P. Buzzelli, L.B. Crowder, L.A. Eby, J.M. Fear, M. Go, B.L. Peierls, T.L. Richardson, and J.S. Ramus. 2001. Ecosystem impacts of 3 sequential hurricanes (Dennis, Floyd and Irene) on the US's largest lagoonal estuary, Pamlico Sound, NC. Proceedings of the National Academy of Science of the United States of America 98(10):5655-5660.

Paerl, H.W., R.L. Dennis, and D.R. Whitall. 2002. Atmospheric deposition of nitrogen: Implications for nutrient over-enrichment of coastal waters. Estuaries 25:677-693.

Paerl, H.W., J. Dyble, L. Twomey, J. L. Pinckney, J. Nelson, and L. Kerkhof. 2002. Characterizing man-made and natural modifications of microbial diversity and activity in coastal ecosystems: Antonie van Leeuwenhoek 81:487-507.

Peierls, B.L., N.F. Caraco, M.L. Pace, and J.J. Cole. 1991. Human influence on river nitrogen. Nature 350:386-387.

Peierls, B.L. and H.W. Paerl. 1997. The bioavailability of atmospheric organic nitrogen deposition to coastal phytoplankton. Limnology and Oceanography 42:1819-1823.

Pihl, L., S.P. Baden, and R.J. Diaz. 1991. Effects of periodic hypoxia on distributions of demersal fish and crustaceans. Marine Biology 108:349-360.

Pinckney, J.L., H.W. Paerl, M.B. Harrington, and K.E. Howe. 1998. Annual cycles of phytoplankton community structure and bloom dynamics in the Neuse River Estuary, NC. Marine Biology 131:371-382.

Pinckney, J.L., H.W. Paerl, P.A. Tester, and T.L. Richardson. 2001. The role of nutrient loading and eutrophication in estuarine ecology. Environmental Health Perspective 109 (Supplements 5): 699-706.

Rabalais, N.N. and R.E. Turner (eds.). 2001. Coastal Hypoxia: Consequences for Living Resources and Ecosystems. Coastal and Estuarine Studies 58. American Geophysical Union. Washington, DC. pp. 454.

Rabalais, N.N., R.E. Turner, D. Justic, Q. Dortch, and W.J. Wiseman, Jr. 2001. Gulf of Mexico Hypoxia Assessment, Topic #1, Characterization of Hypoxia. Report to White House Office of Science and Technology Policy, Committee on Environment and Natural Resources, Hypoxia Work Group, Mississippi River/Gulf of Mexico Watershed Nutrient Task Force, pp. 219.

Renaud, M.L. 1986. Hypoxia in Louisiana coastal waters during 1983. Implications for the fisheries. Fishing Bulletin 84:19-26.

Richardson, K. 1997. Harmful or exceptional phytoplankton blooms in the marine ecosystem. Advances in Marine Biology 31:302-385.

Rizzo, W, G. Lackey and R.R. Christian. 1992. Significance of euphotic, subtidal sediments to oxygen and nutrient cycling in a temperate estuary. Marine Ecology Progress Series 86:51-61.

Rosenberg, R. 1980. Effect of oxygen deficiency on benthic macrofauna in fjords. *In:* Fjord Oceanography, H.L. Freeland, D.M. Farmer and C.D. Levings (eds.). Plenum, New York. pp 499-514.

Rosenberg, R. 1990. Negative oxygen trends in Swedish coastal bottom waters. Marine Pollution Bulletin 21:335-339.

Rudek J, H.W. Paerl , M.A. Mallin, and P.W. Bates. 1991. Seasonal and hydrological control of phytoplankton nutrient limitation in the lower Neuse River Estuary, North Carolina. Marine Ecology Progress Series 75:133-142.

Ryther J.H. and W. Dunstan. 1971. Nitrogen, phosphorus, and eutrophication in the coastal marine environment. Science 171:1008-1112.

Seitzinger, S.P. and A.E. Giblin. 1996. Estimating denitrification in North Atlantic continental shelf sediments. Biogeochemistry 35:235-259.

Seitzinger, S.P and J. Sanders. 1997. Contribution of dissolved organic nitrogen from rivers to estuarine eutrophication. Marine Ecology Progress Series 159:1-12.

Smetacek, V., U. Bathmann, E.-M. Nöthig, and R. Scharek. 1991. Coastal eutrophication: Causes and consequences. *In:* Ocean Margin Processes in Global Change, R. C. F. Mantoura, J.-M. Martin and R. Wollast (eds.). John Wiley & Sons. Chichester. pp. 251-279.

Stanley, D.W. 1988. Historical trends in nutrient loading to the Neuse River Estuary, NC. *In:* Proceedings of the American Water Resources Association Symposium on Coastal Water Resources, W. Lyke and T. Hoban (eds.). AWRA Technical Publication Series TPS-88-1. AWRA. Bethesda, MD. pp 155-164.

Stanley, D.W. and S.W. Nixon. 1992. Stratification and bottom water hypoxia in the Pamlico River Estuary. Estuaries 15:270-281.

Stanley, D. W., M.L. Lebo, H.W. Paerl, M. Borsuk, and C.A. Stow. 1999. Historical trends in nitrogen production and loading in the Neuse River Basin. Abstract, Proceedings Annual Meeting, March 1999. UNC Water Resources Research Institute. Raleigh, NC.

Stolte W., T. McCollin, A. Noodeloos, and R. Riegman. 1994. Effect of nitrogen source on the size distribution within marine phytoplankton populations. Journal of Experimental Marine Biology and Ecology 184:83-97.

Strickland J.H. and T.R. Parsons. 1971. A practical handbook of seawater analysis. Fish Research Board, Canadian Bulletin 167.

Syrett, P. 1981. Nitrogen metabolism in microalgae. *In:* Physiological Bases of Phytoplankton Ecology, T. Platt (ed.). Canadian Bulletin of Fisheries and Aquatic Science 210:182-210.

Tedder, S.W., J. Sauber, J. Ausley, and S. Mitchell. 1980. Working Paper: Neuse River Investigation 1979. Division of Environmental Management, N.C. Department of Natural Resources, Community Development. Raleigh, NC.

Turner, R.E., W.W. Schroeder, and W.J. Wiseman. 1987. The role of stratification in the deoxygenation of Mobile Bay and adjacent shelf bottom waters. Estuaries 10:13-19.

Turpin, D.H. and P.J. Harrison. 1979. Limiting nutrient patchiness and its role in phytoplankton ecology. Journal of Experimental Marine Biology and Ecology 39:151-166.

Valiela, I. 1995. Marine Ecological Processes, 2nd Ed. Springer, New York.

Van Dolah, P. and G. Anderson. 1991. Effects of Hurricane Hugo on salinity and dissolved oxygen conditions in the Charleston Harbor estuary. Journal of Coastal Research 8:83-94.

Van Heukelem, L., A. Lewitus, T. Kana, and N. Craft. 1994. Improved separations of phyto-plankton pigments using temperature-controlled, high performance liquid chromatography. Marine Ecology Progress Series 114:303-313.

Vitousek P.M., H.A. Mooney, J. Lubchenko, and J. M. Mellilo. 1997. Human domination of earth's ecosystems. Science 277:494-499.

Vollenweider, R. A., R. Marchetti, and R. Viviani (eds.). 1992. Marine Coastal Eutrophication, The Response of Marine Transitional Systems to Human Impacts: Problems and Perspectives for Restoration. Proceedings of the International Conference, Bologna, Italy, March, 1990. Elsevier. Amsterdam, The Netherlands.

Welsh, B.L. and F.C. Eller. 1991. Mechanisms controlling summertime oxygen depletion in western Long Island Sound. Estuaries 14:265-278.

Whitall, D.R. and H.W. Paerl. 2001. Spatiotemporal variability of wet atmospheric nitrogen deposition to the Neuse River Estuary, North Carolina. Journal of Environmental Quality 30: 1508-1515.

Yin, K., Z. Ke and S. Liu. 2003. Temporal and spatial distribution of dissolved oxygen in the Pearl River Estuary and adjacent coastal waters near Hong Kong. Continental Shelf Research (in press).

PERSPECTIVES FOR COASTAL MARINE HYPOXIA IN A WARMER WORLD

Dubravko Justić and R. Eugene Turner, [1] Nancy N. Rabalais[2]

ABSTRACT

A coupled biological-physical model was used to simulate monthly responses of the Gulf of Mexico hypoxia to variations in climate and anthropogenic nutrient loading. We examined six hypothetical future scenarios that are based on observed and projected changes in Mississippi River discharge, Mississippi River nitrate concentrations, and ambient water temperatures. In particular, we investigated the implications of a 30% decrease in the Mississippi River nitrogen flux, which was recently proposed by the Mississippi River Watershed/Gulf of Mexico Hypoxia Task Force as a measure to reduce the size of the hypoxic zone. Model simulations indicate that, if potential climatic variations are taken into account, a 30% decrease in the nitrogen flux of the Mississippi River may not be sufficient to accomplish the proposed hypoxia management goal. For instance, a 20% increase in the Mississippi River discharge, projected under a number of climate change scenarios, would offset a decrease in the frequency of hypoxia resulting from a 30% decrease in the anthropogenic nitrogen flux.

INTRODUCTION

A scientific consensus now exists that the buildup of greenhouse gases in the atmosphere is warming the earth (IPCC 2001). The last decade of the 20th century was the warmest on record, and paleo-records indicate that recent warming has no counterpart in the last 1000 years (Crowley 2000). The global Earth's temperatures increased by almost 1 ^{o}C during the last 150 years (Jones *et al.* 1999), and general circulation models (GCMs) have projected further temperature increases of 1-6 ^{o}C over the next 100 years (IPCC 2001). An increase in global temperatures of such a magnitude is expected to produce a general intensification of the hydrologic cycle that would be manifested in increased global precipitation, evapotranspiration and runoff (Miller and Russell 1992).

General circulation models (GCMs) are not consistent in their predictions of the effects of climate change on precipitation and temperature, two important drivers of freshwater inflow to estuaries. A GCM-based study that examined the potential impacts of global warming on the annual runoff of the world's 33 largest rivers (Miller and Russell 1992) suggested that runoff increases are likely for 25 of the 33 studied rivers. The model predicted higher runoffs for all rivers in high northern latitudes, with a maximum increase of 47%. At low latitudes, both increasing and decreasing runoffs were predicted, ranging from a 96% increase to a 43% decrease. In most cases, the increase in river runoff was coincidental with increased rainfall within the drainage basin (Miller and Russell 1992). Based on this GCM study, the average annual Mississippi River discharge would increase about 20% if the concentration of

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atmospheric CO_2 doubles. However, other studies have shown that runoff estimates for the Mississippi River Basin differed greatly between the Canadian GCM model and the Hadley GCM model (Wolock and McCabe 1999). Both models predict an increase in future extreme rainfall and runoff events, but they disagree in terms of both the magnitude and direction of changes in average annual runoff. The average annual runoff of the Mississippi River Basin, for example, was projected to decrease 30% by the Canadian model, but increase 40% by the Hadley model by the year 2099. Estimated changes of water input into major U.S. estuaries projected by the Hadley model by the year 2099 range from -40% to +100%. Similar calculations based on Canadian model projections suggest significantly reduced inflows for all coastal regions except the US Pacific coast (Wolock and McCabe 1999). Thus, it is likely many coastal and estuarine ecosystems will experience changes in freshwater inflow, although it is unclear in which direction these changes will occur. It is also likely that extreme precipitation events will become more common, as either droughts or floods (Easterling *et al.* 2000).

Climate change, if manifested by increased global temperatures and enhanced hydrologic cycles, may influence the development of hypoxia in two major ways. First, the magnitude and seasonal patterns of freshwater and nutrient inputs would be affected, which could have an immediate effect on nutrient-enhanced coastal water productivity and carbon flux to the bottom sediments. Also, the fundamental characteristics of the physical environment would likely change, thereby affecting the susceptibility of coastal ecosystems to hypoxia. Here, we examine the potential implications of future climate change for hypoxia in river-dominated coastal waters. We focus on the northern Gulf of Mexico (Figure 1), a coastal ecosystem dominated by the Mississippi River, where decadal and interannual variations in the size of a large hypoxic zone provide representative examples of anthropogenic and climatic controls on eutrophication.

GULF'S HYPOXIA

A zone of large-scale hypoxia (< 2 mg O_2 l^{-1}) in the northern Gulf of Mexico (Figure 1), recently exceeding 22,000 km^2 (Rabalais *et al.* 2002), overlaps the habitat and fishing grounds of commercially important fish and shrimp species. This phenomenon develops as a synergistic product of high water column stability and high surface primary productivity that manifests in a high carbon flux to the sediment (Rabalais *et al.* 1996). Hypoxia typically occurs from March through October in waters below the pycnocline, and extends between five and 60 km offshore (Rabalais and Turner 2001). Retrospective analyses (Turner and Rabalais 1994, Sen Gupta *et al.* 1996) and model simulations (Justić *et al.* 2002) suggest that the Gulf's hypoxia has intensified during the last five decades as a probable consequence of increased riverine nitrogen inputs (Turner and Rabalais 1991, Goolsby *et al.* 1999) and more balanced nutrient ratios in the fresh water (Turner and Rabalais 1991, Justić *et al.* 1995a). These trends in nutrient concentrations in the Mississippi River are well known. Additionally, the nitrogen input increases occurred coincidentally with the increased use of fertilizer in the watershed (Turner and Rabalais 1991, Howarth *et al.* 1996, Goolsby *et al.* 1999).

Despite the controlling influence of anthropogenic factors on hypoxia, the influence of climatic factors has also been significant. Although the size of the Gulf's hypoxic zone has varied greatly over the past 17 years, it has been significantly larger in wet years compared to dry years (Rabalais *et al.* 1996, Rabalais and Turner 2001). During the drought of 1988 (52 - year low discharge record of the Mississippi River), Gulf bottom oxygen concentrations were significantly higher than average and the formation of a continuous hypoxic zone along the coast did not occur in mid-summer (Figure 1). During the flood of 1993 (62 - year maximum discharge of the Mississippi River for August and September), however, the areal extent of hypoxia doubled with respect to the 1985-1992 average (Rabalais *et al.* 1998). Both the drought of 1988 and the flood of 1993 were caused by anomalous precipitation patterns associated, in part, with the El Nino/Southern Oscillation (ENSO) cycle (Trenberth and Guillemot 1996). During 1988, a particularly strong, cool ENSO phase (La Niña) in the tropical Pacific triggered a series of anomalous circulation events that are believed to be responsible for the drought. In contrast, the 1993 flood was partly the outcome of an extended warm ENSO phase (El Nino).

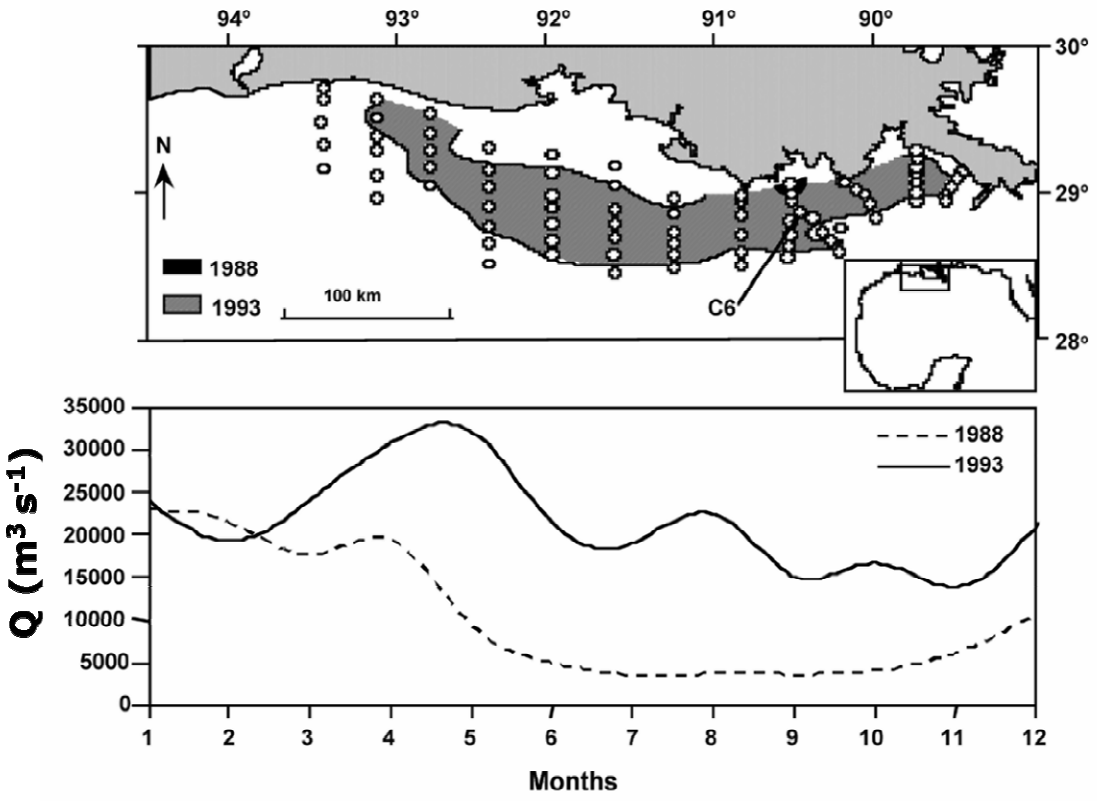

Figure 1. Map of the study area showing the hypoxia monitoring transects and location of reference station C6 (circles). Shaded areas represent the extent of hypoxic (< 2 mg O_2 l^{-1}) bottom waters during August 1988 and July 1993. Note that during August 1988, hypoxia was observed only at the inshore end of transect C. Seasonal patterns in the Mississippi River discharge (Q) during 1988 and 1993 are depicted in the lower panel.

Because the northern Gulf of Mexico is one of the most important areas for U.S. fisheries, hypoxia has received considerable scientific and public attention. In 2001, the Mississippi River Watershed/Gulf of Mexico Hypoxia Task Force set a goal to reduce the 5-year running average of the Gulf's hypoxic zone to less than 5000 km^2 by the year 2015 (Rabalais *et al.* 2002). The proposed action plan suggested that a 30% decrease in nitrogen load was needed to reach this goal, and that the plan's implementation should be based on voluntary, incentive-based strategies applied to the watershed (Mitsch *et al.* 2001). While the linkage between the anthropogenic nutrient inputs and hypoxia was addressed in the proposed action plan, the importance of climatic factors has not received full consideration.

MODEL DESCRIPTION

We used our previously published two-box model (Justić *et al.* 1996, 2002, Figure 2) that assumes uniform properties for the water column layers above and below the average depth of the pycnocline. The applicability of a two-box modeling scheme to the inner section of the Gulf's hypoxic zone is discussed in Justić *et al.* (1996), while a detailed model description is given in Justić *et al.* (2002). Model forcing functions include the relevant climatic and anthropogenic factors affecting the oxygen and carbon budgets of the coastal waters of the northern Gulf of Mexico, namely, the Mississippi River discharge, the Mississippi River nitrate flux and temperature. Although wind speed is not explicitly listed as a forcing function, it is included in the model as an important variable affecting the air-sea oxygen flux (*e.g.*, Equation 2). A brief description of the model theoretical formulations is given below.

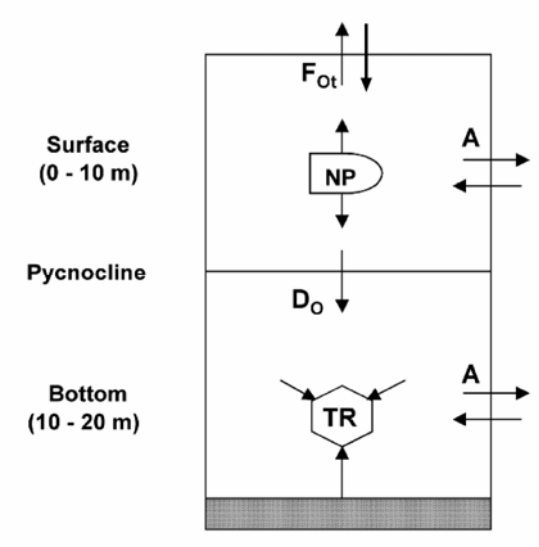

Figure 2. A conceptual model of oxygen cycling in the core of the Gulf's hypoxic zone (from Justić *et al.* 1996). The F_{Ot} denotes the total air-sea oxygen flux. NP is the net productivity. D_O is the diffusive oxygen flux through the pycnocline, A is the horizontal oxygen transport by advection and diffusion, and TR is the total oxygen uptake in the lower water column.

The oxygen concentration in the upper water column changes as a result of biological oxygen production and consumption, oxygen transport in the horizontal and vertical direction, and atmospheric exchanges. In the core of the hypoxic zone, horizontal oxygen transport due to advection and diffusion is small compared to vertical oxygen transport (Justić *et al.* 1996), and the oxygen balance for the upper water column (O_{ts}, g O_2 m^{-2}, 0-10 m) can be described as:

$$\partial O_{ts}/\partial t = - F_{Ot} - D_O + NP \qquad (1)$$

where t is time (days), F_{Ot} is the total air-sea oxygen flux (g O_2 m^{-2} day^{-1}), D_O is the diffusive oxygen flux through the pycnocline (g O_2 m^{-2} day^{-1}), and NP is the net primary productivity expressed in terms of oxygen equivalents (g O_2 m^{-2} day^{-1}).

Oxygen transport through the sea surface was computed using the formulation proposed by Stigebrandt (1991), which takes into account the effect of gas transfer due to bubbles:

$$F_{Ot} = V (O_s - 1.025\ O_2'). \qquad (2)$$

Here F_{Ot} is the total air-sea oxygen flux (g O_2 m^{-2} day^{-1}), V is transfer velocity (m day^{-1}), evaluated as a function of Schmidt number and wind speed (Liss and Merlivat 1986), O_s is the average surface zone oxygen concentration (g O_2 m^{-3}, 0-10 m), and O_2' is the oxygen saturation value at the water temperature (g O_2 m^{-3}). Negative F_{Ot} values indicate that the oxygen flux is directed towards the water column. The O_s value was computed by dividing the O_{ts} value (Equation 1) by the thickness of the upper water column (10 m). The oxygen saturation value was computed from the observed temperature data and estimated salinity values using the equation of Weiss (1970). Surface salinity values for station C6 (Figure 1) were calculated from the Mississippi River runoff data, using a time-delayed linear model (τ = 2 months; $r^2 = 0.8$; $p < 0.001$; Justić *et al.* 1996).

The vertical diffusive flux of oxygen (D_O) was estimated from the equation:

$$D_O = - K_z (\partial O_2/\partial z) \qquad (3)$$

where K_z is the vertical eddy diffusivity (m^2 s^{-1}), O_2 is ambient oxygen concentration (g O_2 m^{-3}), and z is depth (m). ∂O_2 is calculated as the difference in the average oxygen concentrations measured above and below the pycnocline (see "Data" below). The model assumes that the only properties of the stratified water column controlling K_z are the turbulent kinetic energy dissipation rate and the buoyancy frequency (= Brunt-Väisälä frequency). We assumed that the turbulent energy dissipation rate at the depth of 10 m is in the range of 10^{-7} m^2 s^{-3}, which is likely to be an upper estimate (Dillon and Caldwell 1980). Buoyancy frequency was computed from changes in vertical density gradients (Justić *et al.* 1996).

The net productivity of the upper water column (NP; g C m^{-2} day^{-1}) was computed from the time-delayed regression model ($r^2 = 0.73$; $p < 0.001$; Justić *et al.* 1996):

$$NP_t = - 0.34 + 3.93 \times 10^{-7} (N\text{-}NO_3)_{t-1} \qquad (4)$$

where $N\text{-}NO_3$ is the nitrate flux of the Mississippi River (10^6 kg day^{-1}), and subscripts *t* and *t-1* denote the current and preceding month, respectively. Conversion of carbon to oxygen equivalents, so that Equation 1 is dimensionally correct, was carried out using a ratio of 3.47 by weight (mol. C: mol. O_2 = 106: 138, Redfield *et al.* 1963).

We expressed net productivity of the upper water column as a function of riverine nitrate flux for the following reasons: first, nitrogen is often considered to be the limiting nutrient for the growth of estuarine and coastal phytoplankton (*e.g.*, D'Elia *et al.* 1986). Second, the combined discharges of the Mississippi and Atchafalaya Rivers account for 98% of the total nitrogen flux into the northern Gulf of Mexico (Dunn 1996), with nitrate being the predominant form of nitrogen (Goolsby *et al.* 1999). Also, an analysis of the 1985-1991 data subset from the northern Gulf of Mexico (Justić *et al.* 1995a, 1995b) suggested a high incidence of stoichiometric nitrogen limitation at a station within the core of the hypoxic zone.

Because of the high turbidity of continental shelf waters near the Mississippi River, biological oxygen production below the depth of 10 m is low (Lohrenz *et al.* 1990), and may be considered an insignificant term when compared to vertical oxygen transport. Thus, the balance equation for oxygen in the lower water column (O_{tb}, g O_2 m^{-2}, 10-20 m) includes only two terms: oxygen uptake due to benthic and water column respiration (R), and oxygen re-supply from the upper water column via turbulent diffusion (D_O):

$$\partial O_{tb}/\partial t = -R + D_O. \qquad (5)$$

The respiration rate (R; g O_2 m^{-2} day^{-1}) in the lower water column at any given time t can be expressed in terms of the net productivity rate $NP(t)$ at some earlier time t_0 (Officer *et al.* 1984, 1985), so that:

$$R(t) = k(t) \int_{-\infty}^{t} \alpha\, NP(t_0) \exp\left[- \int_{-t0}^{t} k(t_1)\, dt_1 \right] dt_0 \qquad (6)$$

where the proportionality constant α describes the fraction of *NP* that reaches the lower water column.

For the northern Gulf of Mexico, Justić *et al.* (1993) showed that there is a significant correlation ($r = 0.85$; $P < 0.01$) between the net productivity of the upper water column (0-10 m) and oxygen deficit in the lower water column (10-20 m) when there is a time-lag of one month. Rabalais *et al.* (1991) suggested that about 50% of surface primary production may be reaching the bottom (~ 20 m depth on average) in the northern Gulf of Mexico. Based on data for the period 1985-1992, Justić *et al.* (1997) estimated that the average respiration rate (R) of the lower water column (10-20 m) at station C6 accounted for 47% of the *NP* in the upper water column (0-10 m). Accordingly, a value of $\alpha = 0.47$ was used in this study. The respiration constant k was calculated as a function of bottom temperature and bottom oxygen concentration (Justić *et al.* 2002). The average bottom oxygen concentration (O_b; g O_2 m^{-3}, 10-20 m) was computed by dividing the O_{tb} value (Equation 5) by the thickness of the lower water column (10 m). Carbon uptake during respiration was converted to oxygen equivalents using a ratio of 3.47 by weight (mol. C : mol. O_2 = 106 : 138, RQ = 0.77; Redfield *et al.* 1963).

Equation 6 uses net productivity as a surrogate for excess carbon in the upper water column (0-10 m) that is available for export to the lower water column (10-20 m). Accordingly, the balance equation for organic carbon in sediments (C_s, g C m^{-2}) can be written as:

$$\partial C_s / \partial t = S_f(t) - R(t) - E_c \qquad (7)$$

where S_f is the instantaneous vertical carbon flux resulting from the sedimentation of organic material from the upper water column (g C m^{-2} day^{-1}), $R(t)$ is the respiration rate in the lower water column, expressed in terms of carbon equivalents (g C m^{-2} day^{-1}), and E_c (g C m^{-2} day^{-1}) is the loss of sedimentary carbon due to resuspension and export.

The continental shelf of the northern Gulf of Mexico is a highly dynamic system where wind-driven sediment resuspension can be a driving force in exporting sediments to the outer shelf and slope. Seasonal deposition rates can be locally high, but decadal sediment accumulation rates are significantly lower (Wiseman *et al.* 1999). In computing the organic carbon accumulation rates, we assumed that 50% of the sedimented organic carbon is not subsequently decomposed, and is ultimately exported from the study area.

DATA

The data series used in model calibration were collected between June 1985 and November 1993 at station C6 in the core of the Gulf of Mexico hypoxic zone (Figure 1). Biweekly to monthly data series included ambient temperature and salinity measurements, as well as dissolved oxygen concentrations measured throughout the water column at depth intervals of 1-2 m. Standard water column profile data were obtained from a Hydro lab Surveyor or a SeaBird CTD system with a SBE 13-01 (S/N 106) dissolved oxygen meter. The dissolved oxygen measurements were calibrated with Winkler titrations (Parsons *et al.* 1984) periodically carried-out during the hydrologic surveys.

Daily discharge data for the Mississippi River at Tarbert Landing for the period January 1955-May 2000 were obtained from the U.S. Army Corps of Engineers. The monitoring station at Tarbert Landing is 478 km upstream from the Mississippi River delta, and 13 km downstream from the inlet channel to the Old River control structure where one-third of the Mississippi River is diverted to join the Red River and then forms the Atchafalaya River. The discharge at Tarbert Landing accounts for about 70% of the total Mississippi River and Atchafalaya River discharges. Wind speed data for the coastal station at Grand Isle, Louisiana were obtained from the Louisiana Office of State Climatology. Grand Isle is within 65 km of our reference station C6. Monthly nitrate fluxes of the Mississippi River for the January 1955-May 2000 period (Figure 3) were computed using $N\text{-}NO_3$ concentrations measured at St. Francisville, approximately 430 km upstream from the Mississippi River Delta. Data sources and analytical methods used to determine nitrate concentrations are discussed in Turner and Rabalais (1991) and Goolsby *et al.* (1999).

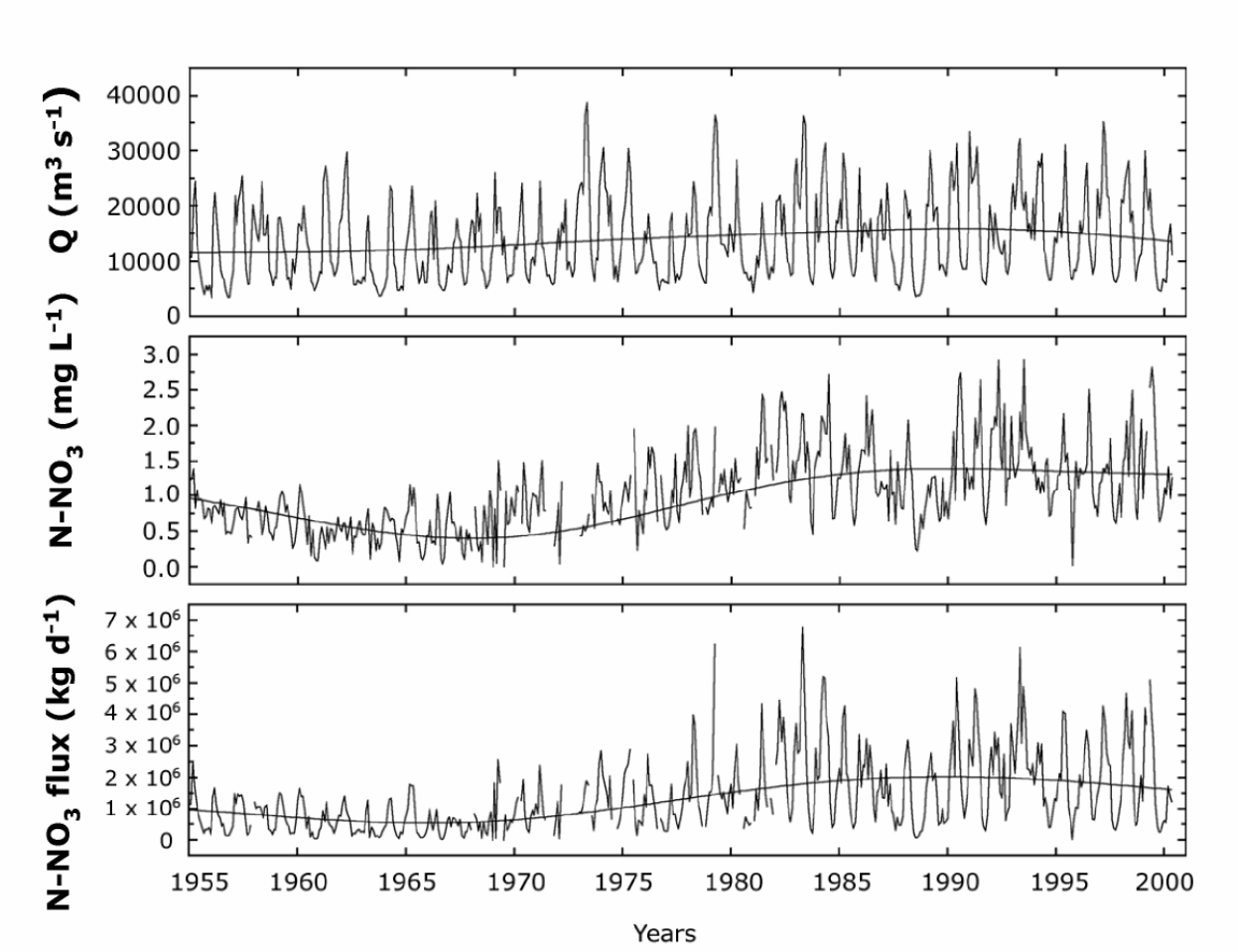

Figure 3. Monthly averages of Mississippi River discharge (Q), nitrate concentration (N-NO_3), and nitrate flux (N-NO_3 flux) for January 1955-May 2000. Smoothed curves were obtained by using a 96-point fast Fourier transform (FFT) filter (from Justić *et al.* 2002).

SIMULATED MODEL SCENARIOS

The forcing functions used in the nominal model simulations included monthly values of Mississippi River runoff (Q), nitrate concentration (N-NO_3) and nitrate flux (N-NO_3 flux) for the January 1955 - May 2000 period (Figure 3). Ambient water column temperatures and surface wind data were not available for the entire 1955-2000 period, and they were replicated from the observed 1985-1993 data. The nominal model was calibrated using the 1985-1993 time-series for station C6. The 1985-1993 period included three average hydrologic years (1985, 1986 and 1989), a record flood year (1993), two years with above average discharge (1990 and 1991), three years with below average discharge (1987, 1988, and 1992), and a record drought year (1988). Given the time-span of the data, as well as the range of observed hydrologic variability, we considered the 1985-1993 data set to be appropriate for model calibration. The parameter estimation process, calibration results, and sensitivity analysis are discussed in Justić *et al.* (2002).

The investigated model scenarios are described in Table 1. These scenarios are based on the available projections of GCMs for the continental U.S., the Mississippi River, and the northern Gulf of Mexico, as well as the proposed nutrient management goals.

Table 1. Investigated model scenarios. In scenarios 1-6, simulated changes denote deviations from the observed values used in the nominal model simulation.

Nominal model	Time series of observed monthly values of the Mississippi River discharge, nitrate concentration, and nitrate flux, for 1955-2000 (Figure 3); observed monthly averages of surface and bottom temperatures at station C6 for 1985-1993; observed monthly wind speed averages at Grand Isle for 1985-1993.
Scenario 1.	30% decrease in the average Mississippi River discharge (a conservative low estimate based on the Canadian model projections; Wolock and McCabe 1999).
Scenario 2.	Mississippi River nitrate concentration is constant and equal to the average for 1955-1967 (= 0.61 mg N l^{-1}).
Scenario 3.	20% increase in the average Mississippi River discharge (based on Miller and Russell 1992), and also supported by the Hadley model projections (Wolock and McCabe 1999).
Scenario 4.	4 ^{o}C increase in the average surface and bottom water temperatures of the northern Gulf of Mexico (based on Giorgi *et al.* 1994 and IPCC 2001).
Scenario 5.	20% increase in the average Mississippi River discharge, and 4 ^{o}C increase in the average surface and bottom water temperatures of the northern Gulf of Mexico (Scenarios 3 and 4 combined).
Scenario 6.	30% decrease in Mississippi River nitrate concentrations (management scenario proposed by the Mississippi River Watershed/Gulf of Mexico Hypoxia Task Force (Rabalais *et al.* 2002); because the Mississippi River discharge is unaffected in this scenario, a 30% decrease in nitrate concentration is equal to a 30% decrease in nitrate flux).

RESULTS

The results of the nominal model simulation indicate that the average oxygen concentrations of the lower water column (10-20 m) decreased from 6.6 mg l^{-1} in 1955-1965 to 4.2 mg l^{-1} in 1990-2000 (Figure 4). The model identified the mid 1970s as the start of recurring hypoxia in the lower water column, and predicted a total of 19 years with hypoxia between 1955 and 2000 (Figure 4). These results are in good agreement with the timing of the first reports

documented hypoxia in the northern Gulf of Mexico (Rabalais *et al.* 2002), and are additionally supported by monitoring studies (Rabalais and Turner 2001) and retrospective analyses of sedimentary records (Eadie *et al.* 1994, Turner and Rabalais 1994).

Model simulations suggest that hypoxia in the northern Gulf of Mexico would not have developed if the average nitrate concentration remained unchanged with respect to the period 1955-1967 (Figure 5; Table 2; Scenario 2). For a scenario with 20% increase in average Mississippi River discharge (Scenario 3), the model predicts a 37% increase in frequency of hypoxia, relative to the nominal model simulation. For a scenario with 4°C increase in average temperatures of the northern Gulf of Mexico and a 20% increase in average Mississippi River discharge (Scenario 5), the model predicts an increase in the frequency of hypoxia of 63%. In contrast, a 30% decrease in Mississippi River nitrate concentration (= 30% decrease in nitrate flux; Scenario 6) would result in a 37% decrease in the frequency of hypoxia (Figure 5; Table 2).

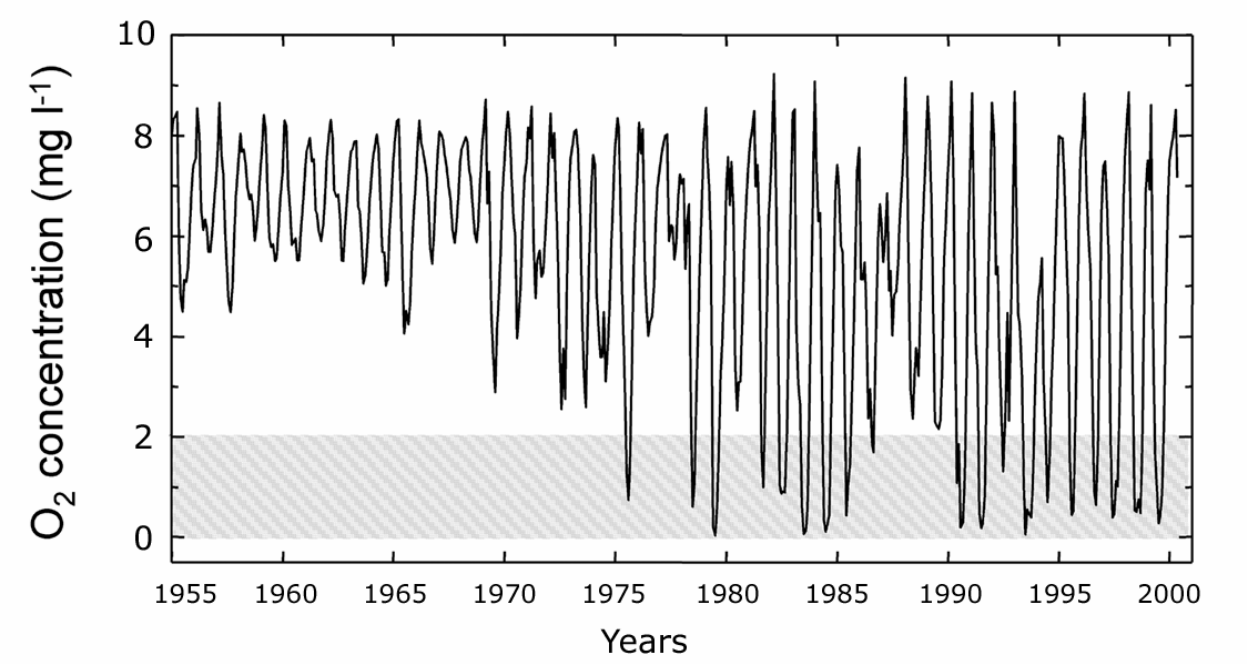

Figure 4. Simulated changes in the average bottom (10-20 m) oxygen concentration at station C6 for the period January 1955 – May 2000. Shaded area denotes hypoxic conditions (< 2 mg O_2 l^{-1}; from Justić *et al*. 2002).

DISCUSSION AND CONCLUSIONS

Assessment of future climate change scenarios for the northern Gulf of Mexico is complicated by the fact that runoff projections for the Mississippi River Basin are highly variable (Wolock and McCabe 1999). It is just as difficult to predict future trends in anthropogenic nutrient loading. While it is likely that global riverine nitrogen flux will continue to increase in response to the increased use of agricultural fertilizers (Tilman *et al.* 2001), future estimates for the Mississippi River Basin are not available. Also, nitrate concentrations in the Mississippi

River may increase in response to an increase in discharge (Goolsby *et al.* 1999, Justić *et al.* 2001), or decrease as a result of nutrient control efforts in the Mississippi River Basin (Mitsch *et al.* 2001, Rabalais *et al.* 2002).

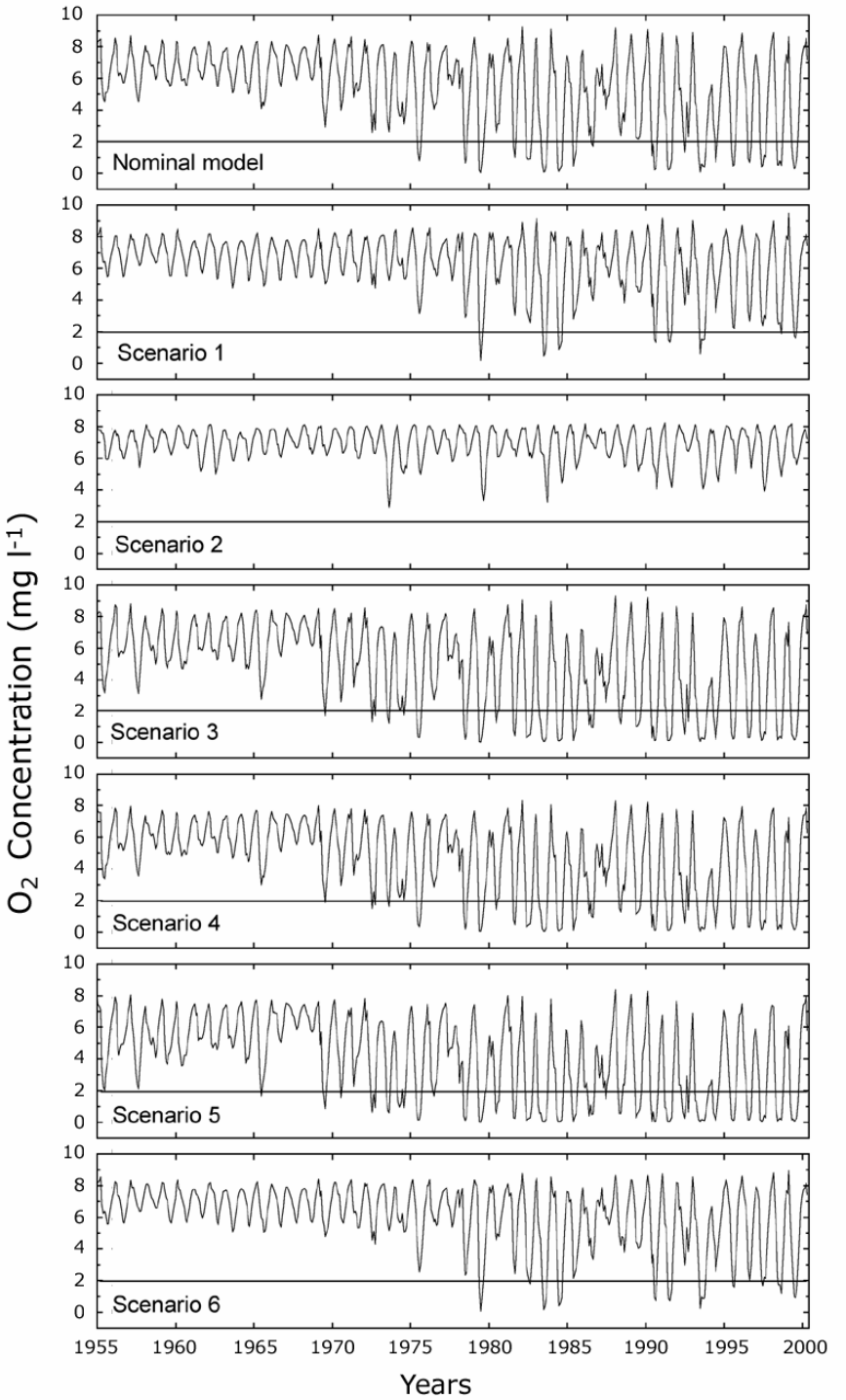

Figure 5. Simulated monthly changes in the average oxygen concentration of the lower water column (10-20 m), at a station within the core of the Gulf's hypoxic zone. A solid line at 2 mg O_2 l^{-1} denotes the upper limit of hypoxia. Model scenarios are explained in Table 1.

Table 2. Simulation results for selected model scenarios described in Table 1. The simulation interval was 45 years (1955-2000).

Model scenario	Number of years with hypoxia (< 2 mg O_2 l^{-1})	% change relative to the nominal model
Nominal model	19	-
Scenario 1 (-30% Q)	8	-58
Scenario 2 (1955-1967 N-NO_3)	0	∞
Scenario 3 (+20% Q)	26	+37
Scenario 4 (+4 ^oC)	25	+32
Scenario 5 (+20% Q, +4 ^oC)	31	+63
Scenario 6 (-30% N-NO_3)	12	-37

Our study strongly suggests that the Gulf's hypoxia is highly sensitive to variations in freshwater discharge, riverine nitrate flux, and ambient water temperature (Table 2). Variations in discharge affect the stability of the water column and vertical oxygen transport (Justić *et al.* 1996), and this effect can be enhanced due to variations in ambient temperature. Also, nitrate concentrations in the Mississippi River are typically an order of magnitude higher than those in the Gulf's coastal waters (Turner and Rabalais 1991, Justić *et al.* 1995a, 1995b), and riverine nitrate flux is generally well correlated with discharge (Goolsby *et al.* 1999). Thus, a change in discharge has the potential to eventually alter both the stability of the water column and nutrient-enhanced productivity, which are the two compulsory factors in the development of hypoxia.

It is difficult to predict changes in the areal extent of hypoxia based on model projections for a single station within the core of the hypoxic zone (C6; Figure 1). In this respect, it was not possible to determine whether a 30% decrease in nitrate flux would reduce the average areal extent of hypoxia below 5,000 km^2, as suggested by the Mississippi River Watershed/Gulf of Mexico Hypoxia Task Force (Rabalais *et al.* 2002). Nevertheless, model simulations indicated that, if potential climatic variations are taken into account, a 30% decrease in the nitrogen flux of the Mississippi River may not be sufficient to accomplish the proposed hypoxia management goal. For instance, a 20% increase in the Mississippi River discharge, projected under a number of climate change scenarios (Table 1), would completely offset a decrease in the frequency of hypoxia resulting from a 30% decrease in the anthropogenic nitrogen flux (Table 2).

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REFERENCES

Crowley, T.J. 2000. Causes of climate change over the past 1000 years. Science 287:270-277.

D'Elia, C.J., J.G. Sanders, and W.R. Boynton. 1986. Nutrient enrichment studies in a coastal plain estuary: phytoplankton growth in large-scale, continuous cultures. Canadian Journal of Fisheries and Aquatic Sciences 43:397-406.

Dillon, T.M. and D.R. Caldwell. 1980. The Batchelor spectrum and dissipation in the upper ocean. Journal of Geophys. Research 85: 910-1916.

Dunn, D.D. 1996. Trends in nutrient inflows to the Gulf of Mexico from streams draining the conterminous United States 1972 – 1993. Water-Resources Investigations Report 96-4113. U.S. Geological Survey, Austin, Texas.

Eadie, B.J., B.A. McKee, M.B Lansing, J.A. Robbins, S. Metz, and J.H. Trefry. 1994. Records of nutrient-enhanced coastal ocean productivity in sediments from the Louisiana continental shelf. Estuaries 17:754-765.

Easterling, D.R., G.A. Meehl, C. Parmesan, A.A. Changnon, T.R. Karl, and L.O. Mearns. 2000. Climate extremes; observations, modeling and impacts. Science 289:2068-2074.

Giorgi, F., C. Shields-Brodeur, and T. Bates. 1994. Regional climate change scenarios produced with a nested regional climate model. Journal of Climate 7:375-399.

Goolsby, D.A., W.A. Battaglin, G.B. Lawrence, R.S. Artz, B.T. Aulenbach, R.P. Hooper, D.R. Keeney, and G.J. Stensland. 1999. Flux and Sources of Nutrients in the Mississippi-Atchafalaya River Basin, Topic 3 Report for the Integrated Assessment of Hypoxia in The Gulf of Mexico. NOAA Coastal Ocean Program Decision Analysis Series No. 17, NOAA Coastal Ocean Program. Silver Springs, Maryland. pp. 130.

Howarth, R.W., G. Bilen, D. Swaney, D. Townsend, N. Jaworski, K. Lajtha, and J.A. Downing. IPCC (Intergovernmental Panel on Climate Change). 2001. Climate Change 2001: The Scientific Basis, Contribution of Working Group I to the Third Assessment Report, Cambridge University Press, Cambridge. pp. 881.

Jones, P.D., M. New, D.E. Parker, S. Martin, and I.G. Rigor. 1999. Surface air temperature and its changes over the past 150 years. Reviews of Geophysics 37:173-199.

Justić, D., N.N. Rabalais, R.E. Turner, and W.J. Wiseman Jr. 1993. Seasonal coupling between riverborne nutrients, net productivity and hypoxia. Marine Pollution Bulletin 26:184-189.

Justić, D., N.N. Rabalais, and R.E. Turner. 1995a. Stoichiometric nutrient balance and origin of coastal eutrophication. Marine Pollution Bulletin 30:41-46.

Justić, D., N.N. Rabalais, R.E. Turner, and Q. Dortch. 1995b. Changes in nutrient structure of river-dominated coastal waters: stoichiometric nutrient balance and its consequences. Estuarine Coastal and Shelf Science 40:339-356.

Justić, D., N.N. Rabalais, and R.E. Turner. 1996. Effects of climate change on hypoxia in coastal waters: A doubled CO_2 scenario for the northern Gulf of Mexico. Limnology and Oceanography 41:992-1003.

Justić, D., N.N. Rabalais, and R.E. Turner. 1997. Impacts of climate change on net productivity of coastal waters: implications for carbon budgets and hypoxia. Climatology Research 8:225-237.

Justić, D., N.N. Rabalais, and R.E. Turner. 2002. Modeling the impacts of decadal changes in riverine nutrient fluxes on coastal eutrophication near the Mississippi River Delta. Ecological Modeling 152:33-46.

Liss, P.S. and L. Merlivat. 1986. Air-sea gas exchange rates: Introduction and synthesis. *In:* The Role of Air-Sea Exchange in Geochemical Cycling, P. Buat-Ménard (ed.). Reidel, Dorecht, Holland. pp. 113-127.

Lohrenz, S.E., M.J. Dagg, and T.E. Whitledge. 1990. Enhanced primary production in the plume/oceanic interface of the Mississippi River. Continental Shelf Research 10:639-664.

Miller, J.R. and G.L. Russell. 1992. The impact of global warming on river discharge. Journal of Geophysical Research 97:2757-2764.

Mitsch, W.J., J.W. Day Jr., J.W. Gilliam, P. M. Groffman, D.L. Hey, G.W. Randall, and N. Wang. 2001. Reducing nitrogen loading to the Gulf of Mexico from the Mississippi River Basin: Strategies to counter a persistent ecological problem. BioScience 51:373-388.

Officer, C.B., R.B. Biggs, J.L. Taft, L.E. Cronin, M. Tyler, and W.R. Boynton. 1984. Chesapeake Bay anoxia: Origin, development and significance. Science 223:22-27.

Officer, C.B., D.R. Lynch, W.M. Kemp, and W.R. Boynton. 1985. Estimation of benthic respiration parameters from field data. Estuarine Coastal and Shelf Science 21:357-364.

Parsons, T.R., Y. Maita, and M. Lalli. 1984. A Manual of Chemical and Biological Methods for Seawater Analyses. Pergamon Press. New York. pp. 173.

Rabalais, N.N., R.E. Turner, W.J. Wiseman Jr., and D.F. Boesch. 1991. A brief summary of hypoxia on the northern Gulf of Mexico continental shelf: 1985-1988. *In:* Modern and Ancient Continental Shelf Anoxia, Tyson, R.V., Pearson, T.H., (eds.). London. Geological Society Special Publication No. 58:35-47.

Rabalais, N.N., R.E. Turner, D. Justić, Q. Dortch, W.J., Jr., Wiseman, and B.K. Sen Gupta. 1996. Nutrient changes in the Mississippi River and system responses on the adjacent continental shelf. Estuaries 19:386-407.

Rabalais, N.N., R.E. Turner, W.J. Wiseman Jr., and Q. Dortch. 1998. Consequences of the 1993 Mississippi River flood in the Gulf of Mexico. Regulated Rivers: Research & Management 14:161-177.

Rabalais, N.N. and R.E. Turner. 2001. Hypoxia in the northern Gulf of Mexico: description, causes and change. *In*: Coastal Hypoxia: Consequences for Living Resources and Ecosystems, N.N. Rabalais, R.E. Turner (eds.). American Geophysical Union, Washington, D.C. Coastal and Estuarine Studies 581-36

Rabalais, N.N., R.E. Turner, and D. Scavia. 2002. Beyond science into policy: Gulf of Mexico hypoxia and the Mississippi River. BioScience 52:29-142.

Redfield, A.C., B.H. Ketchum, and F.A. Richards. 1963. The influence of organisms on the composition of seawater. *In*: The Sea, M.N. Hill, (ed.). John Wiley, New York. Volume 2:26-77.

Sen Gupta, B.K., R.E. Turner, and N.N. Rabalais. 1996. Seasonal oxygen depletion in continental-shelf waters of Louisiana: Historical record of benthic foraminifers. Geology 24:227-230.

Stigebrandt, A. 1991. Computation of oxygen fluxes through the sea surface and net production of organic matter with application to the Baltic and adjacent seas. Limnology and Oceanography 36:444-454.

Tilman, D., J. Fargione, B. Wolff, C. D'Antonio, A. Dobson, R. Howarth, D. Schindler, W.H. Schlesinger, D. Simberloff, and D. Swackhamer. 2001. Forecasting agriculturally driven global environmental change. Science 292:281-284.

Trenberth, K.E. and C.J. Guillemot. 1996. Physical processes involved in the 1988 drought and the 1993 flood in North America. Journal Climate 9:288-1298.

Turner, R.E. and N.N. Rabalais. 1991. Changes in the Mississippi River water quality this century - Implications for coastal food webs. BioScience 41:140-147.

Turner, R.E. and N.N. Rabalais. 1994. Evidence for coastal eutrophication near the Mississippi river delta. Nature 368:619-621.

Weiss, R.F. 1970. The solubility of nitrogen, oxygen and argon in water and seawater. Deep-Sea Research 17:721-735.

Wiseman, W.J., Jr., N.N. Rabalais, M.J. Dagg, and T.E. Whitledge Editors. 1999. Nutrient Enhanced Coastal Ocean Productivity in the Northern Gulf of Mexico. Understanding the Effects of Nutrients on a Coastal Ecosystem. NOAA Coastal Ocean Program, Silver Springs, Maryland. NOAA Coastal Ocean Program Decision Analysis Series 14:156.

Wolock, D.M. and G.J. McCabe. 1999. Estimates of runoff using water-balance and atmospheric general circulation models. Journal of American Water Research Association 35:1341-1350.

SEDIMENT QUALITY ASSESSMENT STUDIES IN NEMUNAS RIVER DELTA AND CURONIAN LAGOON, LITHUANIA

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ABSTRACT

Toxicity of sediments from the Nemunas River Delta and Curonian Lagoon was analyzed using *Vibrio fischeri* bioluminescence quenching tests of pore water and organic extracts, directly and after removal of sulfur with copper. Sulfur removal resulted in a marked decrease in the toxicity of organic extractants, but the remaining toxicity was still remarkable. Toxicity increased with increased concentrations of organic carbon and chlorophyll *a* in most sediment samples. Sediments from the Nemunas River Delta and Curonian Lagoon were analyzed for concentrations of polychlorinated biphenyls (PCBs), organochlorines (DDT, its metabolites, HCH isomers, etc.), and heavy metals (HM). To evaluate possible sediment contamination, data were compared to different sediment quality guidelines (SQG). In general, sediments were relatively clean based on comparison with existing SQGs for PCBs, organochlorines, and HM. A more detailed comparison with consensus-based Sediment Effect Concentrations (SEC) of total PCBs revealed that sediment data could be compared only below screening-level concentrations (SLC) or in the range of threshold effects of SEC. *Vibrio fischeri* bioluminescence quenching tests showed marked toxicity to the IS_3 standard (standard of 3 PCBs), the total extracts of sediment samples, and sample fractions prepared and analyzed for the content of persistent organic pollutants (POPs; *i.e.*, PCBs, DDT and its metabolites, and others) using gas chromatography with electron capture detector (GC-ECD). Although the toxicity of these individual sample fractions did not depend directly on the total amount of PCBs and organochlorines present, in general, samples from the Curonian Lagoon contained higher concentrations of POPs and were up to 10 times more toxic than sediment samples from the Nemunas River Delta. In most sediment samples, the PCB, organochlorine, and HM content increased with increasing concentrations of organic carbon and chlorophyll *a*. A few sampling stations located near known or highly likely sources of anthropogenic pollution had increased amounts of pollutants that were independent of the amount of organic carbon present in sediments. Based on our general analyses of point source pollution in the Nemunas River Delta and Curonian Lagoon, the influence of eutrophication on the concentration of POPs in sediments was evident only in bottom areas that were remote from point sources of pollution, active currents, or the shipping lanes.

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INTRODUCTION

The Nemunas River and its tributaries drain 71% of the territory of Lithuania. The river drains into the Baltic Sea via the Curonian Lagoon (1,584 km^2; separated from the sea by a narrow, sandy spit) and Klaipeda Straits. In the Baltic area, it is the third largest river based on drainage area and the fourth largest in discharge (total annual discharge of approximately 22 km^3) (Environmental Protection Ministry of Lithuania 1995). Despite the existence of more than 40 years of water quality control on Lithuanian rivers and the creation of an extensive monitoring network, there are no data reported in the literature on individual persistent organic pollutants (POPs) from either Curonian Lagoon or Nemunas River Delta sediments. Limited data on individual organic pollutants in sediments (such as atrazine, simazine, 3,4-dichlorobenzoic acid, and a few POPs, including halogenated cyclic hydrocarbon (HCH) isomers, DDT and its metabolites) are presented in the official literature as averages of annual values from certain official sampling points within the Nemunas River and its tributaries (Environmental Protection Ministry of Lithuania 1999, Cetkauskaitė *et al*. 2001). Such methods lead to a high degree of uncertainty and do not satisfactorily reflect the pollution situation in the Nemunas River. In contrast, analyses of pollution in Curonian Lagoon are performed by the Sea Research Center in Klaipeda and Klaipeda University; limited water quality parameters and eutrophication status, including the abundance of phytoplankton species, are analyzed, yet neither sediment quality data nor data on individual organochlorine pollutants are reported to the Ministry of Environment. Midsummer eutrophication is a major problem in the lagoon; large fish kills were recorded in 1979, 1980, and 1993, and bacteriological pollution is recorded there every year (Cetkauskaite *et al*. 2001).

Analyses of POPs only in the water column do not provide enough information to understand all ecosystem processes. Experience and information obtained from valuable projects such as EUCON (Eutrophication and Contamination, 1995–1999) on pollution trends in Baltic Sea water and sediments can be successfully used for the interpretation of POP accumulation and transport trends in estuaries or lagoons (EUCON 1997, Skei *et al*. 2000). Many different aspects of sediment chemical and toxicological analyses were developed and reviewed in articles, workshops, and proceedings over the last ten years, with attempts to clarify guidelines for sediment Ecological Risk Assessments (ERA) (Adams *et al.* 1992, Cubbage *et al.* 1997, Fairey *et al.* 2001, Fernandez *et al*. 2000, Foster *et al.* 2000, Hyland *et al.* 1999, Ingersoll *et al.* 1997, Khim *et al.* 1999, Neff *et al*. 1986, Persaud *et al.* 1993, Porebski *et al*. 1999). According to the Sediment Quality Guidelines (SQGs) or consensus-based Sediment Effects Concentrations (SEGs), however, problems of data comparison and evaluation still exist when data are obtained from chemical analyses and toxicological testing (Swartz and Di Toro 1997, Ingersoll *et al.* 1997, MacDonald *et al.* 2000). Other areas of uncertainty relate to the interpretation and comparison of toxicity and chemical data from different sediment fractions, and make it difficult to come to general conclusions on the trends of toxicity and pollution in sediment of certain bottom areas of rivers, estuaries, and lagoons. Therefore, the goals of this project were: 1) to perform quantitative analyses of POPs, including polychlorinated biphenyls (PCBs), other organochlorine compounds, and heavy metals (HM) in the sediments of the Nemunas River Delta and Curonian Lagoon; 2) to determine whether a consistent distribution of POPs and toxicity exists in the Curonian Lagoon and Nemunas River Delta, and determine whether this is characteristic of other pollutants; 3) to determine whether the POP fraction in sediment is

responsible for acute toxicity in bioluminescence quenching tests; and 4) to compare data from some forest region lake(s) in Sweden, data available in the literature on PCB content in rivers or lakes, and existing sediment quality guidelines for PCBs, organochlorine compounds, and HM. This work was a part of the NATO/CCMS field and toxicity study "Modeling of Nutrient Loads and its Response in River and Delta Systems" performed during expeditions in the Nemunas River Delta and Curonian Lagoon, Lithuania, during 2001 and 2002.

MATERIALS AND METHODS

Geographical Characteristics of Sampling Locations

Material for toxicity tests and chemical analyses was collected during the period of most active vegetative growth and highest summer temperature (July 17-19, 2001), as dissolved oxygen and nutrient/eutrophication problems are usually most severe under these conditions. Five sampling stations were selected in the Curonian Lagoon: station 1, located in the mid-eastern part of the lagoon, west of the Atmata branch input; station 2, located at Preila; station 3, located approximately 11 km northwest of station 1 and approximately 3 km east of station 2; station 4, located 1 km southeast of Nida, a resort town; and station 5, located approximately 4 km southwest of station 1 (Figure 1). Five additional sampling stations were selected in the lower reaches of the Nemunas River Delta: sites 1M and 1P at the mouth of the Nemunas River and the Atmata branch; sites 2M and 2P in the northern, high flow area of the Atmata branch of the Nemunas River at Port Uostadvaris and the mouth of the Minija River; and site 3M in the middle, low flow area of the Rusnaite branch. Lake Havgard, located in the Skane region of southern Sweden and surrounded by forest and agricultural fields, was used as a reference site. Sediment samples were collected at this site in May 2000.

Geological and Geochemical Characteristics

Bottom sediment samples collected in July 2001 from stations in the Curonian Lagoon and the Nemunas River Delta had different granulometric types. Recent data from the lagoon showed there were primarily seven sediment types of different diagenetic and granulometric characteristics, including peat, shells, gravel, coarse sand, fine sand, coarse silt, and fine silty mud (Gulbinskas A., personal communication 2001). As presented in the NATO/CCMS pilot study report (NATO/CCMS 2001), bottom sediments from the surface layer between 0–5 cm depth at station 1 contained coarse silt (median diameter [Md] = 0.05-0.1 mm) and sometimes contained shells. Sediments at lagoon stations 3 and 5 and stations 1M and 1P of the Nemunas River Atmata branch contained fine sand (Md = 0.1-0.25 mm), and lagoon station 2 and 4 samples contained fine silty mud, *i.e.* aleurite, that was primarily of biogenic origin (Md = 0.01-0.05 mm) (NATO/CCMS 2001). Sediments from other sampling locations in the Nemunas River Delta were represented by the following characteristics: muddy sand in sample 2M, and dark silt or clay with organic matter in samples 2P and 3M (NATO/CCMS 2001).

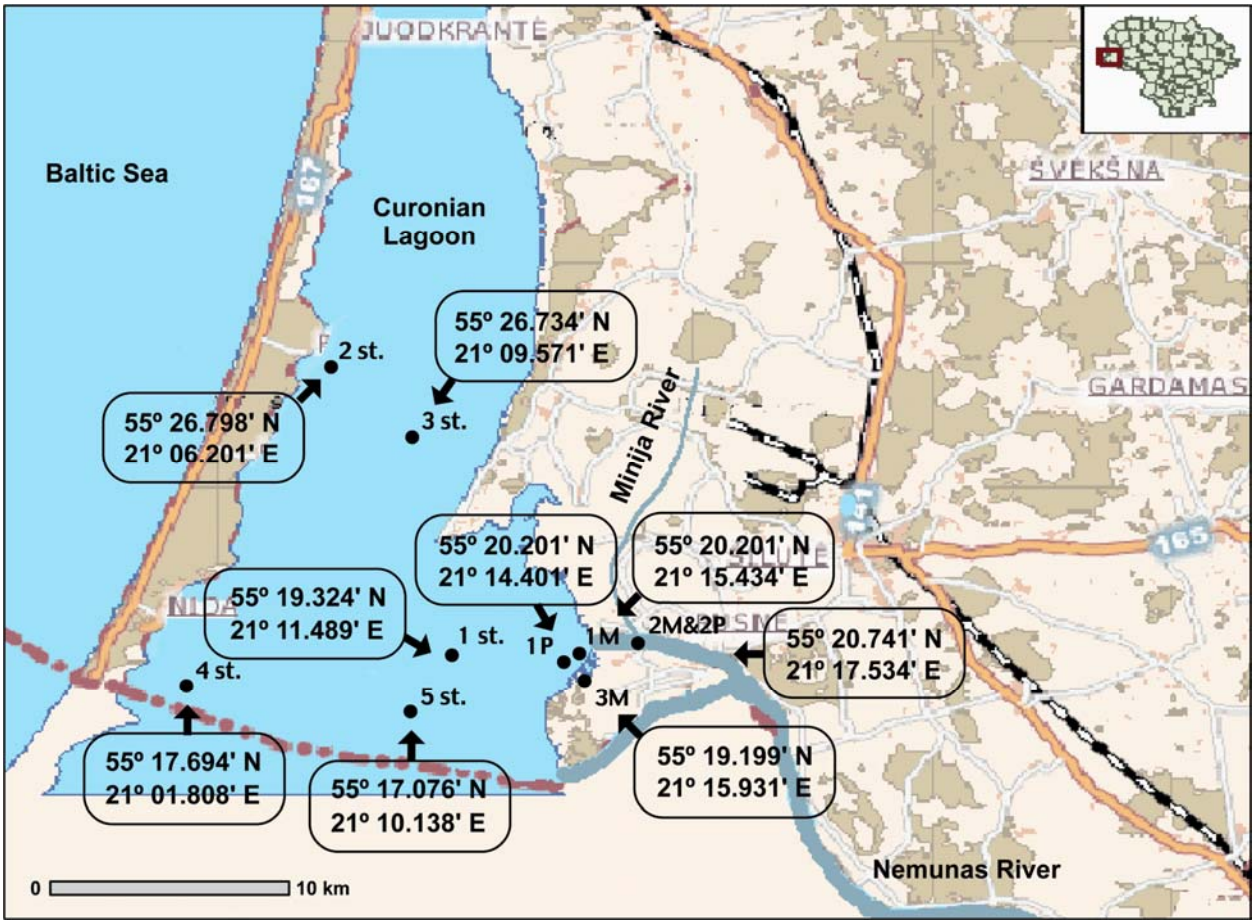

Figure 1. *In situ* research and sampling stations in the Nemunas River Delta, Atmata branch and Curonian Lagoon. Sites 1M, 1P, 2M, 2P and 3M are located near the mouth of the Nemunas River and the Atmata branch; the distance between sampling points 2M and 2P (marked by the same geographical coordinates) is 20 m. Sites 1, 2, 3, 4 and 5 are located in the Curonian Lagoon.

Sampling and Sample Storage

Samples of bottom sediments were collected using a specially-constructed dredge (similar to an Eckman's dredge) that allowed selective collection of the surface (0–3 cm or 0–5 cm) or deeper (5–10 or 10–15 cm) layers of bottom sediment. In this study, the upper sediment surface layer of 0–5 cm depth was collected for analysis. Water samples from selected sampling stations were collected using samplers of different modifications. Sediment and water samples were placed in glass or plastic vessels, transported immediately to the laboratory, and analyzed or prepared for biotests on the day of collection. Sediment and water samples were frozen and kept at –20^{o}C or –80^{o}C for chemical analyses or for preparation of extracts used in toxicity testing, respectively. Deep bottom and low redox potential sediments from the Curonian Lagoon retained a dark (black) color while frozen during storage.

General Water Quality Parameters

Water transparency (Secchi depth) and temperature were measured *in situ;* dissolved oxygen, pH, Eh, salinity, and conductivity were measured by selective electrodes of the WTW MultiLine F/Set 3, a universal portable measuring instrument. Most nutrient compounds, *i.e.* ammonia, nitrite/nitrate nitrogen, and phosphate, were measured using a Hach DR 2010 spectrophotometer in the laboratory.

Chemical Analyses of Elements and Heavy Metal Content in Sediments

Analyses of dry sediments were performed using an Inductively-Coupled Plasma Mass Spectrometer (analysis of heavy metals) and Atomic Emission Spectrometer Perkin Elmer Zeeman 3030 equipped with graphite furnace (analysis of other elements).

Sediment Extraction and Extract Clean-up for POP Analyses

Sediments (10 g wet weight) were placed in Kimax tubes and 100 µl of Internal Standard (IS_3) was added. After addition of 4 ml acetone, samples were shaken on a Vortex for three minutes and then processed for 10 minutes in an ultrasonic bath. Then cyclohexane (3 ml) was added and 10 minutes of extraction in the ultrasonic bath was repeated. Samples were left to stand overnight; 10 ml of 2% NaCl-solution was then added and samples were shaken well and centrifuged at 1000 rpm (Hermle Z510, r=14 cm) for 10 minutes. The cyclohexane phase was transferred to a glass centrifuge tube using a Pasteur pipette. Settled sediments were extracted twice with acetone and cyclohexane by repeating the procedures of Vortex shaking, ultrasonic extraction, and centrifugation. All collected extracts were evaporated under a nitrogen stream to a 0.5 ml volume. For the removal of organic pollutants (other than persistent), samples of cyclohexane extracts were treated with 2 ml of concentrated sulfuric acid and shaken for two minutes on a Vortex, then processed one minute in an ultra-sonic bath. After centrifugation for five minutes, the acid phase was removed with Pasteur pipettes. Sulfuric acid treatment was repeated if the cyclohexane phase still had some color. Before gas chromatography, cyclohexane extracts were left in sealed glass capillary tubes for at least one day with metallic copper and silica-gel for sulfur removal.

Gas Chromatography – Electron Capture Detection Analysis of POPs

Analyses of PCBs and other organochlorines were performed on a Varian 3500 capillary gas chromatograph, equipped with electron capture detector (GC-ECD). The DB-5 column (30 m × 0.25 mm, J&W Scientific) was used under the following conditions: split injection of 1/100; injector temperature of 225^{o}C; detector temperature of 300^{o}C; H_2 carrier gas, flow rate 2 ml/minute; N_2 as the make up gas, flow rate 18 ml/minute; column temperature of 110^{o}C for one minute, then 6^{o}C/minute up to 240^{o}C, followed by 40 minutes at 240^{o}C.

Standards and Other Reagents

The GC analytical standards were: 1) internal standard (IS_3), which consisted of PCB 30 (0.5028 ng/μl), PCB 204(0.2899 ng/μl), and PCB 209 (0.9683 ng/μl); 2) external standard, which consisted of Clophen A50 (0.829789 ng/μl), IS (PCB 30 (0.05 ng/μl), PCB 204 (0.01 ng/μl), and PCB 209 (0.1 ng/μl); and 3) external standard for organochlorines, which consisted of α-BHC (0.025 ng/l), β-BHC (0.100 ng/l), Lindane (0.025 ng/l), Aldrin (0.050 ng/l), Heptachlor (0.025 ng/l), Dieldrin (0.120 ng/l), Heptachlorepoxide (0.080 ng/l), Endrin (0.200 ng/l), p,p'–DDE (0.100 ng/l), o,p'-DDD (0.200 ng/l), o,p'–DDT (0.225 ng/l), p,p'–DDD (0.190 ng/l), and p,p'–DDT (0.260 ng/l). The solvents acetone, hexane, and cyclohexane were from Merck, and were analytical or chromatography grade reagents.

Toxicity Experiments

Vibrio fischeri bioluminescence quenching measurements were performed using the standard method in aqueous solutions (ISO 11348 1994), with up to 1% acetone in blanks and analyzed samples. Extracts were obtained from dry and wet sediments using acetone or/and hexane. Further analyses of the toxicities of these extracts were conducted using copper to remove sulfur. Toxicity of the POP extracts, *i.e.* the organic phase obtained after extract treatment with sulfuric acid, were also measured. Standard exposure times (5, 15, and 30 minutes) and appropriate procedural blanks (controls) were used. Pore water toxicities were also determined. The bioluminescence quenching was measured with a LKB-Wallace 1250 Luminometer as described earlier (Berzinskiene and Cetkauskaite 1996).

RESULTS

Characteristics of the Accumulation of Heavy Metals and POPs in Sediments

Concentrations of individual heavy metals, as well as the total metal accumulations in sediment samples, were higher in fine sediments from the Curonian Lagoon than those from the Nemunas River Delta (Table 1). During both 2001 and 2002, greater accumulations of heavy metals were observed in fine bottom sediments of stations 2 and 4 than in the sediments of other sandy-bottom stations within the lagoon or the Nemunas River Atmata branch at the Minija mouth (stations 2M and 2P). For example, concentrations of zinc, lead, chromium, and copper in Nemunas River sediments were approximately 3.5, 3, 6, and 5 times less, respectively, than those at lagoon stations 2 and 4 (Table 1). Sediments from the Nemunas River had similar amounts of heavy metals as other sandy-bottom locations, *i.e.* lagoon stations 1, 3, and 5. Persistent organic pollutants analyzed in the benthic system during 2001 included PCBs (No. 31 up to No. 180) and other organochlorines (the cyclodiene compounds including DDT, DDE, DDD and other metabolites, α-, β-, γ- HCH isomers, endrin, heptachlor, aldrin, lindane, and dieldrin). The PCBs and all POPs presented in Table 1 show higher accumulations in the bottom sediments of stations 2 and 5 from the Curonian Lagoon, compared to the sandy bottom of station 3 in the lagoon or the bottom sediments of the Nemunas River at Minija (stations 2M and 2P).

Table 1. Sediment quality values for heavy metals (in μg/g dry weight) and organochlorine compounds (in ng/g or μg/kg of dry weight) for marine and estuarine ecosystems, and the values obtained in the study of the sediments of Havgard Lake (Sweden), Nemunas River Delta, and the Curonian Lagoon (Lithuania).

Chemical name	Sediment Quality Guidelines				Data of analysis of sediments from different sampling stations										
	ERM*	PEL**	ERL***	TEL****	Havgard Lake	Curonian Lagoon					Nemunas River Delta				
						Stat. 1	Stat. 2	Stat. 3	Stat. 4	Stat.5	1M	1P	2M	2P	3M
Cadmium	9.6	4.21		7.24	0.50	0.24	0.91	0.04	0.89	0.02	0.02	0.03	0.09	0.05	0.03
Chromium	370	160.4		0.676	16.57	11.28	38.17	2.56	37.29	2.08	2.48	2.84	15.04	4.69	3.44
Copper	270	108.2		18.7	10.58	3.89	15.33	0.67	15.68	4.21	0.42	0.50	5.23	1.52	1.09
Lead	218	112.18		30.2	23.85	4.79	16.00	1.65	15.43	2.61	1.30	1.37	6.00	3.59	1.52
Mercury	0.7	0.696		0.13	0.08	0.03	0.13	0.01	0.14	0.01	0.01	0.01	0.03	0.01	0.01
Nickel	51.6	42.8		15.9	10.24	2.97	10.24	0.17	12.51	0.38	0.66	0.72	6.74	1.55	1.07
Silver	3.7	1.77		0.73											
Zinc	410	271		124.0	57.00	23.23	77.24	4.84	71.41	10.48	4.52	4.84	23.05	10.95	7.63
Total PCBs	180	189	22.7	22.0	24.39	2.89	11.95	0.13	1.40	6.4	0.31	0.30	0.69	1.83	1.06
Total DDT and its metabolites	46.1	51.7	1.58		19.70	1.63	7.55	0.09	0.00	2.0	0.05	0.41	0.45	0.59	0.24
4,4'-DDD (p,p'-DDD)			7.81		10.70	1.04	3.80	0.06	0.00	0.92	0.03	0.25	0.20	0.37	0.15
4,4'-DDE (p,p'-DDE)	27	374			5.23	0.59	3.74	0.03	0.00	0.95	0.02	0.07	0.20	0.16	0.09
4,4'-DDT (p,p'-DDT)		4.77			3.77	0.00	0.00	0.00	0.00	0.13	0.00	0.09	0.05	0.05	0.00
Lindane (Gamma BHC)		0.99													
Dieldrin		4.3			0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Endrin					0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

ERM* - effects range median; (Swartz and Di Toro, 1997; Fairey *et al.*, 2001; Hyland *et al.*, 1999; MacDonald *et al.*, 2000);
PEL** - probable effects levels; (Fairey *et al.*, 2001; Porebski *et al.*, 1999; Hyland *et al.*, 1999; MacDonald *et al.*, 2000);
ERL*** - effects range low (Swartz and Di Toro, 1997; MacDonald *et al.*, 2000);
TEL**** - threshold effects levels (Porebski et al., 1999; MacDonald et al., 2000)

Analyses of PCB content in sediments of the Nemunas River Delta and Curonian Lagoon revealed a PCB concentration range of 0.13-11.95 ng/g (μg/kg dry weight) (Table 1). Concentrations of DDT and related POPs ranged from 0.00-7.55 ng/g (Table 1). The total amount of PCBs was higher in the lagoon than in the delta (Table 1). Quantitative analyses of heavy metals and POPs revealed that concentrations of these pollutants also were higher in lagoon sediments than in delta sediments. Analyses showed a distribution of POPs in the Curonian Lagoon and Nemunas River Delta with the highest concentrations found in the fine sediments of stations 2 and 4 and the sandy sediments of station 5.

Toxicity of Sediment Extracts to *V. fischeri* Bioluminescence and Organic Carbon Content

Toxicity analyses of Curonian Lagoon sediments using *Vibrio fischeri* bioluminescence revealed that concentrated organic extracts of whole sediments from stations 1-4 were very toxic, producing up to 80–98% bioluminescence inhibition (Table 2). Whole sediment samples from station 5 produced the lowest inhibition of bioluminescence (39%) compared to all other sampling stations. However, only 10-fold extract dilutions of Curonian Lagoon sediments revealed differences in toxicity among sediment samples. In contrast, differences in toxicity among Nemunas River Delta sediment extracts were observed without dilution. After removal of sulfur with copper, marked decreases in toxicity were observed, but the remaining toxicity was still significant (data not presented). In most sediment samples, toxicity increased with increases in organic carbon and chlorophyll *a* concentration (Tables 2 and 3). The amount of $H_2S + HS^-$ also changed in accordance with organic carbon concentration and was highest at station 2, the most toxic site within the lagoon. Treatment with copper to eliminate sulfur toxicity produced similar results in undiluted organic sediment extracts from the river delta. Extracts of whole sediments from stations 1M and 1P were non-toxic. Sediment extracts from station 2P were most toxic (up to 92.9% of bioluminescence inhibition), and removal of sulfur diminished inhibition only to 61.8% (Table 3). Once again, in most sediment samples, *i.e.* from stations 2M, 2P, and 3M, toxicity was greatest in sediments with the highest organic carbon and chlorophyll *a* concentrations (Table 3).

Toxicity of Total Fractions of POPs to *V. fischeri* Bioluminescence and the Effect of PCB Content

The POPs in sediment samples were responsible for toxicity in acute toxicity screening tests (bioluminescence quenching) with the marine bacterium *V. fischeri*. The three PCB standards (IS_3) and POP sample fractions from the sediments at stations 2-5 in the Curonian Lagoon showed marked toxicity in quenching tests (Figure 2). Although the toxicity of individual sample fractions did not correlate to the total amount (sum) of PCBs and organochlorines, samples of POPs from the lagoon (up to 10-fold dilutions) were more toxic than those from the Nemunas River Delta (data not shown). This was consistent with data from GC-ECD analyses of total PCBs; the number of peaks and the total concentration of individual PCBs were higher in lagoon than in delta sediments (Tables 1, 2 and 3).

Table 2. Organic carbon, chlorophyll *a*, and total PCB content in undiluted sediment extracts from different sampling sites within the Curonian Lagoon, and bioluminescence inhibition of *Vibrio fischeri* in hexane extracts (10-fold dilutions).

Parameter	Sampling sites in Curonian Lagoon				
	Station 1	Station 2	Station 3	Station 4	Station 5
Chlorophyll *a* content [$D_{\lambda=670\ nm}$ of acetone]	**0.03**	**0.12**	**0.01**	**0.12**	**0.015**
Organic Carbon content [% of dry wt]	**3.18**	**16.35**	**1.21**	**15.61**	**0.84**
H_2S + HS^- * [in upper layer (0-3 cm) of sediments, mg/dm^3]	**168**	**224**	**0**	**152**	**64**
Eh, mV*	**- 233**	**-158**	**+308**	**-83**	**+55**
Total amount of PCBs [ng/g of dry weight]	**2.89**	**11.95**	**0.13**	**1.40**	**6.4**
Total amount of Persistent Organochlorines [ng/g of dry weight]	**4.52**	**19.50**	**0.22**	**1.40**	**8.40**
Toxicity with sulfur: inhibition of *V. fischeri* bioluminescence, %*	**85**	**98**	**80**	**95**	**39**
Toxicity without sulfur: inhibition of *V. fischeri* bioluminescence, %**	**21**	**50**	**25**	**30**	**10**

*- in hexane extracts of sediments (toxicity with sulfur, *i.e.* samples not exposed to metallic copper);
** - in hexane extracts of sediments (toxicity without sulfur, *i.e.* samples exposed to metallic copper).

Table 3. Organic carbon, chlorophyll *a,* and total PCBs in undiluted sediment extracts from sampling sites within the Nemunas River, and toxicity of extracts.

Parameter	Sampling sites in Nemunas River Delta				
	1M	1P	2M	2P	3M
Chlorophyll *a* content [$D_{\lambda=670\ nm}$ of acetone]	**0.01**	**0.015**	**0.1**	**0.07**	**0.093**
Organic Carbon content [% of dry wt]	**0.53**	**0.79**	**3.77**	**2.04**	**1.71**
Total amount of PCBs [ng/g of dry weight]	**0.31**	**0.30**	**0.69**	**1.83**	**1.06**
Total amount of Persistent Organochlorines [ng/g of dry weight]	**0.36**	**0.71**	**1.14**	**2.42**	**1.30**
Toxicity with sulfur: inhibition of *V. fischeri* bioluminescence, %*	**-6.01**	**0**	**52.5**	**92,9**	**76.6**
Toxicity without sulfur: inhibition of *V. fischeri* bioluminescence, %**	**-5.06**	**0**	**39.9**	**61.8**	**27.7**

* - in hexane extracts of sediments (toxicity with sulfur, *i.e.* samples not exposed to metallic copper);
** - in hexane extracts of sediments (toxicity without sulfur, *i.e.* samples exposed to metallic copper).

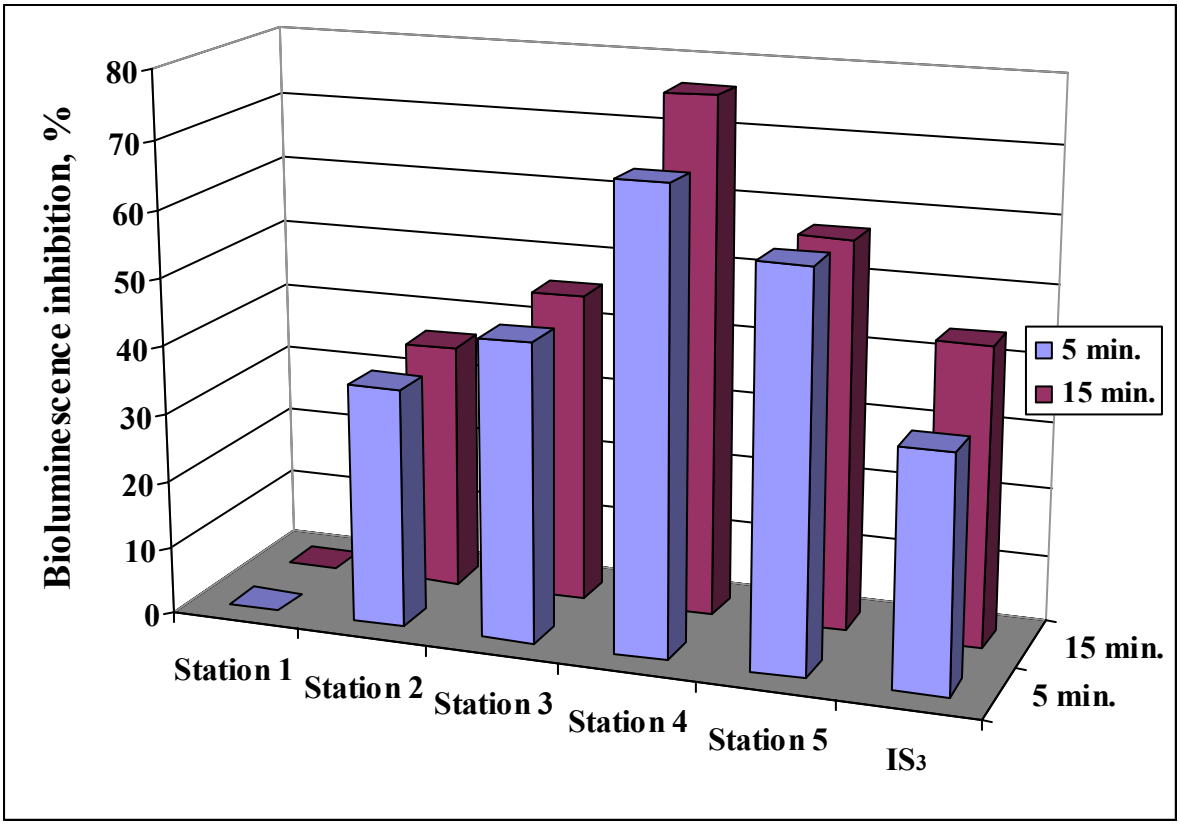

Figure 2. Results from bioluminescence inhibition tests (*V. fischeri*) indicated toxicity of the POP fraction in 10-fold dilutions of Curonian Lagoon sediment extracts. Samples were analysed by GC-ECD after sulfur removal.

From these results we concluded that a consistent pattern of bottom sediment toxicity existed at different locations within the Curonian Lagoon and the Nemunas River Delta. Toxicity was primarily found in extracts of sediment samples of biogenic origin and high organic carbon and chlorophyll *a* concentrations, *i.e.* in areas with fine benthic sediment. The POPs in sediment were responsible for the acute toxicity measured in bioluminescence quenching tests. After elemental sulphur removal, toxicity of the POP fraction was higher in sample extracts from the lagoon than those from the delta.

DISCUSSION

Sediment Particle Type and Pollution Chemistry

In general, sediment samples and cores are used extensively to determine spatial and temporal aquatic pollution, especially of highly hydrophobic persistent pollutants, such as PCBs, polychlorinated dibenzo-dioxins (PCDDs) and polychlorinated dibenzo-furans (PCDFs), (Kjeller and Rappe 1995, Muir *et al.* 1996). In our case, fine sediment from stations 1, 2, 4, and 5 of the Curonian Lagoon had elevated concentrations of total PCBs. After investigation of

bottom sediments from the northern part of the Curonian Lagoon and the Baltic Sea, Galkus and Jokšas (2001) defined geochemical fields ('geochemical pollution' was defined by the authors), where concentrations of fine sediments, organic matter, and heavy metals (*e.g.*, Cu, Pb, Zn, and Hg) were similar. Lithogenic fine sediments form approximately 11% of the bottom sediment of the Curonian Lagoon, whereas the organic phase comprises about 2% (Galkus and Jokšas 2001). Geochemical data show that the water column at stations 2 and 4 overlies a fine aleurite layer (or aleuritic pelite, *i.e.* mud, or mud with clay) and the sediments at these stations have finer grain size than sediments from other lagoon stations. Other geologists state that fine sediments are of biogenic origin and are usually more polluted (Ingersoll 1995, Trimonis 2002). Our data obtained from samples at stations 2 and 4 confirmed that, since they contained increased amounts of chlorophyll *a* and organic carbon, and they accumulated heavy metals and organic substances (POPs, for example). Samples from station 5 were the exception, as they also contained sand with shells. These results suggest zones of sedimentation that formed both geologically and historically. Fine particles - at least 10 times smaller than sand - containing elevated concentrations of pollutants including PCBs, have accumulated along the western coast of the Curonian Lagoon. These western locations within the lagoon are opposite the shores of the Nemunas River mouth and have lower water flow rates and enhanced sedimentation. Greater bioaccumulation by algae of long-range-transport pollutants and non-point source pollution can occur at these locations. Heavy metal concentrations in our sediment samples mimicked those of organic pollutants: sediment samples from stations with lower heavy metal concentrations were located on or above sandy bottoms or coarse silt sediments of the Nemunas River Delta. Sediment samples collected from the Curonian Lagoon during summer 2002 showed that accumulations of PAHs in the fine sediments of stations 2 and 4 were three to six times greater than in other sandy-bottom sites (stations 1, 3, and 5) or in bottom sediments of the Nemunas River Atmata branch at the Minija mouth (stations 2M and 2P). These differences in accumulations of PAHs in bottom sediments were confirmed by PAH bioaccumulation in molluscs: higher concentrations of PAHs were found in benthic molluscs from Curonian Lagoon station 4 than in individuals from the Nemunas River (NATO/CCMS 2001).

Possible Fate of PCBs

PCB partitioning in sediments is controlled by chemical binding to fractions of organic carbon in sediments and dissolved organic carbon in pore water (Farley and Thomann 1998). Other authors cite at least three mechanisms by which PCBs can be transferred from the sediment to water: diffusion from the sediment surface; colloid association and transport; and particle-bound dispersion. The first mechanism causes changes in the congener composition of PCBs (*e.g.*, congeners of higher lipophilicity are sorbed more strongly to sediment than less lipophilic ones), whereas the other mechanisms do not (Brown and Wagner 1990). In our case, a greater number of hydrophobic PCBs were observed at stations 2 and 5 from the Curonian Lagoon (data not presented). It was previously reported that the flux of PCBs from sediment to water is accelerated by higher temperature (*i.e.*, an increase in PCB flux from sediment during summer), bioturbation activity, gas production in sediment, and the amount and quality of lipids in the water column (Larsson 1986, Larsson and Sodergren 1987). Although midsummer sampling occurred during a high-temperature period, these parameters most likely did not influence the content of PCBs in our sediment samples. Despite similar depths at stations 4 (4 m) and 5 (3.3 m), compared to station 2 (2.5 m), sulfide and methane production were higher in

sediments from stations 4 and 2. A more probable explanation for higher PCB concentrations in sediments from stations 1, 2, 4, and 5 was the accumulation of PCBs in areas of fine particles, or in sandy bottom areas in close proximity to fine sediments with a former abundance of filter-feeding molluscs. PCBs or other POPs could spread to these latter areas through bioaccumulation in molluscs, as was possible at station 5. This station was also close to shipping lanes, and thus, industrial non-point sources of pollution by POPs cannot be excluded. We did not find less-chlorinated PCBs in anaerobic zones, *i.e.* deep bottom sediments from stations 2 and 4 of the Curonian Lagoon. Perhaps the biodegradation and/or anaerobic dehalogenation threshold was too low in these areas, as postulated by Farley and Thomann (1998).

Toxicity Analyses: Uncertainty Problems

We used a rapid, acute toxicity screening test, the bioluminescence of the marine bacterium *V. fischeri,* to test toxicity of sediments. The constant question in such experiments is whether the end-point is a relevant method for sediment toxicity screening. Methodological uncertainties include increased variations in test precision, ecological relevance, causality, sensitivity, interference, standardization, discrimination, bioavailability and field validation. Evaluation criteria show great variation in the uncertainty of: (1) the evaluation of sediment phases used in laboratory toxicity tests; (2) endpoints measured in sediment toxicity tests; (3) assessments of benthic community; (4) assessments of bioaccumulation; and (5) uncertainty related to Sediment Quality Guidelines (SQGs). The degree of uncertainty associated with benthic community assessments and endpoints measured in laboratory toxicity testing was documented after the 1995 Pellston Workshop on Sediment ERA (Ingersoll *et al.* 1997). Ecological relevance (ER) is highly complicated in laboratory sediment toxicity tests and has high uncertainty when specific biomarkers are used as endpoints; better estimates of ER are obtained when using growth, behavior, and development as endpoints. The best evaluation of ER is based on survival, reproduction, and time table data (Ingersoll *et al.* 1997). High uncertainty in ER of benthic community assessments originates from a lack of knowledge on community structure and variation among individuals or populations in a system. A better estimate of ER can be obtained by studying the structure of the whole ecosystem (Ingersoll *et al.* 1997). Although problems exist, high-sensitivity bioluminescence tests perhaps are just as valid as other frequently-used end-points.

Toxicity of Sediment Fractions in Acute Toxicity Tests

1. Consistency of Toxicity from Different Places

Toxicity of whole sediment extracts to *V. fischeri* was greatest at stations 2, 3, and 4 in the Curonian Lagoon, even after sulfur removal (see Table 2). In general, results reflected possible contamination of areas of fine sediments by different pollutants, including fatty acids, polyamines and others. Traditional analyses of the amount of accumulated (sorbed) organic matter and pollutants in bottom sediments showed that the concentration depends upon the size of sediment particles (Ingersoll 1995). Bottom sediments from the surface layer (0–5 cm depth) at stations 2 and 4 contained fine silty mud (*i.e.* aleurite; Md = 0.01-0.05 mm), and were primarily of biogenic origin. Surface-layer sediments from stations 3 and 5 of the Curonian

Lagoon and the Nemunas River contained fine sand (Md = 0.1-0.25 mm) and, according to geological classification, were primarily of terrigenic origin (Gulbinskas 2001, NATO/CCMS 2002, Trimonis 2002). German authors reported an increase in concentration (amount per unit of sediment mass) of organic carbon, phosphate, and nitrogen compounds when going from sand to muddy/silty deposits, and the amounts of organic carbon and nutrients increased in the water column above the sand-silt sediment area (Meyer-Reil 2002). Enhanced toxicity and pollution of sandy-bottom areas could be connected only with point-source pollution, as in the case of the port near stations 2P and 2M of the Nemunas River Delta, or with shipping lanes, as was the case near stations 1 and 5 in the Curonian Lagoon.

2. Toxicity of the POP Fraction

The *V. fischeri* bioluminescence quenching test was sensitive to PCB compounds, shown by the marked toxicity of the IS_3 standard and of the sample fractions, prepared and analyzed by GC-ECD. Sample fraction toxicity did not vary with the total amount of PCBs and organochlorines, but in general, samples with higher concentrations of POPs from the Curonian Lagoon were more toxic (up to 10 times) than those from the Nemunas River Delta. The content of high-molecular-weight PCBs, identified by GC-ECD, was equally distributed in toxic and non-toxic sample fractions (data not presented). In addition, planar tetra-congeners of PCBs did not contribute to high values of Toxicity Equivalence Factors. Therefore, the toxicity of highly hydrophobic and persistent compounds in the samples was probably induced by unidentified classes of halogenated POPs in the sulfuric acid treatment fraction, e.g. polybrominated biphenyls, terpenes or PCDDs (dioxins) or even PCDFs (furans).

3. Problem of Sulfur Toxicity

We found that sediment toxicity of organic extracts after sulfur removal was still remarkable in samples from the Nemunas River Delta and the Curonian Lagoon. In most samples, toxicity increased with increasing organic carbon and chlorophyll *a* concentration. The highest concentrations of organic carbon were found at stations 2 and 4 in the lagoon. The observed toxicity in total sediment extracts and after elemental sulfur removal was higher in these samples compared to sediments from stations 1, 3, and 5. Sediment extracts from a river in Florida and other polluted areas were tested in 1992 for toxicity in commercial Microtox test after Solid Phase Extraction with C18 SPE cartridges and elution with acetonitrile and water. When metallic copper powder was used in cartridges or extracts to remove sulfur, the toxicity was markedly diminished, indicating sulfur was responsible for the toxicity (Jacobs *et al.* 1992). It was also possible to extract the sulfur with a mixture of solvents (50% methylene chloride, 47% hexane, 3% acetonitrile (Jacobs *et al.* 1992). Pardos *et al*. (1999) later found that organic extracts (hexane with acetone) were toxic to *V. fischeri*, and the presence of elemental sulfur in the extracts was determined using GC/ECD analysis. Toxicity and sulfur were removed from samples using acid-activated metallic copper. The elemental sulfur was most toxic to Microtox (15 min. EC_{50} = 11.9 μg S/L), but reported EC_{50} values of *Daphnia magna* Straus and *Selenastrum capricornutum* toxicity were much higher: 802.9 μg S/L (48 hr) and > 1,000 μg S/L (96 hr), respectively (Pardos *et al.* 1999).

It has been proposed that elemental sulfur is not soluble in water, and perhaps is not toxic to benthic organisms (Environment Canada 1984). This is doubtful, as hydrophobic sulfur can dissolve in fats and is found in Semipermeable Membrane Devices (SPMDs) filled with triolein (Sabaliūnas 1999). The toxic effects of elemental sulfur have been described for hematological and central nervous systems; possible effects included decreases in SH groups of proteins (Na^+,K^+-ATPase of erythrocytes, nervous cells), decreased amounts of reduced glutathion, and inhibition of blood peroxidase and catalase (Filov *et al.* 1989). As sulfur is an element that is formed during biodegradation of proteins or as an intermediate product in sediment sulfate reduction, it is possible that it has toxic effects on benthic macrofauna.

Quantification of PCB Content in Sediment and Sediment Quality Guidelines

Sediment Quality Criteria, SQC, (Adams et al. 1992; Ingersoll 1995), Sediment Quality Guidelines, SQG, (Swartz and Di Toro 1997) or consensus-based sediment effect concentrations, SECs**,** (MacDonald *et al,* 2000) are numerical concentrations of an individual chemical (μg contaminant/g sediment dry weight) intended to be predictive of biological effects, mostly on benthic organisms, and are to be applicable to a range of natural sediments in lakes, streams, estuaries, and near-coast marine waters (Adams et al. 1992; Ingersoll 1995). All SQC, SQG or SECs for total PCBs were created from data of chemical and ecotoxicological or ecological (species diversity) analysis of natural marine or freshwater ecosystem sediment (Barrick *et al*. 1988, US EPA 1996 and 1997) in comparison to clean reference sediment, or of laboratory-spiked sediment with ranges of concentrations to generate ecotoxic effects (Ingersoll 1995). So, they are presented mostly as databases or data in different reports or articles: up to 195 freshwater and 599 marine and estuarine sediment samples had been analyzed in total up to year 2000 (Barrick *et al*. 1988, USEPA 1996 and 1997, Environment Canada 1992, MacDonald *et al.* 2000). The variety of different terms for SQG or SECs, such as (1) threshold, midrange and extreme effect concentrations (TEC, MEC, EEC), (2) screening level concentrations, SLCs, (3) lowest, moderate and highest-apparent-effect-threshold (LAET, MAET, HAET), and (4) threshold, probable, severe effect levels (TEL, PEL, SEL) emerged from different analytical approaches. These include survival and growth end-points of benthic fauna (midges, amphipods, etc.); the species distribution frequency or sum of biological endpoints, like Microtox, survival of oyster larvae, or structure of benthic community; or biological effects in field and spiked sediments in comparison to chemical analytical data (MacDonald *et al.* 2000). The classification or ranking of PCB effects concentrations in sediment was based on degree or severity of adverse biological effects and the effects ranges or zones were defined quantitatively (MacDonald *et al.* 2000). For example, sediment low or threshold effects concentrations, TECs, of total PCBs corresponded to the great range of values from 3 up to 40 ± 54 (SD) μg/kg dry wt of sediment; (Figure 3); consensus-based MECs (midrange effect concentrations) were those up to 400 ± 330 (SD) μg PCBs per kg dry wt.; and consensus-based EEC (extreme effect concentrations) up to 1700 ± 2000 (SD) μg/kg dry wt of sediment.

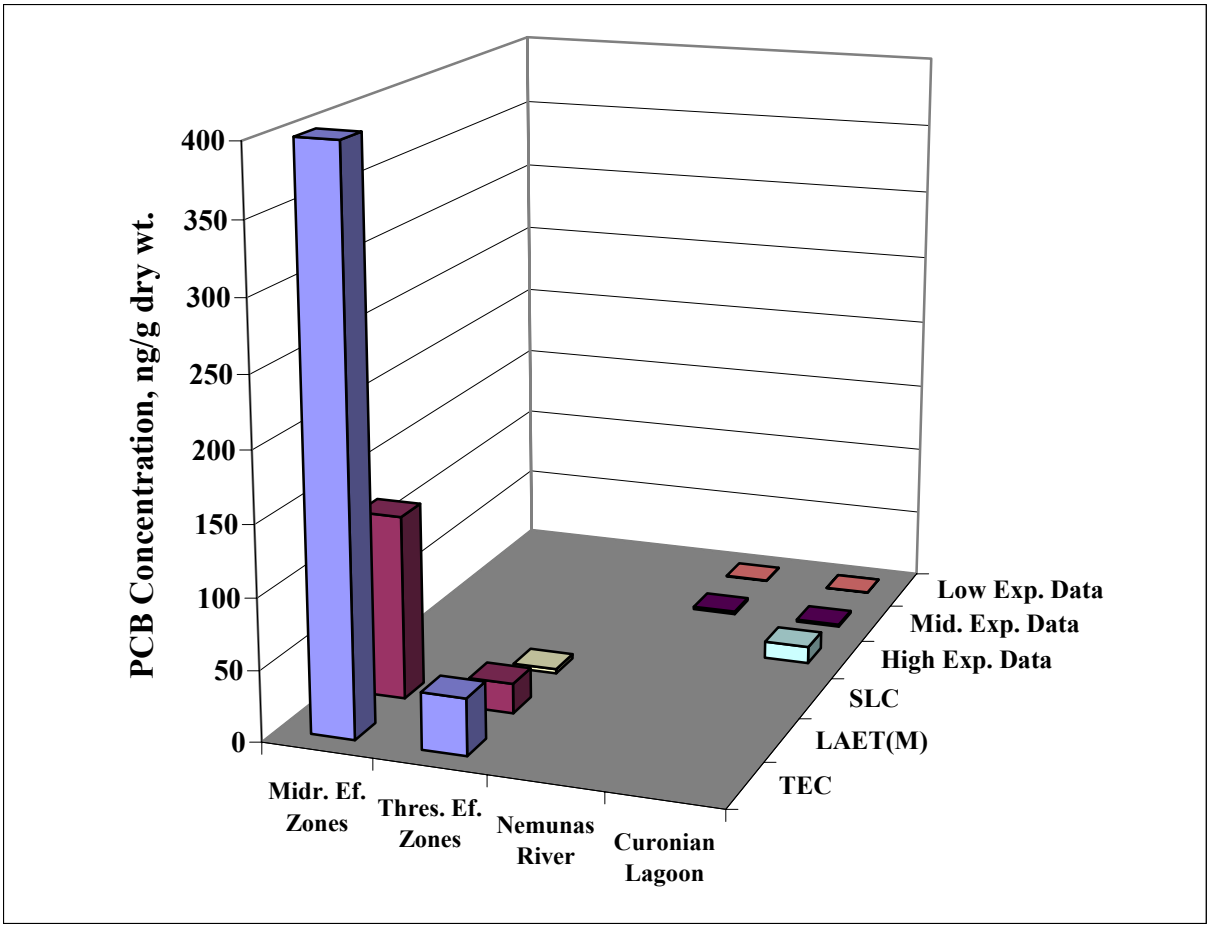

Figure 3. Comparison of consensus-based Sediment Effect Concentrations for total PCBs in sediments and those measured in our field samples. SLC = Screening-Level Concentration; LAET (Microtox) = Lowest-Apparent-Effects Threshold; TEC = Threshold Effects Concentration (MacDonald *et al*. 2000). High Exp. Data**:** Curonian Lagoon (CL) station 2 = 11.95 ng/g dry wt.; Mid. Exp. Data: CL station 4 = 1.4 ng/g dry wt., Nemunas River (NR) station 2P = 1.83 ng/g dry wt.; Low Exp. Data: CL station 3 = 0.13 ng/g dry wt., NR station 1P = 0.3 ng/g dry wt.

The concentrations of PCBs from Curonian Lagoon and Nemunas River Delta sediments were compared to different values and whole ranges of sediment effect concentrations (see Figure 3). Based on comparison of existing SQGs for PCBs, DDT and its metabolites, and HM (Swartz and Di Toro 1997), our sediments were found to be relatively clean. A more detailed comparison of consensus-based SECs of total PCBs revealed that concentrations within our Nemunas River sediment samples and those from stations 1, 3 and 4 in the Curonian Lagoon were less than screening-level concentrations (SLC; 3 μg/kg dry wt.), while samples from stations 2 and 5 in the Curonian Lagoon were lower than threshold and midrange effects of different SEC values (21 – 400 μg/kg dry wt.) (Figure 3). The comparison with Sediment Quality Guidelines (ERM, PEL, ERL, TEL) for DDT and its metabolites and for HM also confirmed the conclusions of low content of total and individual pollutants in sediments from the Curonian Lagoon and the Nemunas River Delta. The comparison of total PCB concentration

with other experimental and literature data revealed the values of PCBs obtained in lagoon sediments (range of concentrations 0.13–11.95 μg/kg dry wt) were less than in some lakes in forested regions of Sweden (Lake Havgard; concentrations up to 24 μg/kg dry wt), and comparable to the Flint River reservoir (rural Georgia, U.S.) or White Rock Lake (urban Texas, U.S), having from 5 to 10 μg/kg of sediment in the period from 1980 to 1995, as dated by ^{137}Cs in sediment cores (Smol 2002).

CONCLUSIONS

Conclusion 1 (Chemistry)

Comparisons of individual and total values of PCBs, POPs, and HM showed that concentrations of these pollutants were higher in sediments from the Curonian Lagoon than those from the Nemunas River Delta. There was a consistent distribution of POPs (including PCBs) and HM in sediments of the lagoon and the delta: higher concentrations were found mostly in bottom areas with fine sediment at stations 2, 4, and 5. Exceptional samples (with relatively high HM and POP concentrations) were collected from the sandy-bottom station 1 of the lagoon beneath a shipping lane and station 2P of the delta within a port area.

Conclusion 2 (Eco-Toxicity)

The toxicity of organic extracts of all pollutants was at least ten times higher in sample extracts from the Curonian Lagoon than in those from the Nemunas River Delta. Toxicity testing of sediment extracts from the Curonian Lagoon in *V. fischeri* bioluminescence quenching tests was possible only after 10-fold sample dilution. Toxicity also was higher in samples with fine sediment and high concentrations of organic carbon from the bottom of the lagoon. Decreased toxicity was observed after sulfur removal from sediment extracts. The POP fraction (amount) in sediment was responsible for some of the toxicity measured in acute toxicity screening tests (bioluminescence quenching).

Conclusion 3 (Quantification and Characterization of Risk)

Based on comparison with existing SQGs for PCBs, DDT and its metabolites, and HM, the sampled sediments were relatively clean. A more detailed comparison of consensus-based SECs of total PCBs revealed that sediments collected in the Curonian Lagoon and the Nemunas River Delta can be compared only below SLC levels or in the range of Threshold Effects of SEC.

REFERENCES

Adams, W.J., R.A. Kimerle, and J.W. Barnett Jr. 1992. Sediment quality and aquatic life assessment. Environmental Science & Technology 26(10):1865–1875.

Barrick, R., S. Becker, R. Pastorok, L. Brown, and H. Beller. 1988. Sediment quality values refinement: 1988 update and evaluation of Puget Sound AET. EPA 68-01-4341. Final Report. U.S. Environmental Protection Agency, Seattle, WA, USA.

Berzinskiene, J. and A. Cetkauskaite. 1996. Chlorinated acetanilides: Biodegradation and Toxicity in Gram Negative Cells. pp. 261-282. *In:* Environmental Xenobiotics, M. Richardson, (ed.). Francis and Taylor, London.492 pp.

Brown, J.F. Jr. and R.E. Wagner. 1990. PCB movement, dechlorination and detoxication in the Acushnet Delta. Environmental Toxicology and Chemistry 9:1215-1233.

Cetkauskaite, A., D. Zarkov, and L. Stoskus. 2001. Water-Quality Control, Monitoring and Wastewater Treatment in Lithuania 1950–1999. Ambio 30(4-5):297-305.

Cubbage J., D. Batts, and S. Briedenbach. 1997. Creation and analysis of freshwater sediment quality values in Washington State. Technical Report. Washington Department of Ecology, Olympia, WA, USA. Publication 97:323.

Environment Canada. 1984. Sulphur: Environmental and technical information for problem spills. Environmental Protection Service, Ottawa, ON, Canada.

Environment Canada and Ministere de l'Environnement du Quebec. 1992. Interim criteria for quality assessment of St. Lawrence River sediment. Final Report. Environment Canada, Ottawa, ON.

Environmental Protection Ministry of Lithuania. 1995. Lithuania's Environment Status, Processes, Trends. Vilnius, 119 pages (pp.38-41). (In English).

Environmental Protection Ministry of Lithuania. 1999. The Chronicles of the Water Quality of Lithuanian Rivers. 64 pp. (In Lithuanian, Cover translation).

EUCON: Interactions between Eutrophication and Contaminants in the Aquatic Environment. Research Programme. 1997. Report 4690, Swedish Environmental Protection Agency. 52 pp.

Fairey, R., E.R. Long, C.A. Roberts, B.S. Anderson, B.M. Phillips, J.W. Hunt, H.R. Puckett, and C.J. Wilson. 2001. An evaluation of methods for calculating mean sediment quality guideline quotients as indicators of contamination and acute toxicity to amphipods by chemical mixtures. Environmental Toxicology and Chemistry 20(10):2276-2286.

Farley, K.J. and R.V. Thomann. 1998. Fate and Bioaccumulation of PCBs in Aquatic Environments. Chapter 119:1581-1593. *In:* Environmental and Occupational Medicine, Third edition, W.N. Rom (ed.). Lippincott-Raven Publishers. Philadelphia, PA. 1880 pp.

Fernandez, M., S. Cuesta, O. Jimenez, M.A. Garcia, L.M. Hernandez, M.L. Marina, and M.J. Gonzalez. 2000. Organochlorine and heavy metal residues in the water/sediment system of the Southeast Regional Park in Madrid, Spain. Chemosphere 41:801-812.

Filov, V.A., A.L. Bandman, N.V. Volkova, and T.D. Grekhova, (Editors). 1989. Harmful Chemical Compounds. Inorganic Chemical Compounds of the Elements of V - VIII groups. The Handbook. Khimija ("Chemistry" - Publishers) Leningrad. pp. 185-187. (total 592 pages).

Foster, G.D., K.A. Lippa, and C.V. Miller. 2000. Seasonal concentrations of organic contaminants at the fall line of the Susquehanna River basin and estimated fluxes to northern Chesapeake Bay, USA. Environmental Toxicology and Chemistry 19(4):992-1001.

Galkus, A. and K. Joksas. 2001. Geochemical thin-dispersed fields in the bottom sediments of Curonian Lagoon and Baltic Sea. *In*: Book of Abstracts. Metals in the Environment. II International Conference, Vilnius, Lithuania, September 23-26. pp 49-50.

Gulbinskas, S. 2001. Personal communication. Department of Marine Research. Institute of Geology and Geography, Vilnius, Lithuania.

Hyland, J.L., R.F. Van Dolah, and T.R. Snoots. 1999. Predicting stress in benthic communities of southeastern U.S. estuaries in relation to chemical contamination of sediments. Environmental Toxicology and Chemistry 18(11):2557-2564.

Ingersoll, C.G. 1995. Sediment Tests. Chapter 8:231-255 *In:* Fundamentals of Aquatic Toxicology. Effects, Environmental Fate, and Risk Assessment. Second edition, G.M. Rand (ed.). Taylor & Francis, New York. 1125 pp.

Ingersoll, C.G., T. Dillon, and G.R. Biddinger, (Editors). 1997. Ecological Risk Assessment of Contaminated Sediments. SETAC Press, Pensacola. 389 pp.

ISO 11348. 1994. Current Document. Water quality – Determination of the inhibitory effects of water samples on the light emission of *Vibrio fischeri*. (Luminescent bacteria test).

Jackobs, M.W., J.J. Delfino, and G. Bitton. 1992. The toxicity of sulfur to Microtox from acetonitrile extracts of contaminated sediments. Environmental Toxicology and Chemistry 11:137-1145.

Khim, J.S., D.L. Villeneuve, K. Kannan, K.T. Lee, S.A. Snyder, C.H. Koh, and J.P. Giesy. 1999. Alkylphenols, polycyclic aromatic hydrocarbons, and organochlorines in sediment from Lake Shihwa, Korea: instrumental and bioanalytical characterization. Environmental Toxicology and Chemistry 18(11):2424-2432.

Kjeller, L.O. and C. Rappe. 1995. Time trends in levels, patterns and profiles for polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls in a sediment core from the Baltic proper. Environmental Science & Technology 29:346-355.

Larsson, P. 1986. Zooplankton and fish accumulate chlorinated hydrocarbons from contaminated sediments. Canadian Journal of Fisheries Aquatic Science 43:1463-1466.

Larsson, P. and A. Sodergren. 1987. Transport of polychlorinated biphenyls (PCBs) in freshwater mesocosms from sediment to water and air. Water, Air, and Soil Pollution 36:33-46.

MacDonald, D.D., R.S. Carr, F.D. Calder, E.R. Long, and C.G. Ingersoll. 1996. Development and evaluation of sediment quality guidelines for Florida coastal waters. Ecotoxicology 5:253-278.

MacDonald, D.D., L.M. Dipinto, J. Field, C.G. Ingersoll, E.R. Long, and R.C. Swartz. 2000. Development and evaluation of consensus-based sediment effect concentrations for polychlorinated biphenyls. Environmental Toxicology and Chemistry 19(5):1403-1413.

Meyer-Reil, L.A. 2002. Microbial Metabolism in Sediments of Coastal Inlets of the Southern Baltic Sea – Response to Gradients of Eutrophication. Pp 43-52 *In*: Baltic Coastal Ecosystems. Structure, Function and Coastal Zone Management, G. Schernewski and U. Schiewer (eds.). Springer, Berlin. 397 pp.

Muir, D.C.G., A. Omelchenko, N.P. Grift, D.A. Savoie, W.L. Lockhart, P. Wilkinson, and G.J. Brunskill. 1996. Spatial trends and historical depositions of polychlorinated biphenyls in Canadian midlatitude and arctic lake sediment. Environmental Science & Technology 30:3609-3617.

Mullin, M.D., C.M. Pochini, McCrindle, M. Romkes, S.H. Safe, and L.M. Safe. 1984. High resolution PCB analysis: synthesis and chromatographic properties of all 209 PCB congeners. Environmental Science & Technology 18:468-476.

NATO/CCMS Pilot Study Report to National Science Foundation of Lithuania. 2001. Modeling of Nutrient Loads and Responses in River and Estuary Systems. Vilnius University, 1,154 pp. (In Lithuanian, Cover translation).

NATO/CCMS Pilot Study Report to National Science Foundation of Lithuania. 2002. Modeling of Nutrient Loads and Responses in River and Estuary Systems. Vilnius University. 45 pp. (In Lithuanian, Cover translation).

Neff, J.M., D.J. Bean, B.W. Cornaby, R.M. Vaga, T.C. Gulbransen, and J.A. Scalon. 1986. Sediment quality criteria methodology validation: calculation of screening level concentrations from field data. Final Report. U.S. Environmental Protection Agency, Washington DC.

Pardos, M., C. Benninghoff, R.L. Thomas, and S. Khim-Heang. 1999. Confirmation of elemental sulfur toxicity in the microtox assay during organic extracts assessment of freshwater sediments. Environmental Toxicology and Chemistry 18:188-193.

Persaud, D., R. Jaagumagi, and A. Hayton. 1993. Guidelines for the protection and management of aquatic sediment quality in Ontario. Final Report. Ontario Ministry of Environment and Energy, Toronto, ON, Canada.

Porebski, L.M., K.G. Doe, B.A. Zajdlik, D. Lee, P. Pocklington, and J.M. Osborne. 1999. Evaluating the techniques for a tiered testing approach to dredged sediment assessment – a study over a metal. Concentration gradient. Environmental Toxicology and Chemistry 18(11):2600-2610.

Sabaliūnas, D. 1999. Semipermeable membrane devices in monitoring of organic pollutants in the aquatic environment. Dissertation. Department of Ecology, Chemical Ecology and Ecotoxicology. Lund University, Sweden. 134 pp.

Schulz, D.E., G. Petrick, and J.C. Duinker. 1989. Complete characterization of polychlorinated biphenyl congeners in commercial Aroclor and Clophen mixtures by multidimensional gas chromatography-electron capture detection. Environmental Science & Technology 23:852-859.

Skei, J., P. Larsson, R. Rosenberg, P. Jonsson, M. Olsson, and D. Broman. 2000. Eutrophication and contaminants in the aquatic ecosystems. Ambio XXIX (4-5):184-194.

Smol, J. P. 2002. Pollution of Lakes and Rivers: A Paleoenvironmental Perspective. Oxford University Press, New York. 280 pp.

Swartz, R.C. and D.M. Di Toro. 1997. Sediments as complex mixtures: an overview of methods to assess ecotoxicological significance. pp. 255-269 *In:* Ecological Risk Assessment of Contaminated Sediments, C.G. Ingersoll, T. Dillon, G.R. Biddinger (eds.). SETAC Press, Pensacola. 389 pp.

Trimonis, E. 2002. Geology of Seas and Oceans. Vilnius University Publishing House, Vilnius, pp.165-166 (372 pp). (In Lithuanian, Cover translation).

US Environmental Protection Agency. 1996. Calculation and evaluation of sediment effect concentrations for the amphipod *Hyalella azteca* and the midge *Chironomus riparius*. EPA 905-R96-008. Chicago, IL.

US Environmental Protection Agency. 1997. An assessment of sediment from the Upper Mississippi River. Final Report. EPA 823-R-97-005. Columbia, MO.

SUSTAINABLE AGRICULTURAL DEVELOPMENT POLICY IN THE BALTIC SEA REGION

Marek J. Gromiec[1]

INTRODUCTION

The Baltic Sea region is the first region in the world with a consensus on goals for sustainable development. The Brundtland report alerted the world to the urgent need to make progress towards economic development that can be sustained without depleting natural resources or harming the environment. In this report, sustainable development is defined as development that meets present needs without compromising the ability of future generations to meet their needs. Seven economic sectors (agriculture, forests, fisheries, industry, energy, transport, and tourism) were defined as crucial to sustainable development in the Baltic Sea region. This paper addresses sustainable development policy for the agricultural and fisheries sectors only. However, policymakers should employ both measurement and financial incentives to strongly encourage implementation of sustainable development in all other economic sectors as well. The restoration of the Baltic Sea environment is a responsibility shared by all countries in this populous region.

LEGAL FRAMEWORK

The 1st Helsinki Convention of 1974 requires the Baltic Sea countries to protect the marine environment. The 2nd Helsinki Convention of 1992, which was signed by the Ministers of Environment from Denmark, Estonia, Finland, Lithuania, Latvia, Germany, Poland, Russia, and Sweden, regulates environmental protection throughout the entire Baltic Sea watershed, including protection of waters, atmosphere, land, and nature. The Convention calls for a moratorium on potentially hazardous pollutants and requires application of the best available technologies (BAT) and the best available practices (BAP) to eliminate pollution. In accordance with the Convention, the "polluter pays" principle is also applied. A separate organization, known as the Helsinki Commission (HELCOM), was created to implement the requirements of the Helsinki Convention. By 2005, HELCOM contracting parties have agreed to reduce the nutrient load to the Baltic Sea to 50% of the late 1980s levels.

Work on the Helsinki Convention revisions occurred at the same time as the preparation of the Joint Program for the Baltic Sea. The Baltic Sea Joint Program, completed in 1992, defines 132 objects and areas (hot spots) causing the most environmental nuisance. Implementation of the Baltic Sea Joint Program has been planned for 20 years and its costs are estimated to be 18 billion ECU.

An Agenda 21 for the Baltic Sea Region (Baltic 21) was initiated in 1996, at the Prime Ministers' meeting in Visby in May and at the meeting of the Foreign Ministers of Affairs in June. The Environment Ministers provided the terms of reference for Baltic 21 in October 1996 in Sallsjöbaden. In June 1998, the Foreign Ministers adopted Agenda 21, which contains goals

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and an action program for sustainable development (HELCOM 1998). The first Baltic 21 progress report was published in 2000 (HELCOM 2000). Various Baltic 21 documents are published on the Baltic 21 website (http://www.ee/baltic21). Baltic 21 participating nations are Denmark, Estonia, Finland, Iceland, Lithuania, Latvia, Germany, Norway, Poland, Russia, and Sweden.

OVERALL GOALS

The Baltic Sea countries have adopted common goals for regional sustainable development. These goals are separated into the following types: overall goals, goals for different sectors, and a goal for special planning. The overall goals and those for the agricultural and fisheries sectors are listed below.

Overall Goals for Baltic Sea Region Sustainable Development

- A safe and healthy life for current and future generations.
- A cooperative and prosperous economy and society for all.
- Local and regional cooperation based on democracy, openness, and participation.
- Restored or maintained biological and ecosystem diversity and productivity.
- Land, water, and atmospheric pollution not in excess of the carrying capacity of nature.
- Efficient use and management of renewable resources with their regeneration capacity.
- Efficient and cyclic flow of non-renewable resources; creation and promotion of renewable substitutes.
- Increased awareness among different stakeholders and social classes of the elements and processes leading to sustainability.

Agricultural Sector Goals

- Farm income sufficient to provide a fair standard of living.
- Ethical aspects of agricultural production secured.
- Non-renewable resources gradually replaced by renewable resources and recirculation of non-renewable resources maximized.
- Utilization of methods that do not threaten human or animal health or degrade the environment or biodiversity, minimization of the environmental problems handed down to future generations.
- Sustainable agriculture that meets societal needs for food and recreation, preserves the landscape, cultural values, and heritage of rural areas, and contributes to stable, well developed and secure rural communities.

Fisheries Sector Goals

- Maintenance of biologically viable fish stocks, the marine and aquatic environment, and associated biodiversity.
- Establishment of maximum fishing limits and selective fishing techniques for harvesting stocks.
- Distribution of the direct and indirect benefits of open sea and coastal fishery resources between local communities in an equitable manner.

INDICATORS

A number of core indicators have been designated to measure the success of overall goals, agriculture sector goals, and fisheries sector goals (HELCOM 1998). The core indicators for the goal of reducing environmental pollution include the following: CO_2, SO_2, and NO_x emissions; land area where depositions are above critical loads for acidification and eutrophication; nutrient load to the Baltic Sea; consumption of ozone depleting substances; and proportion of protected areas.

The agriculture indicators are the loadings of nitrogen and phosphorus, both via rivers and directly, to the Baltic Sea from arable land; nitrogen and phosphorus fertilizer use; livestock units per hectare; ratio of permanent pasture to total arable land; and crop and milk productivity.

Fisheries sector indicators include spawning stock biomass, fishing mortality, recruitment, and landings per country: tons of cod, salmon, herring, and sprat; number of fishing vessels per country operating in the Baltic Sea; average fishing fleet engine power per country; fish consumption per capita per country; and number of full time fishermen per country in the Baltic Sea Region.

IMPACT/DEVELOPMENT OF THE AGRICULTURAL SECTOR

Nutrients enter the Baltic Sea via rivers and the atmosphere; however, rivers and coastal point sources account for most of the nutrient load. In total, some 200 rivers and streams transport nutrients into the Baltic Sea with the top five of these rivers accounting for about 50% of total nitrogen inputs (HELCOM 2001). Nutrients in the rivers derive primarily from point sources and from diffuse loading from agriculture.

The agricultural sector is a significant anthropogenic source of nutrients to the Baltic Sea. The high agricultural nitrogen inputs in some countries are from both nitrogen fertilizers and great livestock densities. Variations in nitrogen inputs reflect the magnitude of the run-off from the various river drainage areas. It is estimated that agriculture accounts for 30-35% of the nitrogen load and 10-15% of the phosphorus load to the Baltic Sea (HELCOM 2000).

Thus, in order to reach the 50% reduction target, measures are directed towards the agricultural sector in all Baltic countries. A general reduction in both nitrogen and phosphorus loading is necessary if the ecological quality of the Baltic Sea is to be restored and maintained. The key elements of the action program for agriculture are listed below.

Action Program for the Agricultural Sector

- Reduction of nutrient losses from agriculture.
- Reduction of risks associated with the use of pesticides and herbicides.
- Protection of ground and surface water for drinking water purposes in agricultural areas.
- Preservation of high quality food and feed production.
- Maintenance and development of biodiversity in rural landscapes.
- Reduction of the usage of fertilizers and antibiotics in plant and animal crops.
- Development of rural infrastructure and promotion of both a high quality of life and the economic conditions of sustainable agriculture in rural areas.
- Promotion of new production alternatives for arable land.
- Other measures for sustainable agriculture development such as transport logistics, markets for sustainable produce, and addressing the use of genetically modified organisms and the greenhouse effect.

The Agenda 21 for the Baltic Sea Region also contains an action program for sustainable development, which includes joint actions, sector actions, and spatial planning actions.

The following projects have been established for the agricultural sector: The Baltic Agricultural Run-Off Action Program (BAAP) and the Elaboration of Codes of Good Agricultural Practice, which outline good agriculture management for farmers. An important project seeks to implement priority actions associated with control of agricultural non-point source pollution (including demonstration sites). The agricultural sector network is also contributing to bilateral projects. For example, a Danish-Polish project on agricultural nutrient control was completed in 2003.

Large differences in farming practices exist for different areas around the Baltic Sea. However, modernization and restructuring of agriculture, together with development of rural areas, is taking place in many countries. For example, some Polish farms have already adopted modern technology and will be prepared for Poland's 2004 entry into the European Union. This will probably lead to an increase in use of mineral fertilizers. Thus, it is important that appropriate measures be adopted concurrently with new agricultural development to avoid increasing pollution from future agricultural activity.

Today, one might think that Poland, with the largest watershed of the Baltic Sea countries, would generate most of the pollution discharged into the Baltic Sea. However, in comparison to other Baltic countries, discharges of nitrogen and phosphorus compounds per person from Poland are among the lowest (Gromiec 2003). Although diffuse agricultural pollution in Poland is not severe, it is well known that significant nutrient, and especially

nitrogen, runoff from intensively farmed areas, such as those in western European countries, can occur or could increase in the future. To address this potential problem, the Polish government elaborated a Code of Good Agricultural Practices in 2002 (Polish Ministry of Agriculture 2002). An agro-environment policy to achieve compliance with the European Union nitrate directive and the Polish legislative work on Water-Law has also been introduced to avoid significant increases of non point source pollution from the agricultural sector.

CONCLUSION

Agricultural practices are fundamentally important to environmental quality. The most important task for the Baltic Sea Region agricultural sector is to combine environmental, social, and economic needs to begin the transition to sustainable development. A high level of agricultural production, achieved through intensive farming, may cause stress on the natural environment and on long-term land productivity. The status of the environment and its resources are an important factor that should determine the conditions for development in all Baltic Sea Region countries. Sustainable development in this region is a challenging and a long-term task. Considerable efforts to reduce pollution from intensive food production, high livestock density, and the use of fertilizers and manure are necessary to meet environmental, social, and economic criteria for a sustainable society. Agricultural policy should take environmental considerations fully into account, and this policy must be based on the concept of long-term sustainability.

REFERENCES

Gromiec, M.J. (ed.). 2003. Water Resources Management in Poland in an Environmental Context. Ministry of the Environment, Republic of Poland, Warsaw. 83 pp.

Helsinki Commission – Baltic Marine Environment Protection Commission. 1998. Agenda 21 for the Baltic Sea Region: Sustainable Development of the Agricultural Sector in the Baltic Sea Region. Helsinki, FI. 123 pp.

Helsinki Commission – Baltic Marine Environment Protection Commission. 2000. Baltic Sea Environmental Proceedings No. 74. Baltic 21 Series No. 1, Biennial Report. Helsinki, FI. 50 pp.

Helsinki Commission – Baltic Marine Environment Protection Commission. 2001. Environment of the Baltic Sea area, 1994-1998. Baltic Sea Environmental Proceedings No. 82. Helsinki, FI. 23 pp.

Polish Ministry of Agriculture and Rural Development/Ministry of the Environment. 2002. Code of Good Agricultural Practice. (In Polish). Warsaw.

CAN WE PREDICT JOINT EFFECTS OF HYPOXIA AND METALS ON FISH SURVIVAL?

Michael C. Newman[1]

ABSTRACT

Fish are suddenly exposed to hypoxic conditions during diverse events such as seiche- or turnover-related water movements, bottom water release from reservoirs, ice-over of eutrophic arctic lakes, and rapid shifts in respiration: photosynthesis associated with cultural eutrophication. In each case, chemical equilibria established under hypoxic conditions that result in metal dissolution and accumulation suddenly shift toward chemical equilibria of oxic conditions. Critical changes in speciation include those determining the free ion activity that, as expressed by the Free Ion Activity Model (FIAM), is often the most bioactive form of a dissolved metal. Metal phase can also change rapidly and, in some cases, result in a precipitate on respiratory surfaces. Exposure of fish gills to metal (and integument of larval or small fish) changes O_2 exchange dynamics. Changes in mucus quality and production and lamellae morphology decrease the amount of effective gill exchange surface and increase the diffusive layer thickness. These changes exacerbate those associated with the reduced O_2 partial pressure gradient. Consequent shifts in blood chemistry (*e.g.*, pH and ion composition) and ventilation also affect metal transport and deposition within fish tissues. Some of these changes have immediate consequences, but others can continue for long periods after the hypoxic conditions pass. Long-term metal effects can influence fish tolerance during future hypoxic episodes.

A joint, similar action model can be applied if the parsimonious assumption is made that asphyxiation constitutes a common mode-of-action for both acute metal effects and hypoxia. Joint action models are applicable based on either conventional dose-effect or survival time approaches. Expansion of such models to a physiologically-based toxicokinetics-toxicodynamics framework (*e.g.*, framed around the Fick equation) would be desirable, provided that model parameter requirements remain realistic. Long-term effects may be better addressed with models such as the binary logistic models used by epidemiologists.

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BACKGROUND

Metal Effects

Metal toxicity to fish is influenced by biogeochemical, cellular, physiological, and anatomical factors. Many of these factors are also influenced by hypoxia.

Metals released from solid phases under anoxic conditions accumulate through time and, during physical mixing with oxic waters, participate in complex chemical reactions. Some (*e.g.*, iron and manganese) can form colloidal phases, and potentially irritate fish gill surfaces. Speciation shifts for other metals that remain in the dissolved phase can elevate free metal ion activity.

Exposure of fish gills to dissolved or colloidal metals can alter gas exchange, ammonia excretion, and ion and osmotic regulation. Irritation from colloids can increase mucus production and, consequently, lower the diffusion rate of gases across the gills. According to Leland and Kuwabara (1985), production and coagulation of excess mucus during acute exposure to dissolved metals results in asphyxiation, *i.e.*, the "coagulation film anoxia hypothesis" for metal lethality. Spaces between primary and secondary gill lamellae fill with coagulated mucus (Figure 1), increasing the effective diffusion distance. For example, colloidal aluminum precipitation on fish gills causes excessive mucus secretion that clogs interlamellar spaces (Poléo 1995).

Metals can also cause swelling of the gill epithelium, a general filling-in between the secondary lamellae, and an increase in numbers of chloride cells. The epithelium of the secondary lamellae, which is composed of two cell layers separated by an intercellular lymphoid space, swells within the lymphoid space to separate the two cell layers. Necrosis and inflammation can also occur (Daoust *et al.* 1984). Blasco *et al*. (2000) noted fusion of secondary lamellae during copper exposure in the Senegalese sole. Jagoe *et al.* (1996) observed thickening of the primary lamellar epithelium to such an extent that the secondary lamellae appeared absent on gills of mercury-exposed mosquitofish, greatly reducing the area available for O_2 exchange. The chloride cell volume density increased with mercury exposure at the expense of pavement cells and, because chloride cells are involved more in ion regulation than with gas exchange, the available gill area for effective respiratory exchange decreased. Chloride cell proliferation can also increase the thickness of the blood-to-water diffusion barrier (Perry 1998) and, as a consequence, impair O_2 diffusion (Greco *et al*. 1995). In turn, these changes produce shifts in ventilation (elevated ventilation amplitude and generally depressed ventilation frequency (Bindon *et al*. 1994). However, Witters *et al.* (1996) noted increased ventilation frequency for brown trout experiencing acute respiratory stress due to aluminum polymerization on their gills. Blood chemistry also shifts.

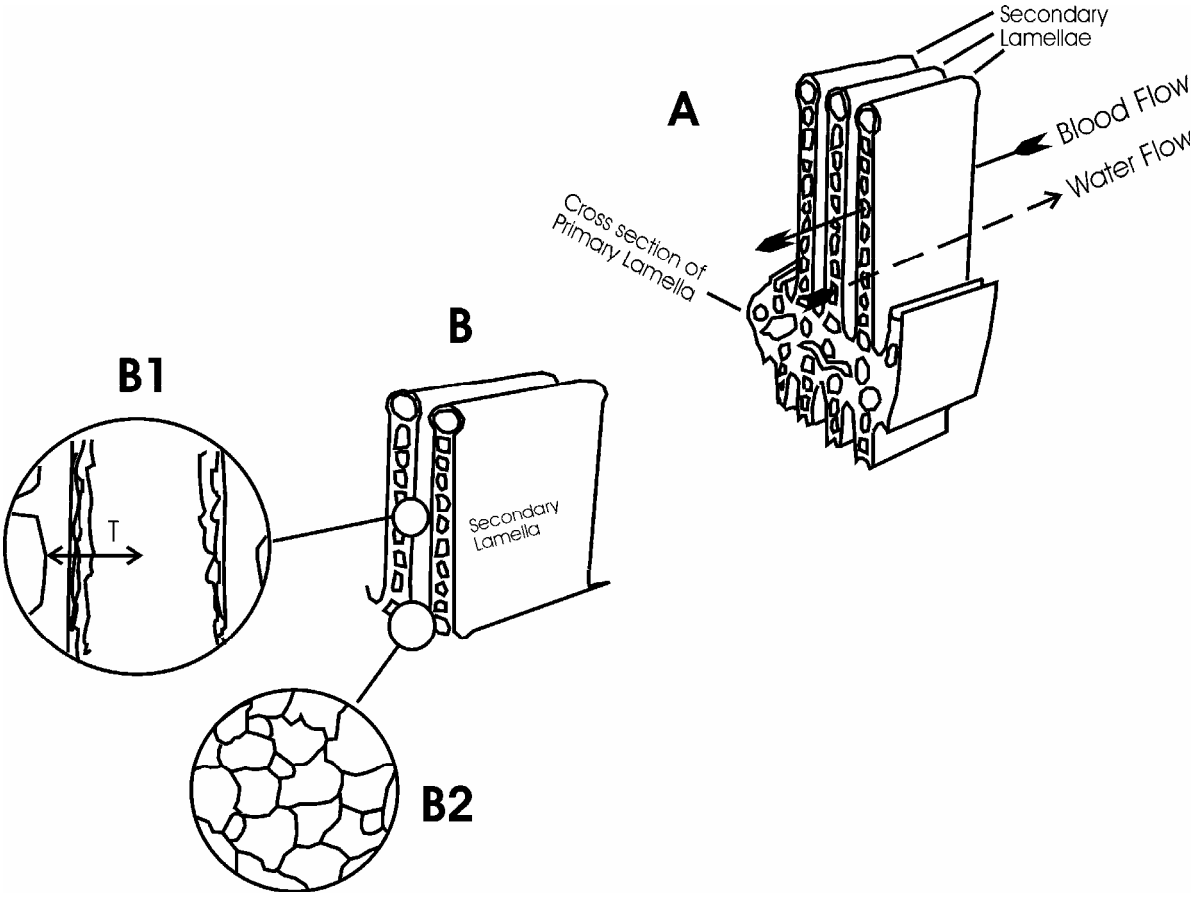

Figure 1. Teleost gill structure. A. Cross section of a primary lamella with three secondary lamellae extending at right angles (upward) from it. Secondary lamellae also extend downward but, in this panel, are not shown completely. Water drawn in via ventilation passes along the surface of each secondary lamella and blood within the lamella flows in the opposite direction (modified from Figure 3 of Randall (1982)). B. Two secondary lamellae with magnified areas B1 and B2. B1. Interlamellar space within which O_2 bearing water passes over the exchange surfaces of the lamellae. Each lamella has a mucus layer and specified thickness. The diffusion distance (T) influences the O_2 diffusion rate across the gill. B2. Cells covering the lamellae. Here cells at the junction of the primary and secondary lamellae are depicted. The pavement cells (white) are the principle cells involved in O_2 exchange. The larger and thicker chloride cells (dark) are involved primarily with ion regulation. Although not depicted, mucus-producing cells on the gill also respond to metal exposure. Pillar cells also reduce the gill area for O_2 exchange. Evans (1998) estimates that as much as 30% of the gill surface is directly above pillar cells and unavailable for respiratory exchange.

Hypoxia Effects

Abrupt exposure to hypoxic conditions results in predictable changes in respiration as the fish adjusts to the change in the O_2 partial pressure gradient across the gill. Initially, ventilation and general activity level change in an attempt to maintain adequate oxygen delivery to tissues (Wu 2002). An extreme example is the eelpout, which becomes immobile under hypoxic conditions (Fischer *et al.* 1992). As an example of change in ventilation, Randall (1982) reports that gill water flow of dogfish is inversely related to arterial O_2 concentration. Increased ventilation volume is generally achieved by large changes in ventilatory stroke volume and smaller changes in ventilation frequency (Gilmour 1998), suggesting respiratory impairment; water softness-induced increases in chloride cells also results in elevated ventilation stroke volume and lowered ventilation frequency (Bindon *et al.* 1994). Heart stroke volume can increase and heart rate decrease under hypoxic conditions (Randall 1982). These changes are energetically efficient, short-term means of coping with hypoxia.

Other compensatory responses to hypoxia can enhance O_2 diffusion rates (Gilmour 1998). Changes in ventilation and thinning of the epithelium due to increased blood pressure tend to reduce the O_2 diffusion barrier thickness. Increased water and blood flow can also modify the difference in O_2 partial pressures across the gill.

Joint Effects of Hypoxia and Metals

There is a commonality in the effects on fishes from hypoxia and metals: asphyxiation. This can be easily described with Fick's model for O_2 diffusion rate (dO_2/dt) (Table 1):

$$\frac{dO_2}{dt} = A \cdot K_{O_2} \cdot \frac{\Delta P_{O_2}}{T}$$

where ΔP_{O2} = difference in O_2 partial pressures across the gill diffusional barrier, K_{O2} = gill barrier diffusion coefficient, A = the effective diffusion area of the gills, and T = the diffusion barrier thickness. By definition, hypoxia changes the gill partial pressure differential (ΔP_{O2}). Modifications to ventilation and cardiac dynamics have the purpose of minimizing changes to this differential. The gill barrier diffusion coefficient (K_{O2}) can be increased by mucus and metal precipitates. The morphological changes to primary and secondary lamellae can decrease the effective diffusion area (A). Combined, the morphological changes and mucus increase the diffusion barrier thickness.

Table 1. Effects summary for hypoxia and acute metal exposures contributing to asphyxiation. Terms in the left column are those of Fick's equation for O_2 diffusion rate.

Term	Hypoxia	Acute Metal Exposure
A (effective diffusion area)	Increased slightly	Decreased
K_{O2} (diffusion coefficient)	Decreased slightly	Increased
ΔP_{O2} (partial pressure difference)	Greatly increased	None
T (diffusion barrier thickness)	Decreased slightly	Increased

Both acute metal exposure and hypoxia reduce the O_2 diffusion rate across the gills. Some metal-induced changes, such as excessive mucus production, might be coincident with those changes associated with hypoxia. A sudden release of hypoxic, metal-rich water from a lake hypolimnion is one situation in which this might occur. In one of many such situations, Baden *et al.* (1995) described the simultaneous exposure of decapods to low oxygen and high manganese conditions during periodic autumnal hypoxia resulting from coastal eutrophication. Some metal-induced changes in gill morphology could occur prior to the hypoxic event of concern and predispose an individual to succumb more quickly. Any exposure to high metal concentration (or soft water (Greco *et al.* 1995, Perry 1998)) prior to a low oxygen event could do this.

MODELING JOINT METAL-HYPOXIA MORTALITY

Assuming that asphyxiation has a common mode-of-action suggests the application of similar joint action models to predict the combined effects of low O_2 and high metal conditions. For concentration-effect experiments in which the proportion of exposed individuals that die is scored at a set exposure duration, a general approach exists for incorporating the joint effects of two or more similarly acting stressors. Finney (1947) established this approach by first observing that similarly acting stressors often have concentration-response curves with identical slopes (b).

$$\Pr obit(P_1) = a_1 + b\ (\log C_1)$$
$$\Pr obit(P_2) = a_2 + b(\log C_2)$$

where, P_1 and P_2 = proportions dying after exposure to stressor 1 or 2, a_1 and a_2 = intercepts for stressors 1 and 2, and C_1 and C_2 = concentrations for stressors 1 and 2.

By combining and then re-arranging these equations, the joint effect of the two stressors (Probit ($P_1 + P_2$)) can be predicted for the binary mixture.

$$\log \phi_2 = \frac{a_2 - a_1}{b}$$

$$\Pr obit(P_1 + P_2) = a_1 + b(\log C_1 + \phi_2(\log C_2))$$

Unfortunately, this conventional model is not directly applicable because the slope for the metal concentration-effect model would be positive and that for the O_2 concentration-effect model would be negative. That is, mortality increases as metal concentration increases or as O_2 concentration decreases. Despite a deviation from convention, the two probit models can be combined and successfully rearranged. Specifically, the O_2 concentration can be expressed as the absolute deviation from normoxic O_2 concentration. The slope for the O_2 concentration-effect model would then be positive.

A more direct model formulation might be possible if a common, physiologically-based metric of stressor intensity were available. Fick's equation suggests O_2 diffusion rate might be the most appropriate metric that reflects the common effect of both low O_2 and high metal concentrations.

$$\log it(P) \; or \; \Pr obit(P) = a + b_1(\log \frac{dO_2}{dt})$$

The O_2 diffusion rate would be estimated with Fick's equation. This model is likely to be appropriate only below a threshold O_2 diffusion rate; thus a threshold value might also need to be estimated. The threshold would reflect the minimum O_2 diffusion rate below which the tissue O_2 demands are not met. A physiologically-based, toxicokinetics-toxicodynamics model could facilitate such a formulation but, in many cases, would likely require more parameter estimates than one has the resources and time to generate.

Instead, a simpler logistic or probit model could be applied with O_2 and metal concentrations as covariates (C_1 and C_2). The coefficients (b_1 and b_2) would have opposite signs in this case. The O_2 concentrations for which the model would be appropriate would be those below some minimum threshold, *i.e.*, below normoxic conditions. Such a model would not strictly require a common mode-of-action.

$$\log it(P) \; or \; \Pr obit(P) = a + b_1(\log C_1) + b_2(\log C_2)$$

Choosing between the logistic (logit) and normal (probit) models would require a goodness-of-fit statistic such as the χ^2 statistic. Although log transformations of both concentration variables are shown here, a χ^2 statistic could also be used to select the best transformations of concentrations. Whether expressed as a logit or probit model, the model could include an interaction term if warranted.

$$\log it(P) \; or \; \Pr obit(P) = a + b_1(\log C_1) + b_2(\log C_2) + b_{12}(\log C_1 \cdot \log C_2)$$

These same models could be used if previous metal exposure had occurred and the deaths associated with an hypoxic event were to be predicted. The fish might be classified with a categorical variable relative to whether it had or had not been previously exposed to high metal concentrations. This common approach taken by epidemiologists (*e.g.*, Ahlbom 1993) to estimate relative risks for etiological factors would allow one to estimate the increase in risk of mortality under hypoxic conditions as a function of past metal exposure. The logistic model including log O_2 concentration and the categorical variable (previously exposed to metal or not) would be the following:

$$Logit(P) = \ln(\frac{P}{1-P}) = a + b_1(\log C_1) + b_2(E)$$

where P = proportion of individuals exposed to hypoxic conditions that die, a = intercept, b_1 = coefficient for the effect of log O_2 concentration, C_1 = oxygen concentration, b_2 = coefficient for metal exposure status effect, and E = a categorical score denoting whether an individual had (1) or had not (0) been previously exposed to high concentrations of metal. The risk of an individual previously exposed to metal relative to that of an individual with no previous metal exposure would be the following (modified from Ahlborn (1993)),

$$e^{b_2} = \frac{\frac{P_e}{1-P_e}}{\frac{P_n}{1-P_n}} = RR$$

where P_e and P_n = the proportion of individuals dying during hypoxic exposure with previous metal exposure (e) and non-previous metal exposure (n) respectively. The relative risk (RR) can be approximated with the estimated b_2 of the logistic model fit to these data.

Another set of models would be useful if survival time were used as the effect metric instead of the proportion dying by a set time. In such a design, individuals are exposed to lethal conditions and the time required to die for each individual is recorded. A rich array of survival time methods is available (see Newman 1995 and Crane *et al.* 2002). Most such methods accommodate censoring, *i.e.*, a time-to-death data set in which some individuals were still alive at the end of the exposure. These methods have been applied to separate effects of oxygen (*e.g.*, Dixon and Newman 1991) and metals (*e.g.*, Newman 1995). Roy and Campbell (1995) used these methods for the joint effects of aluminum and zinc on Atlantic salmon juveniles. There is no apparent reason they could not be applied to the joint effects of hypoxia and metal exposure. As a particularly relevant example, Veldhuizen-Tsoerkan *et al.* (1991) concluded from survival time analyses that mussels previously exposed to metals were less tolerant of anoxia. For this metric, simple parametric models can be generated.

$$\ln TTD = a + b_1(\log\ C_1) + b_2(\log\ C_2) + \varepsilon$$

where TTD = time-to-death and ε = an error term that has a specified distribution. Such a simple model could include O_2 and metal concentrations, or some transformation of these concentrations, as covariates to predict time-to-death during acute, joint exposures. If the situation of concern was survival during exposure to hypoxic conditions for fish with different metal exposure histories (*i.e.*, fish with modified gill structure), a similar model could be applied in which O_2 concentration was included as a continuous variable and past metal exposure status was included as a categorical variable.

CONCLUSION

The parsimonious assumption that asphyxiation is a common mode-of-action for both hypoxia and metal toxicity suggests that a wide range of models could be used to predict the combined effects of hypoxia and high metal concentrations (Figure 2). Such models were described for two relevant scenarios. The first is the simultaneous exposure of fish to high metal and low O_2 concentrations. This might occur during the sudden mixing of oxic and anoxic waters. The second scenario involves an initial exposure to metals resulting in gill morphology changes that make individuals more susceptible to the lethal effects of hypoxia. This might occur in fish surviving a hypoxic/high metal event that are exposed again to low O_2 conditions.

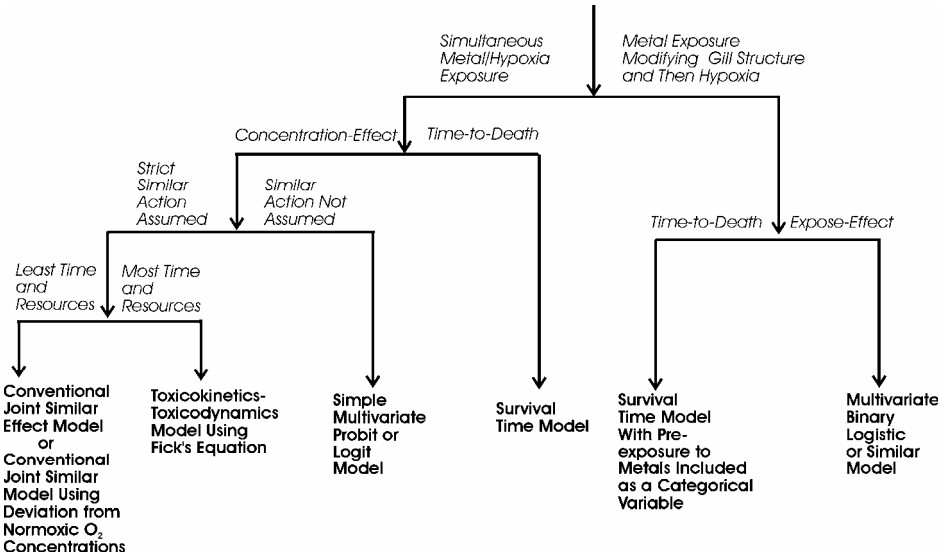

Figure 2. A summary of the potentially useful models for predicting the combined effect of hypoxia and elevated metal concentrations. Models are separated into those dealing with simultaneous exposure to low O_2 and high metal concentrations, and those dealing with low O_2 tolerance after fish gills have been modified during a previous exposure to high metal concentrations. Models that can be derived using conventional concentration-effect or time-to-death data are also shown.

Models for both scenarios were discussed for data sets from conventional concentration-effect and survival time experiments. A wide array of plausible models exists, suggesting that predictive models can be developed for the combined effects of hypoxia and high metal concentrations. However, deciding which model will be the most effective requires the application of candidate models to other appropriate data sets.

REFERENCES

Ahlbom, A. 1993. Biostatistics for Epidemiologists. CRC/Lewis Press. Boca Raton, FL.

Arllano, J.M., J. Blasco, J.B. Oritz, D. Capeta-Da Silva, A. Navarro, M.J. Sanchez-Cel Pino, and C. Sarasquete. 2000. Accumulation and histopathological effects of copper in gills and liver of Senegales sole, *Solea senegalensis* and toad fish, *Halobatrachus didactylus*. Ecotoxicology and Environmental Restoration 3:22-28.

Baden, S.P., S.P. Eriksson, and J.M. Weeks. 1995. Uptake, accumulation and regulation of manganese during experimental hypoxia and normoxia by the decapod *Nephrops norvegicus* (L.). Marine Pollution Bulletin 31:93-102.

Bindon, S.D., K.M. Gilmour, J.C. Fenwick, and S.F. Perry. 1994. The effects of branchial chloride cell proliferation on respiratory function in the rainbow trout *Oncorhynchus mykiss*. Journal of Experimental Biology 197: 47-63.

Crane, M., M.C. Newman, P.F. Chapman, and J. Fenlon. 2002. Risk Assessment with Time to Event Models. Lewis/CRC Press. Boca Raton, FL.

Daoust, P.Y., G. Wobeser, and J.D. Newstead. 1984. Acute pathological effects of inorganic mercury and copper in gills of rainbow trout. Veterinary Pathology 21:93-101.

Dixon, P.M., and M.C. Newman. 1991. Analyzing toxicity data using statistical models for time-to-death: an introduction. *In:* Metal Ecotoxicology, Concepts & Applications, M.C. Newman and A.W. McIntosh, (eds). CRC/Lewis Press. Boca Raton, FL. pp. 207-242.

Evans, D.H. 1998. The Physiology of Fishes, Second Edition. CRC Press. Boca Raton, FL.

Finney, D.J. 1947. Probit Analysis: A Statistical Treatment of the Sigmoidal Response Curve. Cambridge University Press. Cambridge, UK.

Fischer, P., K. Rademacher, and U. Kils. 1992. In situ investigations on the respiration and behavior of the eelpout *Zoarces viviparous* under short term hypoxia. Marine Biology Letters 2:345-351.

Gilmour, K.M. 1998. Gas exchange. *In:* The Physiology of Fishes, Second Edition, D.H. Evans (ed). CRC Press. Boca Raton, FL. pp. 101-127.

Greco, A.M., K.M. Gilmour, J.C. Fenwick, and S.F. Perry. 1995. The effects of softwater acclimation on respiratory gas transfer in the rainbow trout *Oncorhynchus mykiss*. Journal of Experimental Biology 198:2557-2567.

Jagoe, C.H., A. Faivre, and M.C. Newman. 1996 . Morphological and morphometric changes in the gills of mosquitofish (*Gambusia holbrooki*) after exposure to mercury (II). Aquatic Toxicology 34:163-183.

Leland, H.V. and J.S. Kuwabara. 1985. Trace Metals. *In:* Fundamentals of Aquatic Toxicology, G.M. Rand and S.R. Petrocelli, (eds). Hemisphere Publishing Corp. Washington, DC. pp. 374-415.

Newman, M.C. 1995. Quantitative methods in Aquatic Ecotoxicology. Lewis/CRC Press. Boca Raton, FL.

Perry, S.F. 1998. Relationships between branchial chloride cells and gas transfer in freshwater fish. Comparative Biochemistry and Physiology 119A:9-16.

Poléo, A.B.S. 1995. Aluminum polymerization – a mechanism of acute toxicity of aqueous aluminum to fish. Aquatic Toxicology 31:347-356.

Randall, D. 1982. The control of respiration and circulation in fish during exercise and hypoxia. Journal of Experimental Biology 100:275-288.

Roy, R. and P.G.C. Campbell. 1995. Survival time modeling of exposure of juvenile Atlantic salmon (*Salmon salar*) to mixtures of aluminum and zinc in soft water at low pH. Aquatic Toxicology 33:155-176.

Veldhuizen-Tsoerkan, M.B., D.A. Holwerda, and D.I. Zandee. 1991. Anoxic survival time and metabolic parameters as stress indices in sea mussels exposed to cadmium or polychlorinated biphenyls. Archives of Environmental Contamination and Toxicology 20:259-265.

Witters, H.E., S. Van Puymbroeck, A.J.H.X. Stouthart, and S.E. Wendelaar Bonga. 1996. Physicochemical changes of aluminum in mixing zones: mortality and physiological disturbances in brown trout (*Salmo trutta* L.*)*. Environmental Toxicology & Chemistry 15:986-996.

Wu, R.S.S. 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Pollution Bulletin 45:35-45.

HOW BIOLOGICAL RESPONSES TO HYPOXIA AND EUTROPHICATION MODIFY THE EXPOSURE AND TOXICITY TO FISH OF METALS AND OTHER TRACE POLLUTANTS

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ABSTRACT

It is widely recognized that chemical conditions associated with hypoxia and eutrophication can affect the speciation, bioavailability, and toxicity of metals and other pollutants. However, the role of the biological responses of organisms exposed to these conditions in modifying pollutant exposure and toxicity remains largely unexplored. Aquatic organisms experiencing eutrophication stresses (hypoxia, elevated ammonia and nitrate concentrations, etc.) respond with biochemical, physiological, and behavioral changes. Fish typically respond to hypoxic conditions by: 1) behavioral avoidance, 2) increased ventilation volume, and /or 3) increased reliance on anaerobic metabolism. Behavioral avoidance is often size and age specific, resulting in differential exposure and toxicity among age classes or life stages. Behavioral avoidance of hypoxic conditions also results in habitat compression that can enhance inter- and intraspecies competition and increase exposure to predation. Increased ventilation volume can result in increased extraction of contaminants from the water. Increased water flow can also result in enhanced precipitation of dissolved metals at the gills, with associated impacts on gas exchange and ion regulation. Anaerobic metabolism is less efficient, so increased reliance on the impacted gills results in less available energy for maintenance functions and defensive responses. Hypoxia also decreases the rate of protein synthesis that can affect tissue levels of defensive proteins, such as metallothionein and HSP, or the induction of detoxifying enzymes. Integrating the chemical factors and biological responses associated with eutrophication and hypoxia with responses to other pollutants will provide a more realistic understanding of the combined effects of pollutant releases and habitat degradation.

INTRODUCTION

Eutrophication is often caused by discharges of nutrients into surface waters. These nutrients fertilize phytoplankton and macrophytes that are nutrient limited. Initially, biomass increases, but when these plants die their decomposition results in high biological oxygen demand (BOD) causing hypoxic or anoxic conditions. Discharge of bulk organic pollutants, such as sewage, can also increase BOD and decrease dissolved oxygen levels. Such conditions are widespread in lakes and estuaries around the world due to increased inputs of human and animal wastes and agricultural runoff. Anthropogenic changes in water flows, canopy cover, sediment inputs, and other factors can exacerbate hypoxic conditions.

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Eutrophication, hypoxia, and changes in water chemistry that accompany these conditions can alter the chemical speciation and bioavailability of pollutants, as discussed elsewhere in this volume. These chemical changes are predictable, to a large extent, by well-characterized principles in thermodynamics and kinetics. The stresses associated with eutrophication and hypoxia also have profound biological consequences that influence pollutant uptake and subsequent effects on organisms. These biological responses are often less characterized and their effects and implications for pollutant accumulation are much less understood than the chemical changes. Advances in molecular biology in the past decade have stimulated high quality literature on the effects of low oxygen conditions on the physiology of a variety of aquatic organisms. Nonetheless, studies that integrate biological responses to hypoxia with responses to chronic, sublethal concentrations of pollutants are still quite rare.

To address this latter data deficit, this review examines selected responses of fish to eutrophication, hypoxia, and associated stresses, and explores ways that these responses might act to modulate the accumulation and effects of pollutants. This work concentrates on metals, but considers trace organics where appropriate to illuminate principles or illustrate open questions. This is not intended to be an exhaustive survey of biological responses to either hypoxia or pollutants. Instead, it covers a selected variety of areas where recent advances have occurred in the understanding of biological responses to hypoxia. The intent is to cross-fertilize disciplines that do not often interact, specifically the physiology and ecology of low oxygen stress and ecotoxicology. The hope is that this short paper will spark new investigations of the interactions of hypoxia with effects of metals and trace organic pollutants. Clearly, a better understanding of such interactions will improve evaluations of pollutant effects and risks, but may also lead to a clearer picture of the way organisms cope with a range of environmental stressors.

RESPONSES AT THE BEHAVIORAL LEVEL

Avoidance and Habitat Compression

Fish occupy a three dimensional environment whose maximum boundaries are set by the geographic extent of the water body they inhabit and the water depth. In practice, the habitable volume is often significantly smaller than this maximum volume. Factors that influence the habitable volume include physical parameters, such as temperature, salinity, cover, and dissolved oxygen, and biological parameters, such as the presence of food items and relative paucity of predators. The habitable volume is dynamic and changes over time. For example, a change can occur when portions of the total available volume become temporarily anoxic or hypoxic due to seasonal stratification or ice cover, or because of eutrophication. The decrease in habitable volume associated with this type of environmental change has been termed habitat compression or habitat restriction (*e.g.* Eby and Crowder 2002).

There is abundant literature that demonstrates the ability of fishes to detect and avoid hypoxic conditions. For example, Burleson *et al*. (2001) reported that largemouth bass (*Micropterus salmoides*) avoided water where oxygen values were less than 25% air saturation. Eby and Crowder (2002) concluded that 10 estuarine fish species all avoided areas with dissolved oxygen concentrations < 2 mg / L. However, they also noted that this avoidance was highly dependant on other environmental variables. In other words, behavioral avoidance was, to a large extent, context dependant.

An example of context-dependent behavioral avoidance occurs when fish venture into hypoxic areas where they would not otherwise be found in order to feed. Rahel and Nutzman (1994) observed that mudminnows (*Umbra limi*) were regularly captured in hypoxic waters at the bottom of lakes, although caging experiments demonstrated that they could not survive there. They observed that mudminnows captured from bottom waters had midge (*Chaoborus*) larvae in their stomachs, so the fish were entering hypoxic areas for short periods of foraging, even though they were unable to survive there for extended periods. The authors suggested that feeding in hypoxic waters may be common when food is a limited resource in surface waters. Fish might also enter into waters with less suitable oxygen conditions to avoid predators, or because of temperature constraints.

Fish are able to detect and avoid some pollutants through chemical senses, including smell. Farr *et al*. (1995) demonstrated that fathead minnows (*Pimephales promelas*) could detect and avoid fluoranthene concentrations down to 14 μg/L. Hansen *et al*. (1999) found that salmonids of the genus *Onchorhynchus* could detect and avoid low concentrations of Cu and Co, but not high concentrations. They suggested that high concentrations damaged olfactory tissues, thus preventing detection. Scherer and McNichol (1998) observed behavioral avoidance of Cu, Pb, and Zn in lake whitefish *Coregonus clupeaformis*. They also observed that these fish preferred shade when allowed to choose between shaded and illuminated environments. When metal ions were injected into the preferred shaded areas, avoidance of these ions was strongly suppressed, except at the highest concentrations. The latter observation provides another example of context-dependent behavioral avoidance. Although there have been no studies to date investigating joint avoidance response to pollutants and hypoxia, this is a logical area for future investigations, given that avoidance of both anoxia and pollutants can be modified by the physical and biological environment.

The degree of habitat compression produced by anoxia varies with species, especially among those that differ markedly in their ability to tolerate low dissolved oxygen levels. Still, reduction in habitat volume increases interactions, such as predation, by crowding more organisms into less space. Behavioral response to hypoxia decreases the efficiency of some predators, but increases susceptibility of prey to others. For example, Breitburg *et al.* (1994) found that predation of larval goby (*Gobiosoma bosc*) by sea nettles (*Chrysaora quinquecirrha*) increased with hypoxia, but predation by striped bass (*Morone saxitilis*) and adult goby decreased. The latter authors attributed this to different behavioral responses to hypoxia by the predators: striped bass and adult goby avoided the hypoxic areas whereas sea nettles did not. Additionally, hypoxia may have impaired the ability of the larval goby to avoid the nettles. Predation efficiency in some species is also sensitive to hypoxia. Predation efficiency of

juvenile flounder *Platichthys flesus* on benthic invertebrates was significantly lower at 20% and 30% oxygen saturation compared with 40% and 100% oxygen saturation (Tallqvist *et al.* 1999). Breitburg *et al*. (1997) observed that changes in predator-prey interactions reflected variation in physiological tolerance to low oxygen among species and the effects of low oxygen on the escape behavior of prey, as well as on the swimming and feeding behavior of the predators.

Habitat compression changes interactions, such as predation and competition, and so affects the dynamics of pollutants in food webs, especially those that biomagnify. For example, diet shifts and increased availability of prey could increase both growth rate and accumulation of persistent pollutants, such as organochlorine pesticides or methyl mercury, in predatory fish. Also, there is evidence that behavioral avoidance of hypoxia is size related. Burleson *et al*. (2001) found that in largemouth bass (*Micropterus salmoides*), smaller individuals utilized water with lower oxygen levels better than the larger individuals. Thus, in hypoxic conditions, areas were open to foraging by small bass that were avoided by large bass. This could impact the relative growth rates among fish size classes, as well as the accumulation of persistent pollutants. While speculative, such potential relationships suggest the need for further investigations.

RESPONSES AT THE ORGAN AND TISSUE LEVEL

Gill Function and Morphology

It has long been recognized that there are considerable differences among species in tolerance to hypoxia and anoxia. For example, consider the difference between large scombrids like tuna that are obligate aerobes unable to tolerate dissolved oxygen concentrations below near saturation, and the small cyprinid, *Carassius carrasius*. The latter is essentially a facultative anaerobe able to tolerate long periods of hypoxia in ice-capped lakes that are closed-off from the atmosphere. This ability makes *C. carassius* a common fish in northern Europe and Asia, and often the only fish found in eutrophic boreal lakes. Most fish fall between these extremes, and employ a suite of mechanisms to cope with decreased dissolved oxygen availability in addition to the behavioral responses noted previously. These include biochemical and physiological mechanisms evolved to confer tolerance for hypoxic or anoxic conditions, adjustment of ventilatory parameters, or air breathing.

When dissolved oxygen concentrations decline, some fish are able to partially or completely switch to aerial respiration. For example, the facultative air-breathing fish, *Hypostomus regani,* begins aerial respiration when the oxygen tension in the water falls between 50 and 60 torr (Mattias *et al*. 1998), and succumbs to hypoxia if access to the surface is prevented. There are several anatomically and evolutionarily distinct adaptations for air breathing in fishes (Graham 1997). Extreme examples include the South American lungfish of the genus *Lepidosiren,* an obligate air breather. Lungfishes, including *Lepidosiren*, have paired lungs, while other species, such as gars (*Ginglymodi*) and Bowfin (*Halecomorphi*), can absorb atmospheric oxygen by ventilating modified swim bladders. Tarpon (*Megalops atlanticus*) are facultative air breathers that also possess a modified swim bladder for aerial respiration. Juvenile tarpon appear to switch from aquatic respiration to air breathing when pO_2 falls below

40 torr (Geiger *et al*. 2000). Some species of fish are able to gulp an air bubble from the surface into the buccal cavity and absorb oxygen from it. An example is the black mudfish, *Neochanna diversus*, that begins to gulp air at the surface when dissolved oxygen falls below 1-2 mg/L (McPhail 1999).

There are several broad strategies for coping with environmental hypoxia among fish that are not capable of aerial respiration. Some fish are oxygen conformers, able to switch to metabolic pathways that decrease oxygen requirements, such as increased reliance on glycolysis. Cellular and biochemical responses to accomplish this, and the implications of these responses for pollutant uptake, accumulation, and effects, are discussed in the next section of this paper. Other fish are oxygen regulators that employ various strategies to ensure that oxygen delivery to tissues is resistant to changes in external oxygen concentration. Perhaps the most obvious means of maintaining O_2 uptake is to increase the amount of water flowing over the gills when the oxygen concentration in water decreases. In theory, this could be accomplished by increasing ventilation frequency, ventilation volume, or both. In practice, increasing ventilation frequency is energetically expensive and severely constrained by the physical properties of water. However, many teleosts respond to decreased dissolved oxygen tension by increasing ventilation volume. This is accomplished without increasing, and in some cases decreasing, ventilation frequency, and the net result is increased delivery of water to the gills. Tilapia (*Oreochromis sp*.) progressively increase ventilation volume without changing ventilation frequency with progressive hypoxia (Shezifi *et al.* 1997). Some teleosts also employ mixed strategies to cope with decreasing pO_2. For example, turbot (*Scophthalmus maximus*) increase ventilation volume as pO2 decreases, and at very low concentrations of dissolved oxygen transition to anaerobic metabolism, resulting in liver glycogen depletion and lactate production (Pichavant *et al.* 2002).

It should be noted that, in addition to increasing oxygen delivery to the gills, many fish also have adaptations to enhance oxygen delivery to tissues. Mechanisms include increased hematocrit (increased volume of red cells in the blood), and modifications to oxygen binding proteins such as hemoglobin. Wells *et al*. (1997) observed differences in fish hemoglobin properties related to fish tolerance to hypoxia and exploitation of aerial respiration. Some fishes with high tolerance to hypoxia possess multiple, biochemically distinct forms of hemoglobin that differ in oxygen affinity; examples include the South African mudfish, *Labeo capensis* (Frey *et al.* 1998), and New Zealand triplefins (Tripterygiidae, Brix *et al.* 1999). These multiple hemoglobin isoforms enhance oxygen binding over a range of environmental conditions.

Changes in gill ventilation in response to anoxia can have important implications for contaminant uptake and accumulation. For example, uptake of some organic chemicals by rainbow trout, *Onchorhynchus mykiss,* was dependant on changes in gill flow rates in response to hypoxia (Schmieder and Weber 1992). Uptake of a hydrophobic compound (decanol) increased with water flow, while accumulation of a hydrophilic compound (butanol) did not. This has implications for pharmacokinetic-based models of the uptake of polar and nonpolar compounds in fish (Erickson and McKim 1990) where uptake rates may be limited by water flow. McKim *et al.* (1999) measured changes in respiratory parameters and xenobiotic gill fluxes in lake trout

(*Salvelinus namaycush*). For chemicals with octanol-water partition coefficients (K_{ow}) between 3 and 6, increased ventilation volume, caused by hypoxic conditions, shortened the time necessary to reach steady state concentration in tissues, and reduced the time required to accumulate a toxic dose of these waterborne chemicals.

While many fishes respond to hypoxia by increasing the water volume passing over their gill, this is not necessarily the case for fish that can switch to partial or complete aerial respiration. However, regardless of anatomical adaptations, air breathing fish still maintain some gill ventilation activity during hypoxia. Gills are multifunctional organs, responsible for excretion of metabolic wastes (ammonia and carbon dioxide) and osmotic and ionic regulation, in addition to oxygen uptake. Even when the organism is relying on air as an oxygen source, these other gill functions must continue. It has long been recognized that the capacitance for carbon dioxide is much higher in water than in air, so water represents an almost infinite sink for CO_2 excreted at the gill. Likewise, gill ventilation maintains an essential excretion pathway for nitrogenous wastes. Teleost fish do not produce urine, but instead void nitrogenous wastes as ammonia via the gill. Teleosts also maintain a state of ionic and osmotic disequilibrium with their environment. That is, the body fluids of freshwater fish have much higher concentrations of essential ions such as Na^+, Ca^{2+} and Cl^- than the water surrounding the fish and flowing over the gills. This situation is reversed in marine fish, where environmental concentrations of essential ions exceed concentrations in plasma and other fluids. Thus, freshwater fish face problems of passive ion loss by diffusion and uptake of water by osmosis, whereas marine fish experience these fluxes in the opposite direction. These passive ion fluxes are countered by active ion pumping by specialized cells in the gill and by adaptations to decrease passive permeability, particularly at intercellular junctions. Obviously, all of these functions will be affected to some extent by major alterations in gill ventilation volume.

Fluxes of ions and respiratory gases at the gill can modify the chemistry of the inspired water so that conditions in the region near the gill are quite different from those in the surrounding water. In poorly buffered freshwater, the excretion of metabolic wastes can cause substantial changes in pH as water passes across the gill (Playle and Wood 1989a). These shifts in pH can change speciation of metals and affect solubility and metal-gill binding properties (Playle and Wood 1989b, Playle *et al.* 1992). In some cases, decreased solubility can lead to metal precipitation on the gill, with an associated decrease in gas exchange capacity (Playle and Wood 1991). Under hypoxic conditions, where acquiring sufficient oxygen is already a problem, such precipitation would cause additional stress to fish. Precipitation and accumulation of material at the gill is often accompanied by increased mucus production as a protective mechanism; mucus thickening and clogging of interlamellar spaces can further reduce water flows and the effective gill surface area.

It is common to see references to "the gill membrane" in the literature, as though gills are simple membrane monolayers separating the interior of the organism from the environment. In fact, the teleost gill epithelium is composed of a variety of cell types that can be several layers thick. Cells contacting the surface include pavement epithelial cells, mucous cells, and chloride cells. The latter are rich in Na^+/K^+ ATPase and are involved in active ion transport. Internal layers include pillar cells, neurons, rodlet cells, and dividing, undifferentiated cells that mature

into one of the more differentiated cell types. It is important to emphasize that the gill epithelium is a dynamic system, undergoing continual cell loss, renewal, and differentiation. Most gas exchange occurs along the secondary lamellae that are normally covered by thin, squamous pavement cells. Immediately under these cells, capillaries of thin endothelial cells facilitate oxygen uptake by circulating blood in close proximity to the external water. Environmental conditions can modify this arrangement, causing the lamellar epithelium to thicken by hyperplasia, hypertrophy, edema, or some combination of these.

Gill epithelial thickness is a major factor impacting both diffusion of oxygen between inspired water and blood and the passive fluxes of ions along diffusion gradients. It may be useful here to reverse our initial question about the impacts of hypoxia on pollutant exposure and toxicity, and instead consider the impacts of responses to low pH and certain metals on susceptibility to hypoxia. In freshwater fish, the increased functional surface area of the gill that facilitates oxygen uptake also increases passive ion loss, a tradeoff sometimes called the osmoregulatory compromise (Gonzalez and McDonald 1992). Low pH and/or toxic concentrations of some metals increase passive ion losses (Witters *et al*. 1992, Wood 1992, Grippo and Dunson 1996) that can be compensated, to some extent, by increasing the active uptake of ions. Increasing uptake involves increasing the number of ion transport sites that is accomplished by increasing the number of chloride cells. This results in physical thickening of the epithelia as cells proliferate, subsequently decreasing gas exchange capacity and depressing arterial pO_2 (Bindon *et al.* 1994). This response can cause a decreased ability to acquire oxygen, so fish exposed to metal stresses may be at a considerable disadvantage if environmental oxygen concentrations decrease. Given the interplay between responses to ionoregulatory stress and oxygen uptake, it is likely that fish exposed to elevated concentrations of trace metals such as Al, Be, Cu, Hg, or Zn are more susceptible to hypoxia than unexposed fish. Investigation of responses to these multiple stressors, differences in susceptibility among size classes, life stages, or species, and how these responses relate to changes in population or community structure with chronic exposure would all be fruitful areas for future research.

RESPONSES AT THE CELLULAR AND BIOCHEMICAL LEVEL

Protein Turnover and Cell Defenses

A variety of processes can damage proteins or cause them to become nonfunctional. These processes include reactions with intracellular metals or reactive oxygen species, misfolding or substitution of amino acids, and pH conditions that affect hydrogen bonds and alter primary or quaternary structures. In most cases, damaged proteins are not repaired. Instead, they are broken down and reconstructed using amino acids derived from the diet and the breakdown of somatic protein. These degradation and synthesis processes consume cellular resources, such as amino acids and energy, but are essential in maintaining homeostasis and allowing flexible responses to injury, starvation, toxicant exposure, and other stressors.

Protein synthesis and degradation account for a large fraction of the energy consumed during basal metabolism. In hepatocytes, the main energy sinks under normoxic conditions are: 1) protein synthesis; 2) protein degradation; 3) Na^+/K^+ pumping; 4) urea biosynthesis; and 5) glucose biosynthesis; together, these account for essentially 100% of the ATP production expected from oxygen consumption (Hochachka and Lutz 2001). A number of studies have attempted to estimate the fraction of basal metabolism due to protein synthesis and degradation in animals. Estimates range from 10-80%, with higher values in mammals and lower values in poikilotherms (Hawkins 1985, Aoyagi *et al.* 1988, Houlihan *et al*. 1990, Land *et al.*1993, Fuery *et al.* 1998). Estimates for fish range from 10-40% (Houlihan *et al.* 1988, Carter *et al.* 1993). There are also considerable differences among tissues and environmental conditions, and with the nutritional status of the organism.

Hochachka and Lutz (2001) argue persuasively that, among animals tolerant to prolonged exposure to low oxygen concentrations, the first line of defense against hypoxia is the maintenance of stable adenylate concentrations by coordinated suppression of ATP supply and demand pathways. Suppression of these pathways results in a significantly reduced rate of ATP turnover. Decreased ATP production results from increased reliance on glycolytic pathways that do not require oxygen, and decreased reliance on the Krebs cycle. The net result is a lower yield of ATP per unit of metabolized glucose. Decreased ATP demand results from alterations in membrane permeability and neuron firing frequency (resulting in lower energy demands for ion pumping), and from a large decline in protein turnover (Hochachka and Lutz 2001).

A number of recent studies support the idea that protein turnover is greatly reduced under hypoxic or anoxic conditions. Fraser *et al.* (2001) reported that protein synthesis decreased below measurable levels in heart, lungs, liver, brain, intestine, and muscle of slider turtles (*Trachemys scripta elegans*) after one to six hours of anoxia exposure. Mente *et al.* (2003) concluded that protein synthesis in the crab *Carcinus maenas* was limited by arterial pO_2 after observing that low pO_2 blocked protein synthesis after feeding. This blockage, however, progressively dissipated as water (and arterial) pO_2 increased. Smith *et al.* (1999) found that crucian carp (*Carassius carassius*) reduced protein synthesis in liver, heart, and red and white muscle with anoxia, but not in brain tissue. It is important to note that these studies measured gross protein synthesis by incorporation of labeled amino acids, and that this technique cannot determine protein breakdown during the experimental period if the labeled amino acid is recycled into new protein. While protein turnover is a dynamic interaction of synthesis and degradation rates, the decreased incorporation of labeled amino acids is consistent with decreased net turnover.

Cellular responses to pollutants or other xenobiotics often involve induction of defensive proteins and/or up-regulation of enzymatic pathways that detoxify, sequester, or eliminate toxins. Examples include: induction of CYP1A (cytochrome p450:1a) and EROD (7-ethoxyresorufin-O-deethylase) to metabolize organic pollutants such as polyaromatic hydrocarbons (PAHs); induction of molecular chaperones such as heat shock proteins in response to a variety of stressors; increased concentrations of intracellular glutathione upon oxidative stress; and increased intracellular concentrations of metallothionein upon metal exposure. In some circumstances, pollutant stress depletes cellular protein stores, necessitating protein synthesis

as a recovery response. As an example, glutathione reserves can be depleted upon exposure to oxidative substances. Under hypoxic conditions where protein synthesis is down-regulated, the ability of animals to carry out defensive responses that require synthesis of new protein can be compromised.

In contrast to the decrease in protein synthesis that accompanies anoxia or hypoxia, there is evidence that some stresses associated with pollutant exposure increase protein turnover. Wilson *et al*. (1996) found that exposure to Al at low pH increased rates of both protein synthesis and degradation in gills of juvenile rainbow trout. They suggested this might represent a chronic cost of gill repair or an ongoing acclimatory process. Reid *et al.* (1998) observed that juvenile rainbow trout exposed to 70 μM ammonia in hard water increased rates of gill protein synthesis. However, decreased rates of protein synthesis were observed in gills of rainbow trout exposed to low pH alone (Wilson *et al.* 1996, Reid *et al.* 1997). In all of the above studies, dietary ration and temperature were also identified as key variables influencing protein turnover.

While protein turnover has traditionally been measured by injecting captive animals with ^{3}H-labeled amino acid and measuring its incorporation into tissues, recent work suggests an alternate method that may be applicable to free-living animals. The ratio of nitrogen isotopes ^{15}N / ^{14}N, expressed as $\delta^{15}N$, can be used as an indicator of trophic status because amine groups bearing ^{14}N are deaminated or transaminated at faster rates than those containing ^{15}N. This results in an increase in $\delta^{15}N$ of about 3-5 o/oo (per mil) with each trophic level (DeNiro and Epstein 1981). Similarly, $\delta^{15}N$ increases in animals on protein limited diets due to preferential retention of ^{15}N from catabolized protein, and so can serve as an index of nutritional status (Hobson *et al.* 1993). These observations suggest that $\delta^{15}N$ might also be a useful index of relative protein turnover rates among tissues or with different environmental conditions, including pollutant stress (Shaw-Allen 2002). Several studies support this idea, including the observation that $\delta^{15}N$ can be enriched up to 50 o/oo in plants exposed to ozone in the laboratory (Hofmann *et al.* 1997). Most recently, Shaw-Allen (2002) found $\delta^{15}N$ increased in tissues of snowy egrets (*Egretta thula*) fed a diet high in methylmercury compared to those receiving a control diet. She also found that $\delta^{15}N$ increased in the acid soluble protein fraction (which includes metallothionein and glutathione) in livers of largemouth bass (*Micropterus salmoides*) fed a high mercury diet relative to controls. This observation suggests an increased rate of protein turnover in this fraction (Shaw-Allen 2002). These results are consistent with the concept that certain pollutants cause an increase in protein turnover as cellular defenses are marshaled. In contrast, hypoxia and anoxia would be expected to cause relatively lower $\delta^{15}N$ ratios in tissues due to decreased protein synthesis. This suggests that caution should be used when interpreting $\delta^{15}N$ ratios in animals that have experienced chronic anoxia or hypoxia, whether for the purposes of assessing responses to pollutant stress, or for evaluation of trophic position or nutritional status.

To further illustrate protein turnover responses to pollutant exposure, consider the induction of glutathione (GSH) and metallothionein (MT), two proteins involved in cellular defense responses to a number of pollutants. Both MT and GSH are small, acid-soluble peptides composed entirely of non-essential amino acids. MT is typically composed of 60-68 amino acid

residues, including about 20 cysteines; it binds and regulates essential trace metals such as Cu and Zn, and also has a high affinity for non-essential toxic metals such as Hg, Ag, and Cd (Kagi 1991). GSH is a tripeptide (glutamic acid, cysteine, and glycine) that acts as a non-enzymatic reducing agent to neutralize reactive oxygen species and bind metals (Cnubben *et al.* 2001).

The potential for reactive oxygen species to react with and damage macromolecules such as DNA has been well documented. Cells have evolved defenses against oxygen radicals, including enzymatic systems, such as superoxide dismutase and peroxidase, and non-enzymatic systems, such as GSH. The level and redox status of GSH respond to hypoxia and oxygen supersaturation in fish (Ross *et al*. 2001), and recovery from hypoxia or exposure to high concentrations of dissolved oxygen can increase concentrations of tissue GSH and activities of enzymes involved in its synthesis and cycling. GSH concentrations in fish tissues also increase in response to exposure to some metals, including Pb and Cd (Thomas and Juedes 1992, Schlenk and Rice 1998). Defenses against oxidative DNA damage also include induction or activation of DNA repair enzymes. All of these responses are impacted by alterations in protein synthesis due to hypoxia. Liepelt *et al.* (1995) exposed rainbow trout to hypoxic, normoxic, and hyperoxic conditions and observed that frequencies of single-strand breaks in DNA increased in both hypoxic and hyperoxic treatments. While such increases under hyperoxia could be due to increased concentration of reactive oxygen species, increases under hypoxia are consistent with the idea that defensive responses are suppressed.

Tissue MT concentrations in fish increase in response to exposure to a variety of metals, including Zn, Cd, Hg, and Ag (Roesijadi 1992, Cosson 1994, Schlenk *et al.* 1995). Also, there is evidence for increased MT concentrations in response to hypoxia in mammalian tumor cells (Raleigh et al. 1998), although the functional role of this increase remains cryptic. While there are no studies to date regarding the joint effects of hypoxia and toxic metals on MT and GSH responses in fish, known effects of oxygen deprivation on protein synthesis suggest that increases of GSH and MT in response to metal exposure could be delayed or blocked by hypoxic or anoxic conditions. If pollutants cause depletion of GSH reserves, such depletion could be more profound under low oxygen conditions. In the same manner, other responses that involve synthesis of new proteins, such as repair of cellular damage, increased enzymatic activities of Na^+/K^+ ATPase, or the induction of detoxifying systems, might be impaired by the down-regulation of protein turnover accompanying hypoxia.

Finally, Wu *et al*. (2003) recently found that hypoxia impaired reproductive function in fish, including decreased gonadal development and spawning success. They reported that hypoxia decreased serum triiodothyronine, estradiol, and testosterone and suggested hypoxia-induced decreases in expression of cytochrome p450 as a possible mechanism. Regardless of the exact mechanism, this finding is also consistent with the concept of hypoxia-induced down-regulation of protein synthesis. Additionally, hypoxia-induced suppression of the p450 pathway would impact responses to xenobiotics that are metabolized by this pathway (Kim and Sheen 2000). This latter statement emphasizes an important consideration when interpreting responses to potential endocrine disruptors in the environment: chronic exposures to hypoxia and other stressors must be considered when establishing causal relationships between endocrine disruption and xenobiotic exposure.

CONCLUSION

In most field situations, fish are likely to experience multiple stressors in a complex environment. Given the widespread distribution of eutrophic and hypoxic environments, combined with ongoing discharges of metals through mining and industrial operations, there is a good chance of overlapping exposures to both these conditions. While the interactions of hypoxia and trace pollutants, such as heavy metals, are often poorly understood, recent work in diverse fields suggests possible responses at multiple levels of biological organization. Knowledge of these interactions and responses can improve assessment of risks and our understanding of the consequences of pollutant exposures in complex, real-world environments

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REFERENCES

Aoyagi, Y., I. Tasaki, J.I. Okumura and T. Mutramatsu. 1988. Energy cost of whole body protein synthesis measured *in vivo* in chicks. Comparative Biochemistry and Physiology 91A:765-768.

Bindon, S.D., K.M. Gilmour, J.C. Fenwick and S.F. Perry. 1994. The effects of branchial chloride cell proliferation on respiratory function in the rainbow trout *Onchorynchus mykiss*. Journal of Experimental Biology 197: 47-63.

Breitburg, D.L., N. Steinberg, S. DuBeau, C. Cooksey and E.D. Houde. 1994. Effects of low dissolved oxygen on predation on estuarine fish larvae. Marine Ecology Progress Series 104:235-246.

Breitburg, D.L., C.A. Pacey, and A. Gerstein. 1997. Varying effects of low dissolved oxygen on trophic interactions in an estuarine food web. Ecological Monographs 67:489-507.

Brix, O., K.D. Clements and R.M.G. Wells. 1999. Haemoglobin components and oxygen transport in relation to habitat distribution in triplefin fishes (Tripterygiidae). Journal of Comparative Physiology Series B, Systematic and Comparative Physiology 169:329-334.

Burleson, M.L., D.R. Wilhelm and N.J. Smatresk. 2001. The influence of fish size on the avoidance of hypoxia and oxygen selection by largemouth bass. Journal of Fish Biology 59:1336-1349.

Carter, C., D.F. Houlihan, J.G. Brechlin and I. McCarthy. 1993. The relationships between protein intake and protein accretion, synthesis and retention efficiency for individual grass carp *Ctenopharyngodon idella* (Val.). Canadian Journal of Zoology 71:393-400.

Cnubben, N.H.P., I.M.C.M. Rietjens, H. Wortelboer, J. van Zanden and P.J. van Bladeren. 2001. The interplay of glutahione-related processes in antioxidant defense. Environmental Toxicology and Pharmacology 10:141-152.

Cosson, R.P. 1994. Heavy-metal intracellular balance and relationship with metallothionein induction in the liver of carp after contamination by silver, cadmium and mercury following or not following pretreatment by zinc. Biometals 7:9-19.

DeNiro, M.J. and S. Epstein. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochimica et Cosmochimica Acta 45:341-351.

Eby, L.A. and L.B. Crowder. 2002. Hypoxia-based habitat compression in the Neuse River Estuary: context-dependant shifts in behavioral avoidance thresholds. Canadian Journal of Fisheries and Aquatic Sciences 59:952-965.

Erickson, R.J. and J.M. McKim. 1990. A model for the exchange of organic chemicals at fish gills: flow and diffusion limitations. Aquatic Toxicology 18:175-198.

Farr, A.J., C. Chabot, C. and D.H. Taylor. 1995. Behavioral avoidance of fluoranthene by fathead minnows (*Pimephales promelas*). Neurotoxicology and Teratology 17:265-271.

Fraser, K.P.P., H. D.F., P.L. Lutz, S. Leone-Kabler, L. Manuel, and J.G. Brechin. 2001. Complete suppression of protein synthesis during anoxia with no post-anoxia protein synthesis debt in the red-eared slider turtle *Trachemys scripta elegans*. Journal of Experimental Biology 204:4353-4360.

Frey, B.W., R.E. Weber, W.J. van Aardt, and A. Fago. 1998. The haemoglobin system of the mudfish, *Labeo capensis*: adaptations to temperature and hypoxia. Comparative Biochemistry and Physiology 120B:735-742.

Fuery, C.J., P.C. Withers, and M. Guppy. 1998. Protein synthesis in the liver of *Bufo marinus*: cost and contribution to oxygen consumption. Comparative Biochemistry and Physiology 119A:459-467.

Geiger, S.P., J.J. Torres, and R.E. Crabtree. 2000. Air breathing and gill ventilation frequencies in juvenile tarpon, *Megalops atlanticus*: responses to changes in dissolved oxygen, temperature, hydrogen sulfide and pH. Environmental Biology of Fishes 59:181-190.

Gonzalez, R.J. and D.G. McDonald. 1992. The relationship between oxygen consumption and ion loss in a freshwater fish. Journal of Experimental Biology 163:317-322.

Graham, J.B. 1997. Air Breathing Fishes: Evolution, Diversity and Adaptation. Academic Press, San Diego.

Grippo R.S. and W.A. Dunson. 1996. The body ion loss biomarker .1. Interactions between trace metals and low pH in reconstituted coal mine-polluted water. Environmental Toxicology and Chemistry 15:1955-1963.

Hansen, J.A., J.C.A. Marr, J. Lipton, D. Cacela, and H.L. Bergman. 1999. Differences in neurobehavioral responses of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) exposed to copper and cobalt: behavioral avoidance. Environmental Toxicology and Chemistry 9:1972-1978.

Hawkins, A.J.S. 1985. Relationships between the synthesis and breakdown of protein, dietary absorption and turnovers of nitrogen and carbon in the blue mussel *Mytilis edulis* L. Oecologia 66:42-49.

Hobson, K.A., R.T. Alisaukas, and R.G. Clark. 1993. Stable nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analysis of diet. Condor 95:388-394.

Hochachka, P.W. and P.L. Lutz. 2001. Mechanism, origin and evolution of anoxia tolerance in animals. Comparative Biochemistry and Physiology 130B:435-159.

Hofmann, D., K. Jung, J. Bender, M. Gehre, and G. Schuurmann. 1997. Using natural isotope variations of nitrogen in plants as an early indicator of air pollution stress. Journal of Mass Spectroscopy 32:855-863.

Houlihan, D.F., S.J. Hall, C. Gray, and B.S. Noble. 1988. Growth rates and protein turnover in Atlantic cod, *Gadus morhua*. Canadian Journal of Fisheries and Aquatic Sciences 43:951-964.

Houlihan, D.F., C.P. Warnig, F. Mutters, and C. Gray. 1990. Protein synthesis and oxygen consumption of the shore crab *Carcinus maenas* after a meal. Physiologial Zoology 63:735-756.

Kagi, J.H.R. 1991. Overview of Metallothionein. *In:* Methods in Enzymology: Metallobiochemistry Part B, J.F Riordan and B.F. Vallee, (eds.). Academic Press, Orlando FL. Pp. 613-636.

Kim, J.E. and Y.Y. Sheen. 2000. Inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-stimulated Cyp1a1 promoter activity by hypoxic agents. Biochemical Pharmacology 59:1549-1566.

Land, S.C., L.T. Buck, and P.W. Hochachka. 1993. Response of protein-synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. American Journal of Physiology 265:R41-R48.

Liepelt, A., L. Karbe, and J. Westendorf. 1995. Induction of DNA strand breaks in Rainbow Trout *Onchorhynchus mykiss* under hypoxic and hyperoxic conditions. Aquatic Toxicology 33:177-181.

Mattias, A.T., F.T. Rantin, and M.N. Fernandes. 1998. Gill respiratory parameters during progressive hypoxia in the facultative air-breathing fish, *Hypostomus regani* (Loricariidae). Comparative Biochemistry and Physiology 120A:311-315.

McKim, J.M., G.J. Lien, A.D. Hoffman, and C.T. Jenson. 1999. Respiratory-cardiovascular physiology and xenobiotic gill flux in the lake trout Salvelinus namaycush. Comparative Biochemistry and Physiology 123A:69-81.

McPhail, J.D. 1999. A fish out of water: observations on the ability of black mudfish, *Neochanna diversus*, to withstand hypoxic water and drought. New Zealand Journal of Marine and Freshwater Research 33:417-424.

Mente, E., A. Legeay, D.F. Houlihan, and J.C. Massabuau. 2003. Influence of oxygen partial pressures on protein synthesis in feeding crabs. American Journal of Physiology, Regulatory, Integrative and Comparative Physiology 284:500-510.

Pichavant, K., V. Maxime, M.T. Thebault, H. Ollivier, J.P. Garnier, B. Bousquet, M. Diouris, G. Boeuf, and G. Nonnotte. 2002. Effects of hypoxia and subsequent recovery on turbot *Scophthalmus maximus*: Hormonal changes and anaerobic metabolism. Marine Ecology Progress Series 225:275-285.

Playle, R.C., R.W. Gensamer, and D.G. Dixon. 1992. Copper accumulation on gills of fathead minnows - influence of water hardness, complexation and pH on the gill microenvironment. Environmental Toxicology and Chemistry 11:381-391.

Playle, R.C. and C. M. Wood. 1991. Mechanisms of aluminum extraction and accumulation at the gills of rainbow trout Onchorhynchus mykiss (Walbaum) in acidic soft water. Journal of Fish Biology 38:791-805

Playle, R.C. and C. M. Wood. 1989a. Water chemistry changes in the gill microenvironment of rainbow trout - experimental observations and theory. Journal of Comparative Physiology Series B, Systematic and Comparative Physiology 159:527-537.

Playle, R.C. and C. M. Wood. 1989b. Water pH and aluminum chemistry in the gill microenvironment of rainbow trout during acid and aluminum exposures. Journal of Comparative Physiology Series B, Systematic and Comparative Physiology 159:539-550

Rahel, F.J. and J.W. Nutzman. 1994. Foraging in a lethal environment: fish predation in hypoxic waters of a stratified lake. Ecology 75:1246-1253.

Raleigh, J.A., S.C. Chou, L. Tables, S. Suchindran, M.A. Varia, and M.R. Horsman. 1998. Relationship of hypoxia to metallothionein expression in murine tumors. International Journal of Radiation Oncology 42:727-730.

Reid, S.D., J.J. Dockray, T.K. Linton, D.G. McDonald, and C.M. Wood. 1997. Effects of chronic environmental acidification and a summer global warming scenario: Protein synthesis in juvenile rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Sciences 54:2014-2024.

Reid, S.D., T.K. Linton, J.J. Dockray, D.G.McDonald, and C.M. Wood. 1998. Effects of chronic sublethal ammonia and a simulated summer global warming scenario: protein synthesis in juvenile rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Sciences 55:1534-1544.

Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquatic Toxicology 22:81-114.

Ross, S.W., D.A. Dalton, S. Kramer, and B.L. Christensen. 2001. Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. Comparative Biochemistry and Physiology 130C:289-293.

Scherer, E. and R.E. McNichol. 1998. Preference-avoidance responses of lake whitefish (*Coregonus clupeaformis*) to competing gradients of light and copper, lead, and zinc. Water Research 32:924-929.

Schlenk, D., Y.S. Zhang, and J. Nix. 1995. Expression of hepatic metallothionein messenger RNA in feral and caged fish species correlates with muscle mercury levels. Ecotoxicology and Environmental Safety 31:282-286.

Schlenk, D. and C.D. Rice. 1998. Effect of zinc and cadmium treatment on hydrogen peroxide-induced mortality and expression of glutathione and metallothionein in a teleost hepatoma cell line. Aquatic Toxicology 43:121-129.

Schmeider, P.K. and L.J. Weber. 1992. Blood and water flow limitations on gill uptake of organic chemicals in the rainbow trout *Onchorynchus mykiss*. Aquatic Toxicology 24:103-121.

Shaw-Allen, P.L. 2002. Delta 15-N distribution as a marker linking altered metabolism of protein resources to mercury exposure. Ph.D. Dissertation, University of Georgia, Athens GA.

Shezifi, Y., E. Kimmel and A. Diamant. 1997. The electrophysiological response of fish to hypoxia. Aquacultural Engineering 16:253-259.

Smith, R.W., D. Houlihan, G.E. Nilsson, and J. Alexandre. 1999. Tissue-specific changes in RNA synthesis in vivo during anoxia in crucian carp. American Journal of Physiology, Regulatory, Integrative and Comparative Physiology 46:690-697.

Tallqvist, M., E. Sandberg-Kilpi., and E. Bonsdorff. 1999. Juvenile flounder, *Platichthys flesus* (L.), under hypoxia: effects on tolerance, ventilation rate and predation efficiency. Journal of Experimental Marine Biology and Ecology 1:75-93.

Thomas, P. and M.J. Juedes. 1992. Influence of lead on the glutathione status of Atlantic croaker tissues. Aquatic Toxicology 23:11-30.

Wells, R.M.G., J. Baldwin, R.S. Seymour, and R.E. Weber. 1997. Blood oxygen transport and hemoglobin function in three tropical fish species from northern Australian freshwater billabongs. Fish Physiology and Biochemistry 16:247-258.

Wilson, R.W., C.M. Wood, and D.F. Houlihan. 1996. Growth and protein turnover during acclimation to acid and aluminum in juvenile rainbow trout (*Oncorhynchus mykiss*). Canadian Journal of Fisheries and Aquatic Sciences 53:802-811.

Witters, H.E., S. Vanpuymbroeck, and O. Vanderborght. 1992. Branchial and renal ion fluxes and transepithelial electrical potential differences in rainbow trout *Onchorynchus mykiss* - effects of aluminum at low pH. Environmental Biology of Fishes 34:197-206.

Wood, C.M. 1992. Flux measurements as indices of H^+ and metal effects on freshwater fish. Aquatic Toxicology 22:239-264.

Wu, R.S.S., B.S. Zhou, D.J. Randall, N.Y.S. Woo, and P.K.S. Lam. 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environmental Science and Technology 37:1137-1141.

HOW MIGHT HYPOXIA AFFECT METAL SPECIATION, ACCUMULATION AND TOXICITY – SOME SPECULATION

Peter G.C. Campbell[1]

ABSTRACT

The Free-Ion Model, and its derivative the Biotic Ligand Model (BLM), are designed to predict how (dissolved) metals interact with, and eventually affect, aquatic organisms. Given a reasonably complete set of water quality parameters (*e.g.*, total dissolved metal concentrations, pH, [Ca], [Mg], $[CO_3^{2-}]$, $[SO_4^{2-}]$, $[Cl^-]$, [dissolved organic matter]), it is now possible to predict the toxicity of many metals to common (freshwater) test species. However, virtually all these fish studies have been carried out in the laboratory, under well-oxygenated conditions. How might hypoxia affect these relationships? To explore this question, we considered two aspects: (i) redox chemistry associated with the development of hypoxia, and its effects on metal speciation and bioavailability; and (ii) respiratory response associated with decreased dissolved oxygen concentrations, and its effects on metal uptake.

The development of reducing conditions may affect the metal itself (*e.g.*, Fe, Mn, Cu, Hg) and the nature of the metal-binding ligands present in the water column (*e.g.*, $SO_4 \rightarrow S_2O_3^{2-} \rightarrow S^{2-}$). Hypoxic layers often overlie truly anoxic conditions, and thus are subject to a continual influx of reduced metal species such as Fe(II) and Mn(II), leading to marked increases in their aqueous concentrations and raising the possibility that Fe or Mn can compete with toxic metals for uptake. For a redox-sensitive trace metal such as Hg, the development of hypoxic conditions can lead to a change in oxidation state, *e.g.* Hg(II) $\rightarrow$ Hg(0), with obvious implications for the bioavailability and inherent toxicity of the metal. Hypoxic conditions also influence the redox chemistry of sulfur. Sulfate reduction can give rise to various metastable intermediates, including thiosulfate and more reduced forms of sulfur, including polysulfides and sulfide clusters. The tendency of these forms to react with metals increases markedly as the sulfur becomes more reduced (*i.e.*, $K_{M\text{-}SO4} < K_{M\text{-}S2O3} < K_{M\text{-}S}$). Thus, even the speciation of redox-insensitive metals can be affected by the development of hypoxia. The overall implications of these changes on metal uptake and toxicity are discussed.

In addition to the redox chemistry associated with the development of hypoxia, there are also biological consequences of decreased ambient oxygen concentrations. Low dissolved oxygen levels are known to affect the ventilation rates of filter-feeding organisms and fish, and thus the rate at which water is moved past the gills. Within the construct of the BLM, it is (tacitly) assumed that metal uptake and toxicity are unaffected by ventilation rates. Examples from the literature are used to examine the validity of this assumption.

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INTRODUCTION

Hypoxia[2] is not uncommon in freshwater ecosystems. Low oxygen conditions are found in eutrophic lakes subject to stratification (where the hypolimnion will often become anoxic during summer, leading to hypoxic conditions in the metalimnion), in lakes in northern climates during late winter (where there is minimal re-oxygenation under ice cover), and in freshly flooded reservoirs (where the biodegradation of terrestrial organic matter leads to an increased benthic oxygen demand). Similarly, running waters that receive large inputs of biodegradable organic matter typically develop hypoxic conditions in the mixing zone downstream. Such conditions of oxygen deficit are particularly prevalent in slow-moving, low-gradient rivers in summer months, where they can experience marked diurnal fluctuations.

Given the high incidence of hypoxia in freshwater systems, it seems worthwhile to explore how the presence of hypoxic conditions might affect the bioavailability of trace metals to aquatic organisms. Metals of concern are those that exist in natural waters as dissolved cations (*e.g.*, Cd, Cu, Fe, Mn, Zn); we will not deal with metalloids such as As or Se, nor with oxyanions such as CrO_4, MoO_4 or VO_4. First, we will review how metals interact with aquatic organisms, then present the Biotic Ligand Model (BLM) approach and outline current thinking about how metal speciation affects metal bioavailability. In the second section, we will consider the redox chemistry associated with the development of hypoxia and its effects on metal speciation and bioavailability. The final section is focused on the physiological / respiratory response associated with the development of hypoxia and its effects on metal uptake.

BIOTIC LIGAND MODEL

The Biotic Ligand Model (BLM) is designed to predict how (dissolved) metals interact with, and eventually affect, aquatic organisms (Di Toro *et al.* 2001, Gorsuch *et al.* 2002). The basic premise underlying the model is that a metal must first interact with an external biological membrane if it is to accumulate within an organism and/or provoke a biological effect. For water-breathing aquatic organisms and waterborne metals, the gill will normally be the primary uptake site and, in addition, will often be the site of toxic impact (Wood 2001). This metal-gill interaction can be envisaged as a surface complexation reaction, involving the metal and a range of potential binding sites. These sites can be divided into two classes: physiologically inert sites, where the metal can "collect" without inducing any toxicity, and physiologically active sites, where metal binding leads to deleterious effects. In this latter case, metal binding can directly affect the organism's metabolism if the binding site corresponds to a membrane-bound ion transporter, or indirectly, if the bound metal is subsequently transported across the plasma membrane into the gill. Once within the gill, the metal can interact with a variety of intracellular sites, resulting in either positive or negative consequences.

[2] Hypoxia defined as conditions with low ambient dissolved oxygen concentrations, *e.g.* $[O_2] < 2$ mg/L.

Within this general model (Figure 1), the interaction of a waterborne metal with an aquatic organism will involve the following steps: (i) diffusion of the metal from the bulk solution to the gill surface; (ii) sorption/surface complexation of the metal at passive binding sites within the protective layer (*e.g.*, mucus), or at sites on the outer surface of the apical gill membrane; and (iii) uptake or "internalization" of the metal (transport across the apical membrane, followed in many cases by transport across the basolateral membrane and entry into the circulatory system). Given this construct, one normally makes a number of simplifying assumptions (Campbell 1995, Van Leeuwen 1999, Di Toro *et al.* 2001):

a. metal transport in solution towards the gill membrane, and the subsequent surface complexation reaction occur "rapidly", such that an equilibrium is established between metal species in the bulk solution and those at the biological surface ("rapid" = faster than metal uptake, faster than the expression of the biological response);

b. the plasma membrane is the primary site for metal interactions with living organisms (*i.e.*, the "physiologically active" sites referred to earlier are embedded in the plasma membrane), and this interaction occurs via a ligand exchange reaction, yielding M-X-gill, (Figure 1, equilibria K_2 or K_3);

c. the biological response, whether it be metal uptake or toxicity, is dependent on the concentration of the M-X-gill surface complex {M-X-gill}; in those cases where X-gill corresponds to a membrane transport site, metal internalization involves cation transport;

d. variations of {M-X-gill} as a function of $[M^{z+}]$ in solution follow a Langmuir-type adsorption isotherm; provided the concentration of free sites $\{^{-}X\text{-gill}\}$ remains relatively constant in the range of metal concentrations of interest, variations in {M-X-gill} will follow those of $[M^{z+}]$ in solution;

e. during exposure to the metal of interest, the nature of the gill surface remains constant (*i.e.*, the metal does not induce any changes in the nature of the plasma membrane).

If these assumptions are valid, then at constant pH and constant hardness the biological response of the exposed organism should vary as a function of the free-metal ion activity in the exposure solution. Indeed, in the first formal presentation of this conceptual model of metal-organism interactions, Morel (1983) suggested that "the most important result to emerge is the universal importance of the free-metal ion activities in determining the uptake, nutrition and toxicity of all cationic trace metals." Recent results suggest the need to attenuate this statement (*e.g.*, Campbell *et al.* 2002), but nevertheless, the importance of the free-metal ion activity as a predictor of metal bioavailability remains indisputable.[3]

[3] In hindsight, the use of the expression "Free-Ion Activity Model", or "Free-Ion Model", to describe metal-organism interactions was perhaps regrettable, since it focused attention on the free-metal ion alone, to the exclusion of other factors such as pH and water hardness ($[Ca^{2+}]$, $[Mg^{2+}]$) that are also known to affect metal bioavailability. One of the advantages of the Biotic Ligand Model is that it subtly shifts the emphasis from the exposure solution to the biological receptor, *i.e.* from the free-metal ion activity in solution to the biotic ligand to which the metal binds (*i.e.*, {M-X-gill} or {M-X-membrane}).

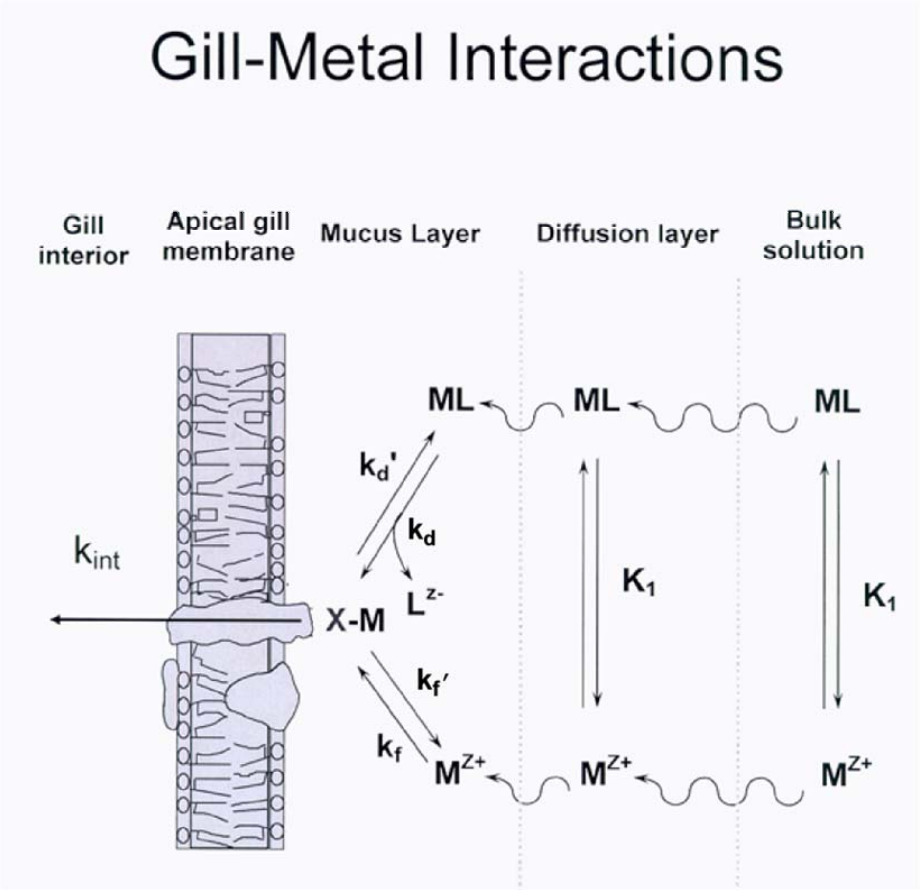

Figure 1. Conceptual model of gill-metal interactions. M^{z+} = free-metal ion; ML = metal complex in solution; K_1 = equilibrium constant for the formation of ML; M-X-membrane = surface metal complex; k_f, k_f' = rate constants for formation of the surface complex; k_d, k_d' = rate constants for dissociation of the surface complex; k_{int} = rate constant for "internalization" or transport of the metal across the biological membrane. Charges on ligand not shown for simplicity. [Modified from Campbell 1995].

Many early insights in this area came from studies with marine and freshwater algae (Campbell 1995), but in recent years researchers have shown that a similar approach can be applied to predict acute toxicity of metals to fish (Gorsuch *et al.* 2002). Given a reasonable set of water quality parameters (*e.g.* total dissolved metal concentrations, pH, [Ca], [Mg], $[SO_4^{2-}]$, $[CO_3^{2-}]$, $[Cl^-]$, dissolved organic carbon concentration), it is now possible to predict the toxicity of many metals to common freshwater test species (Table 1). However, all these fish studies were carried out in the laboratory, under well-oxygenated conditions. How might the development of hypoxia affect these relationships? To explore this question, we will consider two aspects: (i) the redox chemistry associated with the development of hypoxia, and its effects on metal speciation and bioavailability; and (ii) the respiratory response associated with the development of hypoxia, and its effects on metal uptake and toxicity.

Table 1. Summary of metals for which the "Biotic Ligand Model" approach has been developed.

Metal	Organism	Reference	Remarks
Ag	rainbow trout (*Oncorhynchus mykiss*)	McGeer *et al.* 2000 Schwartz and Playle 2001 Paquin *et al.* 2002	
	daphnid (*Daphnia magna*; *D. pulex*)	Bury *et al.* 2002	
Cu	rainbow trout (*O. mykiss*)	MacRae *et al.* 1999 Di Toro *et al.* 2000	
	fathead minnow (*Pimephales promelas*)	Santore *et al.* 2001	
	daphnid (*D. magna*)	De Schamphelaere and Janssen 2002, De Schamphelaere *et al.* 2002	includes field validation
	daphnid (*D. pulex*; *Ceriodaphnia dubia*)	Santore *et al.* 2001	
	unicellular alga (*Pseudokirchneriella subcapitata*)	De Schamphelaere *et al.* 2003	
Ni	fathead minnow (*P. promelas*)	Pyle *et al.* 2001 Meyer *et al.* 1999	larval stage
Pb	rainbow trout (*O. mykiss*)	MacDonald *et al.* 2002	
Zn	rainbow trout (*O. mykiss*)	Santore *et al.* 2002	
	fathead minnow (*P. promelas*)	Santore *et al.* 2002	
	daphnid (*D. magna*)	Santore *et al.* 2002 Heijerick *et al.* 2002a	
	unicellular alga (*P. subcapitata*)	Heijerick *et al.* 2002b	

REDOX CHEMISTRY

Although the redox potential of a water body can be a useful qualitative characterization of a system, there is no single system redox potential that represents all various redox couples. Oxygen is the ultimate electron acceptor in waters containing even less than 1 mg $O_2 \cdot L^{-1}$. A measure of its tendency to oxidize other solutes is given by the pε of the oxygen/water couple:

$$¼\ O_2 + H^+ + e^- = ½\ H_2O; \qquad p\varepsilon^o = 20.75$$

At pH 7 and $p_{O2} = 10^{-0.7}$:

$$p\varepsilon = p\varepsilon^o - \log \{1 / [(P_{O2})^{-1/4} \times (H^+)]\} = 13.6 \text{ (}i.e.\text{, } p\varepsilon = 20.6 - pH)$$

In anoxic systems the redox potential will normally be governed by the oxidation/reduction reactions of sulfur. Turner *et al.* (1981) defined the lower pε limit for such waters by the expression:

$$p\varepsilon = (34 - 9\ pH)/8$$

However, because of rather slow reaction kinetics, the redox potentials calculated from the various couples of redox-sensitive elements rarely show any direct relationship with oxygen concentrations. In effect, the rates of transformation between different oxidation states are highly variable (often much slower than the rates of complexation/dissociation reactions), and they are often strongly affected by such environmental factors as temperature, pH, UV irradiation, the presence of catalytic surfaces, and the concentration/nature of the dissolved organic matter present. A key point is the interplay between the time scales for physical mixing (advection) and chemical reactions (oxidation / reduction) (see discussion in Morgan and Stone 1985).

Metals

The development of reducing conditions can affect the metal itself (*e.g.*, Fe, Mn, Cu, Hg) and the nature of the metal-binding ligands present in the water column. We will consider first the metal itself. For Fe and Mn, oxidation and/or reduction rates have been determined under typical environmental conditions, either in the laboratory (simulated conditions) or in field experiments. These experiments reveal important differences in the redox chemistries of Fe and Mn: reduction of Mn(IV) oxides to dissolved Mn(II) occurs at higher redox potentials (*i.e.*, higher O_2 concentrations) than does the Fe(III) to Fe(II) reduction; when oxygen is reintroduced into the system, the oxidation of dissolved Fe(II) to particulate Fe(III) occurs much more rapidly than does the oxidation of Mn(II) to Mn(IV) oxide. As a result of these differences, Fe and Mn have different temporal and spatial distributions in systems that undergo fluctuating oxygen depletion events (Sholkovitz 1985).

There is, however, a further complication. In the laboratory under axenic conditions, the oxidation-reduction reactions of Fe and Mn often exhibit slow reaction kinetics. In nature, however, these reactions are often mediated by micro-organisms and consequently can be accelerated. These kinetic uncertainties make metal speciation particularly difficult to predict under hypoxic conditions. In general terms, one might anticipate reduction of Mn(IV) to Mn(II) and Fe(III) to Fe(II), leading to marked increases in their aqueous concentrations and raising the possibility that they compete with toxic metals for uptake. As mentioned previously, Mn(II) is only slowly re-oxidized and tends to persist longer than Fe(II) under hypoxic conditions. Hypoxic layers often overlie truly anoxic conditions, and thus are subject to a continual influx of

reduced species such as Fe(II) and Mn(II). The re-oxidation of Fe(II) to amorphous Fe(III) oxyhydroxides will generate colloidal and particulate material that, in turn, will tend to adsorb various cationic species and remove them from solution (*cf.* studies on the environmental impacts of dredging; Brannon *et al.* 1980).

For a redox-sensitive trace metal such as Cu or Hg, the development of hypoxic conditions leads to a change in oxidation state, *e.g.* Cu(II) → Cu(I) or Hg(II) → Hg(0), with obvious implications for the bioavailability and inherent toxicity of the metal (see Table 2). However, for these metals very few pertinent kinetic data are available (for Hg, see Amyot *et al.* 1997, Morel *et al.* 1998).

Table 2. Influence of the oxidation state of metals on their geochemical mobility and bioavailability (adapted from NRCC 1988).

Metal	Mobility / bioavailability ranking	Remarks
Fe	Fe(II) >> Fe(III)	Fe(III) species insoluble; if present, Fe(III) competes strongly for available ligands
Mn	Mn(II) >> Mn (IV)	Mn(IV) species highly insoluble; Mn(II) persistent in (sub)oxic environments
Cu	Cu(I) ~ Cu(II)	Cu(I) highly polarizable ("soft" cation); high affinity for reduced sulfur
Hg	Hg(0) >> Hg(II)	Hg(0) volatile, lost to atmosphere; Hg(II) highly polarizable ("soft" cation) with high affinity for reduced sulfur
Cr	Cr(III) << Cr(VI)	Cr(III) strongly adsorbed; very low dissolved concentrations; Cr(VI) exists as oxyanion, in solution

Ligands

In addition to affecting metal geochemistry *per se*, the development of hypoxic conditions also influences the nature of metal-binding ligands present in the water column. Of particular interest are the various sulfur-containing ligands (*e.g.*, $SO_4 \rightarrow S_2O_3^{2-} \rightarrow S^{2-}$). Sulfate reduction can give rise to various metastable intermediates, including thiosulfate ($S_2O_3^{2-}$) and more reduced forms of sulfur, including polysulfides and sulfide clusters. The tendency of these forms to react with metals increases markedly as the sulfur becomes more reduced (*i.e.*, $K_{M\text{-}SO4} < K_{M\text{-}S2O3} < K_{M\text{-}S}$). Sulfate itself forms only weak, outer-sphere complexes with cationic

metals, but thiosulfate and sulfide form stable, inner-sphere complexes. These latter complexes are particularly important for polarizable (*i.e.*, "soft" or "class B") cations such as Ag, Cd, and Hg.

Although sulfate reduction will not occur until all the ambient oxygen has been consumed, the products of sulfate reduction will often be present under suboxic conditions. As was discussed previously in the case of Mn(II) and Fe(II), hypoxic and anoxic environments often are juxtaposed, and the hypoxic compartment is subject to a continual influx of reduced species from the anoxic layer. Until recently, geochemists generally assumed that the lifetime of sulfides in oxic environments was very short. It is now recognized, however, that some fully reduced sulfur(II) species persist for long periods in oxic natural waters, despite their inherent thermodynamic instability in the presence of oxygen (Rozan *et al.* 1999, 2000). The precise nature of these species remains controversial, but laboratory experiments suggest that their stability in the presence of oxygen is due to their association with zinc or other metals, *i.e.* free sulfide is rapidly oxidized in the presence of oxygen, but only very slowly in the presence of such metals as Cu and Zn (Wang *et al.* 2003).

The steady-state concentrations of these reduced sulfur species, measured in oxic surface waters and expressed nominally as $[S^{2-}]$, appear to be sufficient to interact with ambient levels of mercury, silver, cadmium, copper and zinc (Rozan *et al.* 2000, Smith *et al.* 2002, Bianchani and Bowles, 2002). Thus, the speciation of "soft", redox-insensitive trace metals is also affected indirectly by the development of hypoxia.

Physiological/Biological Consequences

What are the biological consequences of the geochemical changes described in the previous section? From a purely geochemical point of view, one could argue that suboxic conditions will normally lead to decreased metal bioavailability because of the following factors: (i) the presence of metastable cationic reduced species, such as Fe^{2+} and Mn^{2+}, will compete with the trace metals for binding to the "biotic ligand" and thus render the trace metals less bioavailable; (ii) the oxidation of the reduced forms of Fe and Mn, yielding particulate Fe and Mn oxyhydroxides, will tend to scavenge trace metal cations from the solution (analogous to dredging operations) and thus reduce their bioavailability; or (iii) the persistence of reduced sulfur species, *e.g.*, as polysulfide clusters (possibly coated with, or buried within, natural organic matter), will also tend to scavenge "soft" metals from the solution. The best examples of effects (ii) and (iii) come from the sediment dredging literature, where studies in the plume downstream from an active dredging operation consistently show decreased concentrations of dissolved trace metals relative to the conditions upstream (Peddicord, 1987).

There are, however, at least two instances where suboxic conditions might result in enhanced metal bioavailability. Such an effect might be observed if the changes in metal speciation induced by hypoxia favor metal bioavailability / uptake (*e.g.*, by association with thiosulfate as a rogue ligand), or if the higher ventilation rates associated with hypoxia favor metal uptake. We will discuss these two examples in the final section.

EFFECTS OF METAL SPECIATION ON METAL UPTAKE

Thiosulfate

As described in the BLM section of this paper, in aquatic toxicology it is generally accepted that complexation of a metal leads to a decrease in its bioavailability. In effect, most dissolved ligands that bind metals form hydrophilic complexes, $ML_n^{\pm}$, and in such systems metal uptake, nutrition, and toxicity normally vary as a function of the concentration of the free-metal cation in solution. Exceptions to this simple model of metal toxicity generally involve ligands that form lipophilic complexes, ML_n^0 that can bypass normal metal transport mechanisms and cross biological membranes by simple diffusion. However, a number of intriguing metal toxicity experiments have also been reported in the literature where the metal's "residual" bioavailability in the presence of hydrophilic $ML_n^{\pm}$ complexes exceeded that which would have been predicted on the basis of the free-metal ion concentration at equilibrium.

Most of these latter exceptions to the BLM involve assimilable organic ligands, and this has led to the suggestion that "accidental" metal transport may occur in their presence (*i.e.*, the ligand is assimilated as a metal-ligand complex and the metal "comes along for the ride"). In principle, uptake of intact hydrophilic metal-ligand complexes could also occur with inorganic ligands such as chloride or sulfate / thiosulfate. Uptake systems for such essential nutrient anions exist at biological interfaces; if these transport systems could be "fooled" into binding and transporting the intact metal-anion complex, then the metal would find its way into the cell "accidentally". We recently demonstrated such "accidental" uptake of the Ag-thiosulfate complex by freshwater unicellular algae (Fortin and Campbell 2001).

The binding of Ag by thiosulfate ($AgS_2O_3^-$, $Ag(S_2O_3)_2^{3-}$: $\log K_1 = 8.82$, $\log \beta_2 = 13.50$) reduces the free Ag^+ concentration and, according to the BLM, should reduce Ag bioavailability. We reasoned that if Ag could cross biological membranes as the Ag-thiosulfate complex via an anion transporter, then Ag uptake would exceed that predicted by the BLM. We set out to test this "molecular mimicry" hypothesis using a unicellular alga as our biological model. Since algae are known to possess membrane-bound transport systems for the assimilation of sulfate (Hodson *et al.* 1968, Perez-Castiñeira *et al.* 1998), they were appropriate models for testing the hypothesis that thiosulfate (and 1:1 Ag-thiosulfate complexes) can mimic sulfate and enter the cells via the same pathway.

Our metal uptake and toxicity experiments were carried-out with *Chlamydomonas reinhardtii*, a unicellular green alga, as described in Fortin and Campbell (2001). The term "metal uptake" refers to intracellular metal; Ag adsorbed to the algal surface at the end of the exposure periods was displaced by exchange with non-radioactive metal. Short-term (<30 min) Ag uptake was determined in three media, all of which contained 10 nM free Ag^+ (Table 3). Medium A contained nitrate, but no thiosulfate, and virtually all the Ag was present as free aquo-ion; medium B contained both Ag and thiosulfate (114 nM), with the total concentration of Ag increased (104 nM) so as to maintain the desired free Ag concentration (10 nM); medium C was identical to B except that the sulfate normally present in the algal medium (81 μM) was removed.

Table 3. Media used to determine short-term (<30 min) Ag uptake in the unicellular green alga, *Chlamydomonas reinhardtii*.

Medium ($[Ag^+]$=10 nM)	total dissolved Ag (nM)	thiosulfate (nM)	sulfate (μM)
A	10	0	81
B	104	114	81
C	104	114	0

Since the free Ag concentration was identical in each exposure medium, the BLM predicted that Ag uptake should be identical in all three experiments. Contrary to this prediction, Ag uptake by *C. reinhardtii* was strongly affected by the anions present in the exposure medium (Figure 2). Silver accumulation after 25 min increased in the following order: 1 (control) < 5 ($S_2O_3^{2-}$, normal sulfate) < 14 ($S_2O_3^{2-}$, no sulfate). We concluded (i) that the enhanced uptake observed in the presence of thiosulfate is the result of Ag-thiosulfate complexes being transported across the plasma membrane via sulfate / thiosulfate transport systems, and (ii) that this membrane transport mechanism is affected by the external sulfate concentration. Note that removal of sulfate did not affect Ag uptake when chloride was used as the complexing ligand, a result that clearly supports the contention that a sulfate / thiosulfate transporter is involved in Ag uptake in the presence of thiosulfate. In the absence of thiosulfate, Ag is taken-up via a cation transporter (probably via a Cu(I) transport system) and this transporter is unaffected by changes in ambient sulfate concentrations. In the thiosulfate media, however, a second parallel pathway for Ag uptake is introduced, involving the accidental transport of Ag-thiosulfate complexes via one or more sulfate / thiosulfate transporters (Figure 3).

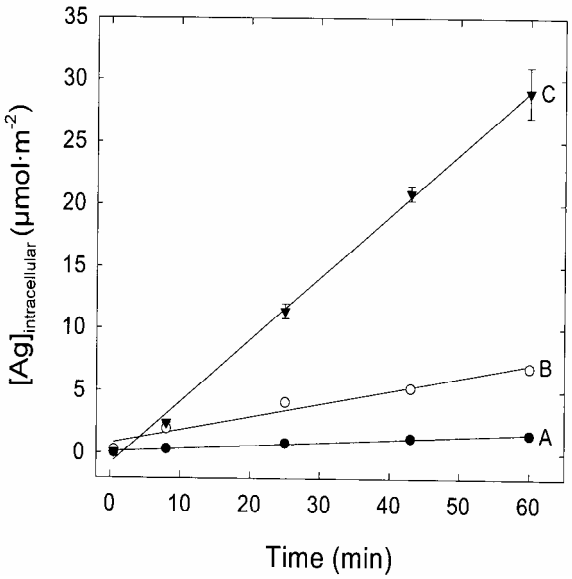

Figure 2. Time course of silver uptake by the unicellular alga *Chlamydomonas reinhardtii* at constant Ag^+ (10 nM). (A) Uptake in the absence of thiosulfate and the presence of sulfate. (B) Uptake in the presence of both thiosulfate and sulfate. (C) Uptake in the presence of thiosulfate but the absence of sulfate. Error bars represent standard deviations from the average of three measurements. [Modified from Fortin and Campbell 2001].

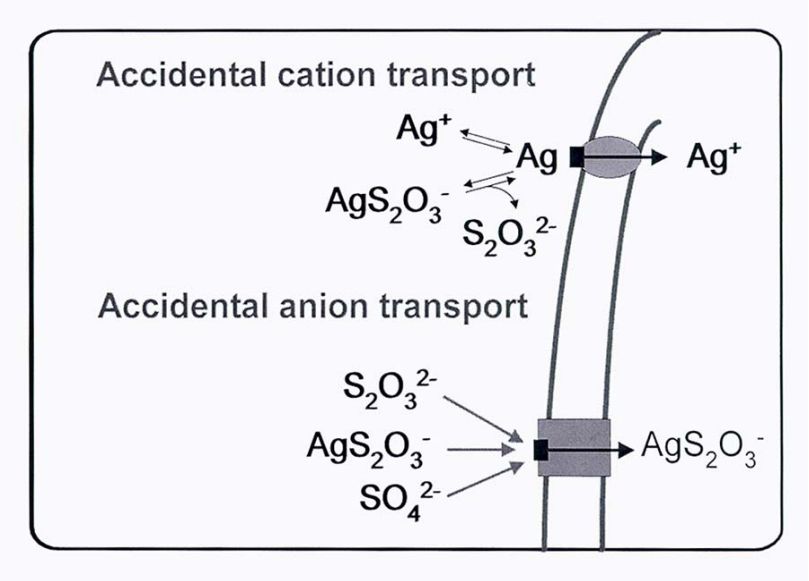

Figure 3. Conceptual model of silver interactions with transport systems at an algal membrane in the presence of sulfate and thiosulfate [Modified from Campbell *et al.* 2002].

The prevailing paradigm for metal uptake by aquatic organisms, *i.e.* the BLM, assumes that metals enter living cells via facilitated cation transport. Most known exceptions to the BLM involve either ligands that form lipophilic complexes, $M\text{-}L_n^o$, that can bypass normal metal transport mechanisms and cross biological membranes by simple diffusion, or "chaperone" ligands that are synthesized by living micro-organisms specifically to complex essential metals and facilitate their eventual uptake (*e.g.*, the role of siderophores in iron nutrition). In contrast, evidence of metal uptake through anion transport systems is scarce to non-existent. To our knowledge, the results with Ag thiosulfate represent the first hard evidence for metal transport into cells via an inorganic anion transport system.

It is likely that uptake of the anionic Ag-thiosulfate complex will occur in other algal species and bacteria that have sulfate / thiosulfate transport systems or thiosulfate-specific transport systems. Indeed, there are several reports in the literature that sulfate and thiosulfate share a common membrane transport system in bacteria (Sirko *et al.* 1995) and algae (Hodson *et al.* 1968). A competitive effect between sulfate and thiosulfate has been noted in sulfate uptake experiments with *C. reinhardtii* (Perez-Castiñeira *et al.* 1998); thiosulfate is an efficient inhibitor of sulfate uptake. In addition, several algal species can grow on thiosulfate as a sole sulfur source (Hodson *et al.* 1968, Perez-Castiñeira *et al.* 1998).

Extrapolation of the thiosulfate example from microorganisms to higher organisms is difficult, since little is known about sulfate transport mechanisms in epithelial cells (*i.e.*, gills, intestine). However, in laboratory water-borne exposure experiments, Ag accumulation by rainbow trout, *Oncorhynchus mykiss*, was inexplicably enhanced in the presence of thiosulfate (Hogstrand *et al.* 1996, Wood *et al.* 1996). It is tempting to explain this greater-than-expected Ag accumulation by generalizing our conceptual model of Ag accumulation, but thiosulfate uptake by fish remains to be demonstrated.

Sulfide Clusters

The recent discovery of metal-rich reduced sulfur species in oxic waters has stimulated some exploratory experiments designed to determine their bioavailability (the assumption is that sulfur ligands should bind soft metals and reduce the free metal concentration in solution). Specifically, experiments were carried-out in the laboratory to determine the effect of "reactive sulfides" on the acute toxicity of Ag (I) to *Daphnia magna* (Bianchani and Bowles 2002, Bianchani *et al.* 2002a, Bowles *et al.* 2002b). Zinc sulfide clusters were synthesized in the laboratory (Bowles *et al.* 2002a) and used as a surrogate for the metal-rich, reduced sulfur species found in natural waters. These clusters are thought to include a range of molecular conformations with basic molecular components of possibly Zn_3S_3 and $Zn_4S_6^{2-}$ (see Luther *et al.* 1999).

Silver toxicity was determined in acute toxicity tests (48 h) performed with *D. magna* neonates in the absence (< 5 nM) and presence of low (~25 nM) or high (~250 nM) concentrations of zinc sulfide clusters under oxic conditions. The high sulfide concentrations (~250 nM) completely protected against toxicity up to the highest Ag concentration tested (19 nM). In the presence of environmentally realistic sulfide levels (~25 nM), acute Ag toxicity was about 5.5 fold lower (Figure 4). This decrease in toxicity was attributed to the binding of Ag to the zinc sulfide clusters, yielding non-filterable forms of Ag (chemisorbed Ag-sulfide forms). Interestingly, the presence of sulfide did not prevent the bioaccumulation of Ag by *D. magna*. On the contrary, Ag bioaccumulation was greater in daphnids exposed to Ag in the presence of sulfide than in its absence, even though acute toxicity was not observed under the former conditions. In control experiments with daphnids that had been killed by exposure to low temperature before Ag exposure, Ag accumulation was very low and unaffected by the presence of sulfide in the test solution. Subsequent work with live daphnids exposed to Ag in the presence of sulfide showed that the Ag was incorporated into the daphnids' intestinal tract, not simply adsorbed to their surface (Bianchini *et al.* 2002b). This disconnect between Ag toxicity and Ag accumulation (or Ag body burden) remains to be clarified.

EFFECTS OF HYPOXIC CONDITIONS ON VENTILATION RATES, AND ON METAL UPTAKE RATES

Fish

There is a general view in the toxicological literature that higher ventilation rates should lead to higher metal uptake rates, but there are very few hard data to back up this contention. Lloyd (1961) and Lloyd and Herbert (1962) studied the relationship between the toxicity of metals and oxygen levels in water. Rainbow trout were exposed to graded concentrations of Zn at different dissolved-oxygen concentrations, yielding a series of log survival time *versus* log [Zn] curves for each oxygen concentration (Figure 5). Note that none of the oxygen concentrations were low enough to be lethal in the absence of Zn. From this and similar experiments with Cu and Pb, these authors extracted values of the ratio X_s/X, where X_s is the threshold metal concentration with oxygen at the air-saturation value and X is the lethal

threshold metal concentration at a lower oxygen concentration (Figure 6). In all cases, the ratio increased as [O_2] decreased, *i.e.* when the lethal threshold metal concentration was low, the metal was more toxic.

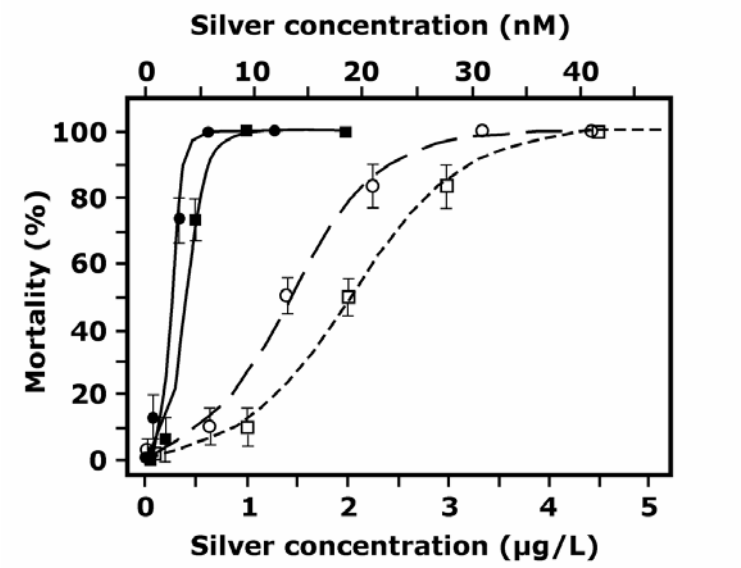

Figure 4. Mortality of *Daphnia magna* neonates after 48-h exposure to silver added as $AgNO_3$ in the absence (<5 nM; solid lines and closed symbols) or presence (~25 nM; dashed lines and open symbols) of zinc sulfide clusters in synthetic hard water. Squares correspond to nominal concentrations of silver, whereas circles represent measured silver concentrations. [From Bianchini *et al.* 2002a].

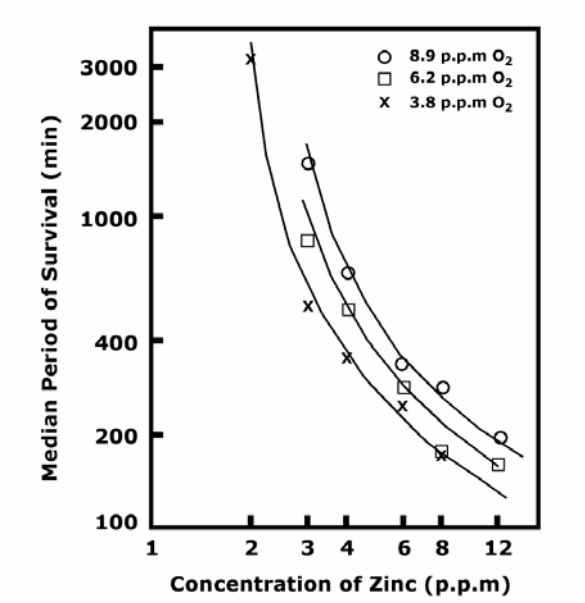

Figure 5. Effect of the concentration of dissolved oxygen on the toxicity of zinc to rainbow trout. Circles = 8.9 mg $O_2 \cdot L^{-1}$; squares = 6.2 mg $O_2 \cdot L^{-1}$; crosses = 3.8 mg $O_2 \cdot L^{-1}$. [From Lloyd and Herbert 1962].

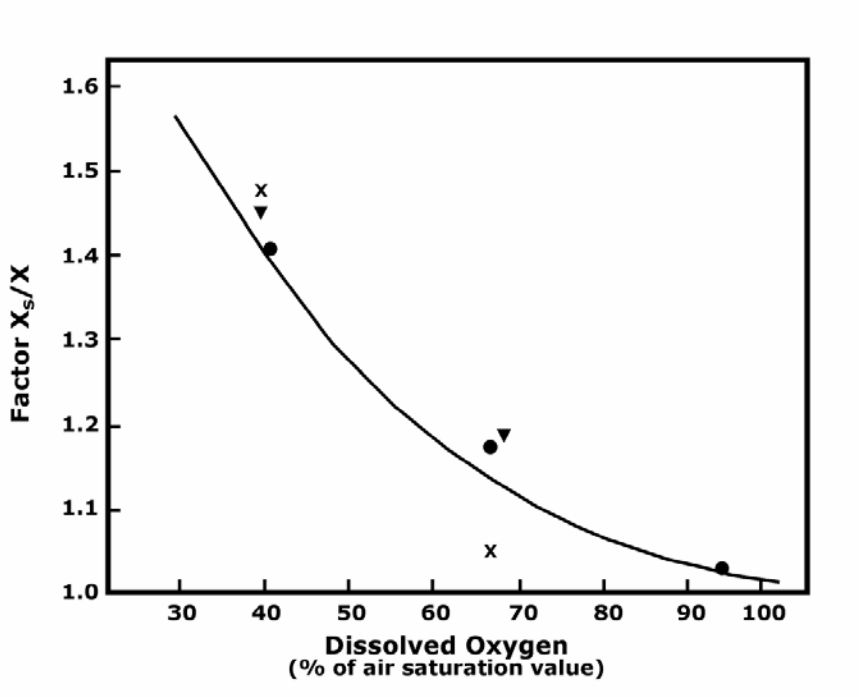

Figure 6. Effect of the concentration of dissolved oxygen on the toxicity of copper (crosses), lead (inverted triangles) and zinc (circles) to rainbow trout. Values of the factor X_s/X that are greater than 1 correspond to an increase in toxicity: X_s is the lethal threshold metal concentration with oxygen at the air-saturation value and X is the lethal threshold metal concentration at a decreased oxygen concentration [From Lloyd and Herbert 1962].

Similar curves were found for all three metals (the increase in toxicity was about 40%) with the greater toxicities noted at the lower oxygen conditions. The authors attributed this trend to greater ventilation rates in fish living under hypoxic conditions. In other words, the increased toxicity of Zn in waters containing little oxygen was related to the increased volume of water coming into contact with the fish gills due to the response of the fish to hypoxia. To quote the authors (Lloyd and Herbert 1962): "An hypothesis to account for this common increase in toxicity is based on the fact that trout increase the flow of water over their gills as the oxygen content of the water decreases … the increased flow of water over the gills brings more metal into contact with the fish in a given time."

This argument was reprised by Hughes and Flos (1978). They exposed rainbow trout to four different treatments: normoxic clean water; hypoxic clean water; normoxic water with 10 mg Zn/L for 10 h; and hypoxic water with 10 mg Zn/L for 10 h. In the hypoxic treatments, the oxygen partial pressure was reduced to 60 mm Hg by means of a vacuum apparatus (normoxia = 760 mm X 0.2 = 150 mm). Gill Zn accumulation, measured for each of four gill arches on each side of the fish and corrected for variations in exposure times, was greater in the Zn-exposed fish than in the controls, but there was no significant difference between normoxia and hypoxia. The interpretation of results was complicated, however, by differences in survival time, especially for fish exposed to Zn with decreased oxygen (in the hypoxic group, all but one fish died within

10 h, whereas in the normoxic group only one fish died during this timeframe). Fish in normoxic waters survived longer and thus had the opportunity to accumulate more Zn. To adjust for different survival times (6.5 versus 10 h), the authors assumed a linear relationship between exposure time and Zn accumulation, and normalized all Zn accumulation data to 10 h.

Hughes and Flos (1978) refer to evidence from physiological and morphological studies that different regions of the fish gill are ventilated and perfused to varying degrees as the oxygen partial pressure decreases. Accordingly, in their experiment they checked for heterogeneity in the distribution of Zn in different parts of the gill system in order to test whether changes in this distribution could be correlated with differences in the pattern of water flow through the gills. In the normoxic and hypoxic groups of untreated fish, there were small differences in zinc concentration between different gill arches; in general, normoxic fish had slightly higher concentrations than the hypoxic specimens and values obtained for the posterior arches tended to be greater than for other gill arches. Following treatment, differences between individual arches became more exaggerated (concentrations in the first arch were always lower than the other arches, and the third arch contained the maximum concentration of Zn). However, the relative importance of each arch (*e.g.* each arch expressed as a percent of the fourth and lowest arch) was unaffected by hypoxia.

Given the dearth of studies on the effect of ventilation rate on metal uptake and toxicity in fish, it seems appropriate to stray briefly into the domain of organic contaminants. Yang *et al.* (2000) recently reported on links between respiration rate and uptake of lipophilic contaminants in fish confined to a respirometer and forced to swim at predetermined velocities (three fish species; small, medium, and large specimens). They demonstrated a significant correlation between fish oxygen consumption and the amount of organic contaminant absorbed (tetrachlorobenzene, tetrachloroguaiacol, dichlorobenzenediol; see Figure 7). Similarly, a significant correlation was found between the toxicant uptake rate constant (k_1) and fish oxygen consumption, regardless of fish size and species. This correlation improved when fish toxicant load was expressed as percent body lipid. However, these authors also point out that "oxygen uptake can only be used as an indicator of toxicant transfer under normoxic conditions. Altered fish ventilation rate or diffusion capacity without changes in whole animal metabolic rate, which may occur during hypoxia or hyperoxia, will change the rate of toxicant movement across fish gills without changes in oxygen uptake. In other words, toxicant absorption may be greatly enhanced under these conditions without there being any significant changes in oxygen consumption."

Invertebrates

A second series of studies, carried-out not on fish but rather with the freshwater clam *Corbicula fluminea*, also showed enhanced metal (Cd) uptake under hypoxic conditions (Tran *et al.* 2000, 2001). In their initial study, carried-out in the absence of any metal, these workers studied the basic adaptation mechanisms that allow *C. fluminea* to maintain constant oxygen consumption under resting conditions when the partial pressure of O_2 (P_{O2}) in the ambient water varied from 4 to 40 kPa (cf. 20-21 kPa in the holding tanks for the bivalves). Ventilation rates

were determined by the volume of water cleared of algal cells per unit time in a transiently closed system; reasonably high plankton concentrations were used such that ventilation rates were affected only by P_{O2} and were independent of the concentration of algal cells in the exposure medium.

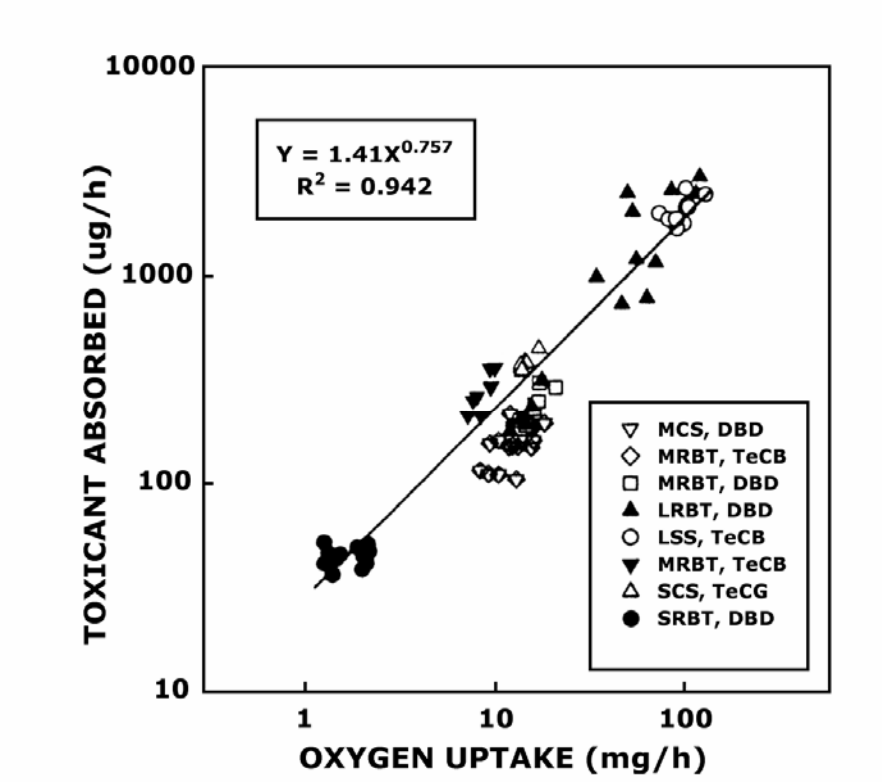

Figure 7. Relationship between uptake of organic contaminants and oxygen absorption in fishes of different species and sizes. Fish code: CS = coho salmon; L = large; LSS = largescale sucker; M = medium; RBT = rainbow trout; S = small. Contaminant code: DBD = 4,6-dichlorobenzene; TeCB = 1,2,4,5-tetrachlorobenzene; TeCG = 3,4,5,6-tetrachloroguaiacal. [From Yang *et al.* 2000].

Steady-state values of O_2 consumption, P_{O2} and O_2 concentration in the arterial and venous blood, P_{O2} in the expired water, and ventilatory and circulatory blood flow were determined after 24-h periods of exposure to selected P_{O2} values. After one day of acclimation, the maintenance of O_2 consumption was achieved exclusively by ventilatory adjustment, with no change in the oxygenation status of the internal milieu (Figure 8). The authors concluded that *C. fluminea*, like numerous other physiologically different water-breathers including fishes and crustaceans, maintained its O_2 consumption under resting conditions by continuously adjusting its ventilatory activity. Under normoxia, the ventilatory rate was < 0.4 $L \cdot d^{-1} \cdot g^{-1}$ fresh weight (not including shell), much lower than earlier literature estimates. The authors attributed these low resting values to their particular protocols used to isolate the animals from laboratory stimulation.

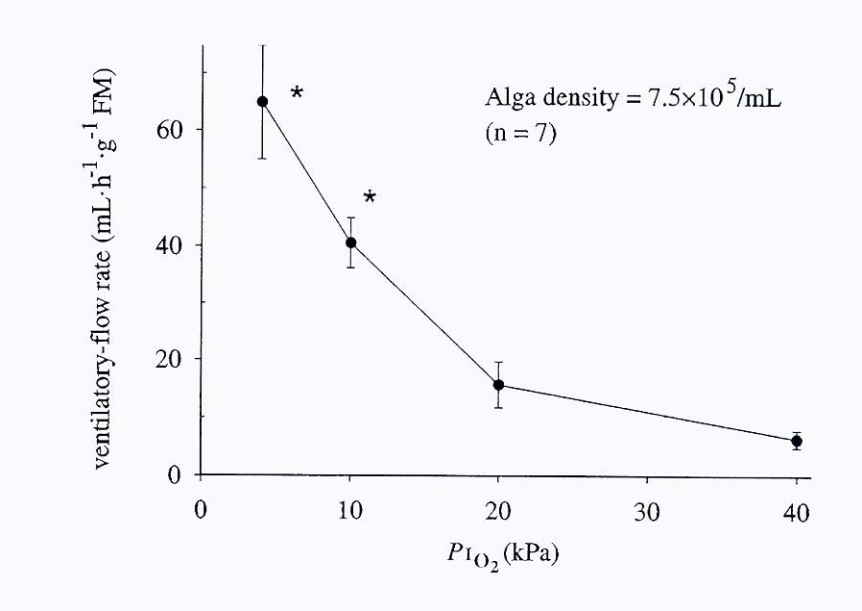

Figure 8. Ventilatory flow rates as a function of the partial pressure of oxygen, P_{O2} in the freshwater mollusc *Corbicula fluminea*, measured after 22-24 h acclimation. Ventilation rates were determined on the basis of water volume cleared of algal cells per unit time in a transiently closed system. Values with an asterisk are significantly different from the reference value in normoxia ($P < 0.05$, n=7). [From Tran *et al.* 2000].

Having studied the response of *C. fluminea* to varying oxygen concentrations, Tran *et al.* (2001) then introduced a metal (Cd) into their system (0.5 or 2 μg·L^{-1}) and studied metal uptake as a function of ventilatory activity over 15 d exposures. As before, three P_{O2} values were used (4, 20 and 40 kPa, corresponding to hypoxia, normoxia, and hyperoxia). The results showed that a low P_{O2} strongly enhanced the rate of accumulation of Cd in the soft tissues of the clam (Figure 9) and modified the distribution pattern and relative metal burdens in the gills and visceral mass (but not in the mantle, foot or abductor muscle). The apparent extraction coefficient of Cd (E_{WCd}) from the ventilated water was calculated:

E_{WCd} = (total amount of accumulated Cd)/(total amount of Cd inspired); and

the concentrations of Cd in the expired water were then calculated from the equation:

$$M_{Cd} = V_W(\text{inspired } [Cd]_W - \text{expired } [Cd]_W)m,$$

where M_{Cd} is the Cd accumulation during the 15 d exposure period (μg/g FW), M is the fish weight, V_W is the ventilatory flow rate over the same period of time, and inspired $[Cd]_W$ is the measured value in the exposure medium). Expired $[Cd]_W$ values calculated in this manner remained very close to those in the inspired water, regardless of P_{O2}; E_{WCd} varied from 2 to 12%.

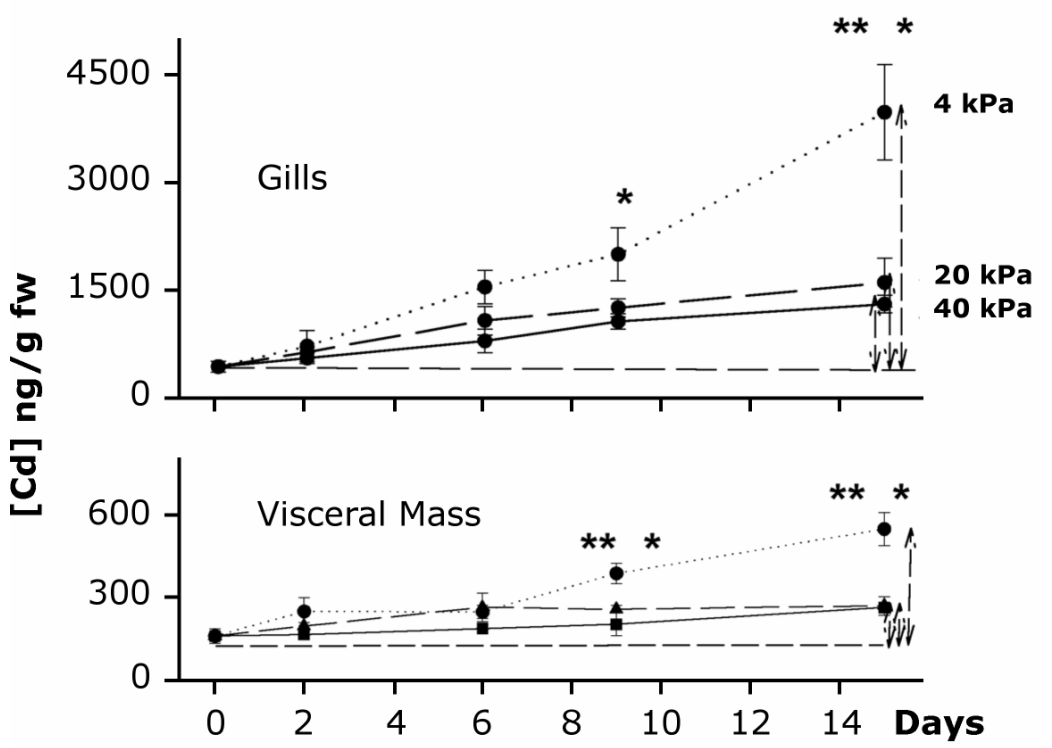

Figure 9. Time course of cadmium uptake ([Cd]) in the gills and visceral mass of *Corbicula fluminea* exposed to dissolved Cd ($[Cd]_w = 2 \pm 0.3\mu g/L$) over a 15-d period, as affected by the partial pressure of oxygen, P_{O2}. Asterisks indicate significant differences ($P < 0.05$) from water P_{O2} = 40* and 20 kPa**, respectively. [From Tran *et al.* 2001].

The authors argue convincingly that their results are environmentally relevant: the effect of changing O_2 concentration was at least as important as the better-recognized effect of changing temperature; *C. fluminea* is a benthic invertebrate that inhabits the interface between the sediment and the water column where low-oxygen microenvironments are not uncommon.

SUMMARY

Within the construct of the BLM, it is (tacitly) assumed that metal uptake and toxicity are unaffected by ventilation rates. The respiratory surface is assumed to be in equilibrium with the exposure solution (see BLM section), *i.e.* the slowest step in metal uptake is assumed to be transfer across the gill membrane. Under such conditions, a metal concentration gradient will not develop in the boundary layer between the gill surface and the bulk inspired solution, and thus arguments based on increased ventilation rates, thinner boundary layer, and increased diffusion rates from the bulk solution to the gill surface do not apply. How then can we explain the scattered evidence in the literature that metal uptake and toxicity do indeed increase when

gill ventilation rates increase? It seems likely that the answer lies elsewhere in the complex physiological responses of water-breathing organisms to low oxygen levels. One possible explanation is that under low oxygen stress the gill is better irrigated, thus exposing a greater gill surface area for metal uptake. Alternatively, if the concentration of excreted CO_2 at the gill surface is lower when the oxygen concentration of the water is reduced (Lloyd 1961), then the pH in the boundary layer would also rise (Randall *et al.* 1991); the BLM would predict less competition from the proton under such conditions, resulting in greater metal uptake and toxicity.

REFERENCES

Amyot, M., G.A. Gill, and F.M.M. Morel. 1997. Production and loss of dissolved gaseous mercury in coastal seawater. Environmental Science & Technology 31:3606-3611.

Bianchini, A. and K.C. Bowles. 2002. Metal sulfides in oxygenated aquatic systems: implications for the biotic ligand model. Comparative Biochemistry and Physiology C 133:51-64.

Bianchini, A., K.C. Bowles, C.J. Brauner, J.W. Gorsuch, J.R. Kramer, and C.M. Wood. 2002a. Evaluation of the effect of reactive sulfide on the acute toxicity of silver (I) to *Daphnia magna*. Part 2: Toxicity results. Environmental Toxicology and Chemistry 21:1294-1300.

Bianchini, A., C. Rouleau, and C.M. Wood. 2002b. Silver accumulation in *Daphnia magna* in the presence of reactive sulfide. 23rd Annual Conference, Society of Environmental Toxicology and Chemistry. Salt Lake City, Utah. November 2002, Abstract #395.

Bowles, K.C., R.A. Bell, M.J. Ernste, J.R. Kramer, H. Manolopoulos, and N. Ogden. 2002a. Synthesis and characterization of metal sulfide clusters for toxicological studies. Environmental Toxicology and Chemistry 21:693-699.

Bowles, K.C., A. Bianchini, C.J. Brauner, J.R. Kramer, and C.M. Wood. 2002b. Evaluation of the effect of reactive sulfide on the acute toxicity of silver (I) to *Daphnia magna*. Part 1: Description of the chemical system. Environmental Toxicology and Chemistry 21:1286-1293.

Brannon, J.M., R.H.J. Plumb, and I.J. Smith. 1980. Long-term release of heavy metals from sediments. *In*: Contaminants and sediments, R.A. Baker (ed.). Ann Arbor Science Publishing. Ann Arbor, Michigan. Pp. 221-266.

Bury, N.R., J. Shaw, C. Glover, and C. Hogstrand. 2002. Derivation of a toxicity-based model to predict how water chemistry influences silver toxicity to invertebrates. Comparative Biochemistry and Physiology C 133:259–270.

Campbell, P.G.C. 1995. Interactions between trace metals and organisms: critique of the free-ion activity model. *In*: Metal Speciation and Bioavailability in Aquatic Systems, A. Tessier, D.R. Turner (eds.). J. Wiley and Sons. Chichester, UK. Pp. 45-102.

Campbell, P.G.C., O. Errécalde, C. Fortin, V.P. Hiriart-Baer, and B. Vigneault. 2002. Metal bioavailability to phytoplankton - applicability of the Biotic Ligand Model. Comparative Biochemistry and Physiology C 133:185-202.

De Schamphelaere, K.A.C. and C.R. Janssen. 2002. A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium and pH. Environmental Science Technology 36:48-54.

De Schamphelaere, K.A.C., D.G. Heijerick, and C.R. Janssen. 2002. Refinement and field validation of a biotic ligand model predicting acute copper toxicity to *Daphnia magna*. Comparative Biochemistry and Physiology C 133:243-258.

De Schamphelaere, K.A.C, F.M. Vasconcelos, D.G. Heijerick, F.M.G. Tack, K. Delbeke, H.E. Allen, and C.R. Janssen. 2003. Development and field validation of a predictive copper toxicity model for the green alga *Pseudokirchneriella subcapitata*. Environmental Toxicology and Chemistry 22: (in press).

Di Toro, D.M., H.E. Allen, H.L. Bergman, J.S. Meyer, R.C. Santore, and P. Paquin. 2000. The Biotic Ligand Model. A Computational Approach for Assessing the Ecological Effects of Copper and Other Metals in Aquatic Systems. International Copper Association, Environment Program. New York, NY, USA, viii + 102 p.

Di Toro, D.M., H.E. Allen, H.L. Bergman, J.S. Meyer, P.R. Paquin, and R.C. Santore. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environmental Toxicology and Chemistry 20:2383-2396.

Fortin, C. and P.G.C. Campbell. 2001. Thiosulfate enhances silver uptake by a green alga: role of anion transporters in metal uptake. Environmental Science & Technology 35:2214-2218.

Gorsuch, J.W., C.R. Janssen, C.M. Lee, and M.C. Reiley Editors. 2002. Special Issue: The Biotic Ligand Model for Metals - Current Research, Future Directions, Regulatory Implications. Comparative Biochemistry and Physiology C 133:1-343.

Heijerick, D.G., K.A.C. de Schamphelaere, and C.R. Janssen. 2002a. Predicting acute zinc toxicity for *Daphnia magna* as a function of key water chemistry characteristics: development and validation of a Biotic Ligand Model. Environmental Toxicology and Chemistry 21:1309-1315.

Heijerick, D.G., K.A.C. de Schamphelaere, and C.R. Janssen. 2002b. Biotic Ligand Model predicting Zn toxicity to the alga *Pseudokirchneriella subcapitata*: Possibilities and limitations. Comparative Biochemistry and Physiology C 133:207-218.

Hodson, R.C., J.A. Schiff, and A.J. Scarsella. 1968. Studies of sulfate utilization by algae. 7. In vivo metabolism of thiosulfate by *Chlorella*. Plant Physiology 43:570-577.

Hogstrand, C., F. Galvez, and C.M. Wood. 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. Environmental Toxicology and Chemistry 15:1102-1108.

Hughes, G.M. and R. Flos. 1978. Zinc content of the gills of rainbow trout (*S. gairdneri*) after treatment with zinc solutions under normoxic and hypoxic conditions. Journal of Fish Biology 13:717-728.

Lloyd, R. 1961. Effect of dissolved oxygen concentrations on the toxicity of several poisons to rainbow trout (*Salmo gairdneri* Richardson). Journal of Experimental Biology 38:447-455.

Lloyd, R. and D.W.M. Herbert. 1962. The effect of the environment on the toxicity of poisons to fish. Institute for Public Health Engineering Journal 61:132-145.

Luther, G.W., S.M. Theberge, and D.T. Rickard. 1999. Evidence for aqueous clusters as intermediates during zinc sulfide formation. Geochimica Cosmochimica Acta 63:3159-3169.

MacDonald, A., L. Silk, M. Schwartz, and R.C. Playle. 2002. A lead–gill binding model to predict acute lead toxicity to rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology C 133:227-242.

MacRae, R.K., D.E. Smith, N. Swoboda-Colberg, J.S. Meye, and H.L. Bergman. 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) gills. Environmental Toxicology and Chemistry 18:1180-1189.

McGeer, J.C., R.C. Playle, C.M. Wood, and F. Galvez. 2000. A physiologically based biotic ligand model for predicting the acute toxicity of waterborne silver to rainbow trout in freshwaters. Environmental Science & Technology 34:4199-4207.

Meyer, J.S., R.C. Santore, J.P. Bobbitt, L. Debrey, C.J. Boese, P.R. Paquin, H.E. Allen, H.L. Bergman, and D.M. Di Toro. 1999. Binding of nickel and copper to fish gills predicts toxicity when water hardness varies, but free-ion activity does not. Environmental Science Technology 33:913-916.

Morel, F.M.M. 1983. Principles and Applications of Aquatic Chemistry. J. Wiley and Sons Ltd, New York, NY.

Morel F.M.M., A.M.L. Kraepiel, and M. Amyot. 1998. The chemical cycle and bioaccumulation of mercury. Annual Review of Ecology and Systematics 29:543-566.

Morgan, J.J. and A.T. Stone. 1985. Kinetics of chemical processes of importance in lacustrine environments. *In:* Chemical Processes in Lakes, W. Stumm (ed.). J. Wiley & Sons, New York. Chapter 17:389-426.

NRCC. 1988. Biologically available metals in sediments, P.G.C. Campbell, A.G. Lewis (eds.). National Research Council of Canada, Associate Committee on Scientific Criteria for Environmental Quality, NRCC report No. 27694, 298 p.

Paquin, P.R., V. Voltay, R. P. Winfield, K. B. Wu, R. Mathew, R. C. Santore, and D. M. Di Toro. 2002. Extension of the biotic ligand model of acute toxicity to a physiologically-based model of survival time of rainbow trout (*Oncorhynchus mykiss*) exposed to silver. Comparative Biochemistry and Physiology C 133:305-343.

Peddicord, R.K. 1987. Overview of the influence of dredging and dredged material disposal on the fate and effects of sediment-associated chemicals. *In*: Fate and Effects of Sediment-Bound Chemicals in Aquatic Systems, K.L. Dickson, A.W. Maki, and W.A. Brungs (eds.). Pergamon Press, Oxford, UK. Pp. 317-326.

Perez-Castiñeira, J.R., J.L. Prieto, J.G. González-Arroyo, and J.M. Vega. 1998. Kinetic properties of sulfate uptake in two types of eukaryotic green microalgae. Journal of Plant Physiology 153:324-331.

Pyle, G.G., S.M. Swanson, and D.M. Lehmkuhl. 2001. The influence of water hardness, pH, and suspended solids on nickel toxicity to larval fathead minnows (*Pimephales promelas*). Water Air Soil Pollution 133:215-226.

Randall, D.J., H. Lin, and P.A. Wright. 1991. Gill water flow and the chemistry of the boundary layer. Physiology and Zoology 64:26-38.

Rozan, T.F., G. Benoit, and G.W. Luther. 1999. Measuring metal sulfide complexes in oxic river waters with square wave voltammetry. Environmental Science & Technology 33:3021-3026.

Rozan, T.F., M.E. Lassman, D.P. Ridge, and G.W. Luther. 2000. Evidence for iron, copper and zinc complexation as multinuclear sulphide clusters in oxic rivers. Nature 406:879-882.

Santore, R.C., D.M. Di Toro, P.R. Paquin, H.E. Allen, and J.S. Meyer. 2001. Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and *Daphnia*. *Environ.* Toxicology and Chemistry 20:2397-2402.

Santore, R.C., R. Mathew, P.R. Paquin, and D. DiToro. 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*. Comparative Biochemistry and Physiology C 133:271-285.

Schwartz, M.L. and R.C. Playle. 2001. Adding magnesium to the silver-gill binding model for rainbow trout (*Oncorhynchus mykiss*). Environmental Toxicology and Chemistry 20:467-472.

Sholkovitz, E.R. 1985. Redox-related geochemistry in lakes: alkali metals, alkaline earths elements and ^{137}Cs. *In:* Chemical Processes in Lakes, W. Stumm (ed.). J. Wiley & Sons. New York. Chapter 6:119-142.

Sirko, A., M. Zatyka, E. Sawody, and D. Hulanicka. 1995. Sulfate and thiosulfate transport in *Escherichia coli* K-12: evidence for a functional overlapping of sulfate and thiosulfate-binding proteins. Journal of Bacteriology 177:4134-4136.

Smith, D.S., R.A. Bell, and J.R. Kramer. 2002. Metal speciation in natural waters with emphasis on reduced sulfur groups as strong metal binding sites. Comparative Biochemistry and Physiology C 133:65-74.

Tran, D., A. Boudou and J.-C. Massabuau. 2000. Mechanism for maintaining oxygen consumption under varying oxygenation levels in the freshwater clam *Corbicula fluminae*. Canadian Journal of Zoology 78:2027-2036.

Tran, D., A. Boudou and J.-C. Massabuau. 2001. How water oxygenation level influences cadmium accumulation pattern in the Asiatic clam *Corbicula fluminae*: a laboratory and field study. Environmental Toxicology and Chemistry 20:2073-2080.

Turner, D.R., M. Whitfield, and A.G. Dickson. 1981. The equilibrium speciation of dissolved components in freshwater and seawater at 25 C and 1 atm pressure. Geochimica Cosmochimica Acta 45:855-881.

Van Leeuwen, H.P. 1999. Metal speciation dynamics and bioavailability: inert and labile complexes. Environmental Science & Technology 33:3743-3748.

Wang, F., A. Tessier, K. Sukola, and C. DeVries. 2003. Metal complexes with reduced sulfur species in surface waters. MITE-RN 4th Annual Research Symposium, Metals in the Environment Research Network. Ottawa, February 2003, abstract.

Wood, C.M. 2001. Toxic responses of the gill. *In:* Target organ toxicity in marine and freshwater teleosts, D. Schlenk and W.H.Benson (eds.). Taylor & Francis. London Pp. 1-89.

Wood, C.M., C. Hogstrand, F. Galvez, and R.S. Munger. 1996. The physiology of waterborne silver toxicity in freshwater rainbow trout (Oncorhynchus mykiss). 2. The effects of silver thiosulfate. Aquatic Toxicology 35:111-125.

Yang, R., C.J. Brauner, R. V. Thurston, J. Neuman, and D.J. Randall. 2000. Relationship between toxicant transfer kinetic processes and fish oxygen consumption. Aquatic Toxicology 48:95-108.

THE FISH OLFACTORY SYSTEM: ITS ROLE IN NORMAL BIOLOGY AND IN TOXICOLOGICAL RESEARCH

Kjell B. Døving[1]

ABSTRACT

The sense of smell plays a major role in the normal biology of fishes. This chemosensory system is mandatory in homing behavior, is crucial in reproductive behavior, and mediates food search and alarm reactions. Recent studies in our laboratory have revealed the organization of the olfactory system in crucian carp *Carassius carassius* L. The sensory neurones with long dendrites and cilia mediate the alarm reaction in carp fishes, and connect to secondary neurones with axons in the medial part of the medial olfactory tract. The sensory neurones with dendrites of intermediate length bear microvilli and respond to food odors, and they connect to secondary neurones with axons in the lateral olfactory tract. The crypt cells probably respond to sex pheromones. In crucian carp, each of the three bundles of the olfactory tract mediates a distinct behavior pattern related to alarm, food search, and reproduction.

The high sensitivity of the fish olfactory system, its major role in essential life processes, and its direct exposure to the environment makes it an interesting target organ for studies of the impact of toxic substances. This review will expose different methods that can be used for studies of normal olfactory functions and how these methods can be applied in toxicological investigations.

INTRODUCTION

Chemosensory systems in fish represent a unique asset in our approach to study water-borne toxic effects. The reasons for this statement are the following: they are directly exposed to the environment; they mediate a large part of the behavioral repertoire; and they are developed early in life. In the present review, I shall deal with the olfactory system only, leaving out the gustatory system (Kasumyan and Døving 2003) and the oligovillous chemosensory system (Kotrschal 1996). The main reason for concentrating on the olfactory system is, of course, that it mediates a series of different and distinct behavior patterns. A more comprehensive discussion of different behavior patterns in fish and their use in toxicological studies is given by Døving (1986, 1991).

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Organization of the Olfactory System

Sensory neurones of the fish olfactory system can be divided into three types, according to their morphology. They are: 1) crypt cells; 2) microvillous neurones; and 3) ciliated neurones (Thommesen 1982, Hansen *et al.* 1997). Crypt cells have short dendrites and thus their cell bodies are situated in the upper layer of the olfactory epithelium (Figure 1). Microvillous neurones have longer dendrites and their cell bodies are in the intermediate layers of the epithelium (Hamdani *et al.* 2001a). Ciliated neurones have long dendrites and consequently their cell bodies are found close to the basal lamina in the epithelium (Figure 1; Hamdani and Døving 2002).

Recent studies have shown that these neurones project to different regions of the olfactory bulb in crucian carp. There they make synaptic connections with secondary neurones, the axons of which form three distinct bundles of the olfactory tract. Our studies have shown that each bundle of the tract mediates a specific behavior (Døving and Selset 1980). Thus, the lateral olfactory tract (LOT) is devoted to feeding behavior (Hamdani *et al.* 2001b), the medial part of the medial olfactory tract (mMOT) participates in the alarm reaction (Hamdani *et al.* 2000), and the lateral part of the medial olfactory tract (lMOT) in reproductive behavior (Weltzien *et al.* 2003). In short, the organization suggested by our recent work is summarized in Figure 1.

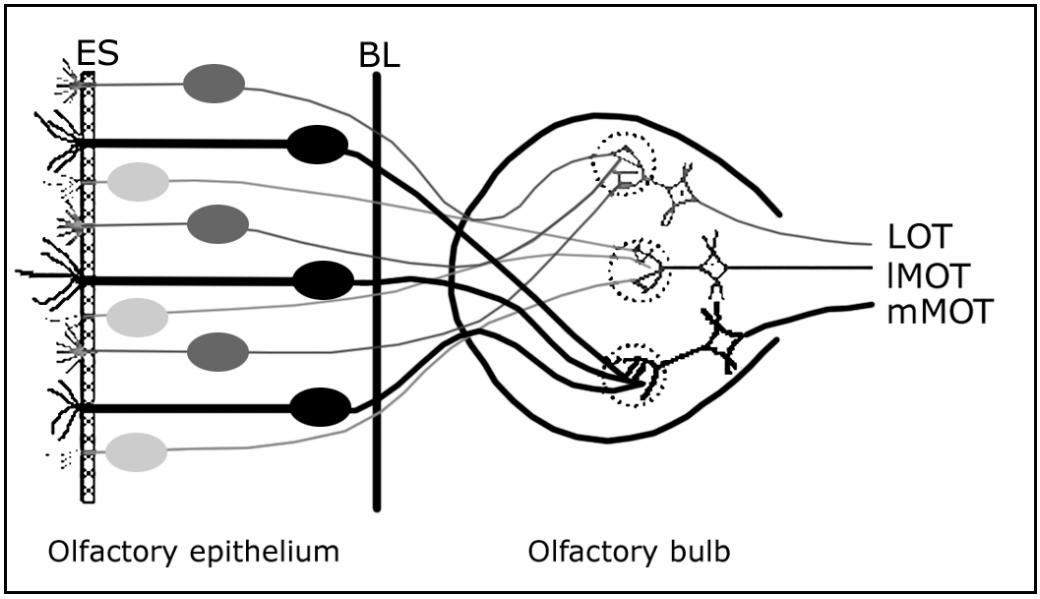

Figure 1. Schematic drawing of the organization of the olfactory system in crucian carp. ES, epithelial surface. BL, basal lamina. LOT, lateral olfactory tract. mMOT, medial part of the medial olfactory tract. lLOT, lateral part of the medial olfactory tract. The ciliated sensory neurones with long dendrites are in black, the sensory neurones with microvilli are in grey, and the crypt cell in light grey. See text for details.

The Electro-olfactogram, EOG

The olfactory receptor neurones are depolarized as a result of stimulation with odorants. Thus, the potential recorded at the surface of the olfactory epithelium becomes more negative upon stimulation. This electric response was named the electro-olfactogram, or EOG, and was discovered and analyzed in detail in frogs and mammals by Ottoson (1956). It has also been possible to record the EOG from olfactory organs of freshwater fish *e.g.* Winberg *et al.* (1992), and it is now routine to record the EOG by the method indicated in Figure 2. It is relatively difficult to obtain good signals in marine fish due to the shunting effects of salt water. However, exceptions can be found (Døving and Holmberg 1974).

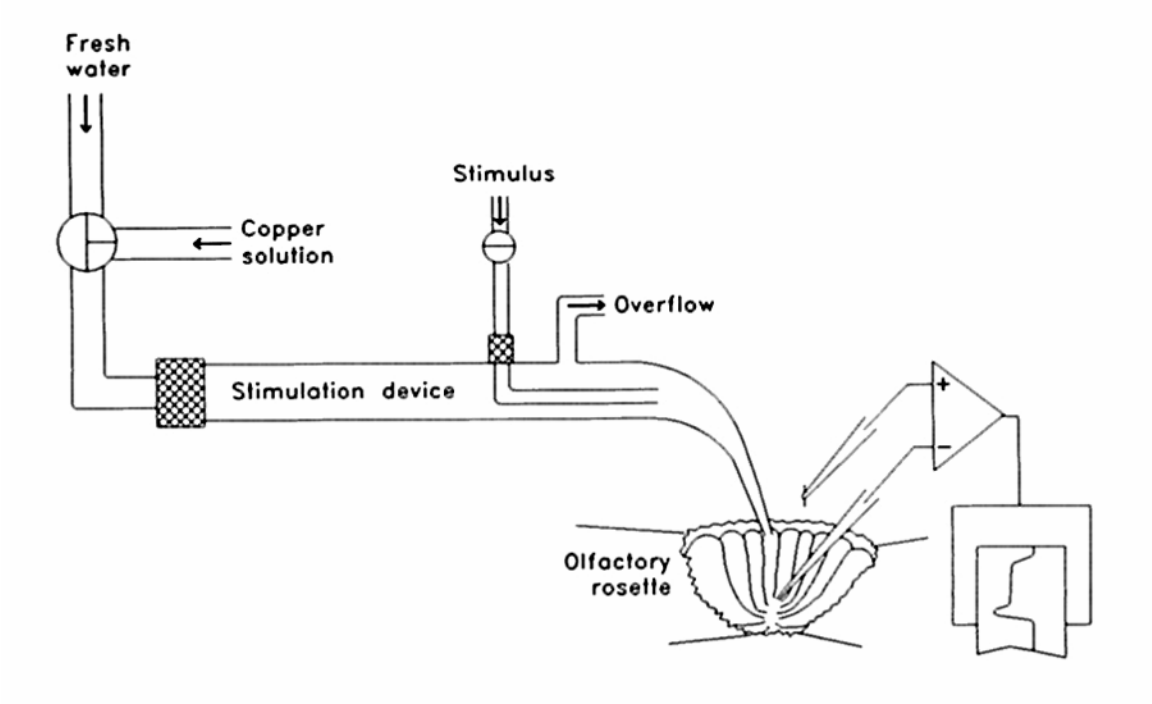

Figure 2. Schematic drawing of the experimental design for recording the EOG

In response to stimulation with an odorant, the epithelium is depolarized, giving an initial peak amplitude followed by a sustained potential at a lower amplitude (Figure 3). It is possible to follow the changes in response of the olfactory organ by exposing the sensory epithelium to different substances.

TOXICANT EFFECTS

Effect of Mercury

Exposure of the olfactory organ of fish to doses of two mercurials, mercuric chloride ($HgCl_2$) and methyl mercuric chloride (CH_3HgCl), has dramatic effects on Atlantic salmon *(Salmo salar* L.) EOG (Figure 3). EOG responses were evoked by stimulating the olfactory epithelium with 340 µM-alanine for 10 seconds every second minute during a one hour period.

Three experimental series, each comprising six fish, were carried-out. In the first series, the olfactory rosette was irrigated solely with artificial "fresh water." In the second and third series, a five-minute exposure to mercury ($HgCl_2$ or CH_3HgCl, at 10^{-5} M) was included after 10 minutes and a 15-minute exposure after 45 minutes, respectively. Mercuric ion (Hg^{2+}) eliminated the peak response within two minutes and suppressed the sustained response by about 35%. During subsequent irrigation with mercury-free fresh water, both EOG components regained about 50% of their initial amplitudes. In contrast, methyl mercury induced a steady and parallel decline of both the peak and sustained responses that were not reversed by rinsing the epithelium with fresh water. The results of this study demonstrated the vulnerability of the olfactory receptor function in fish to mercury exposure. Also, they revealed very different effects of inorganic and organic mercurials on the EOG (Baatrup *et al.* 1990).

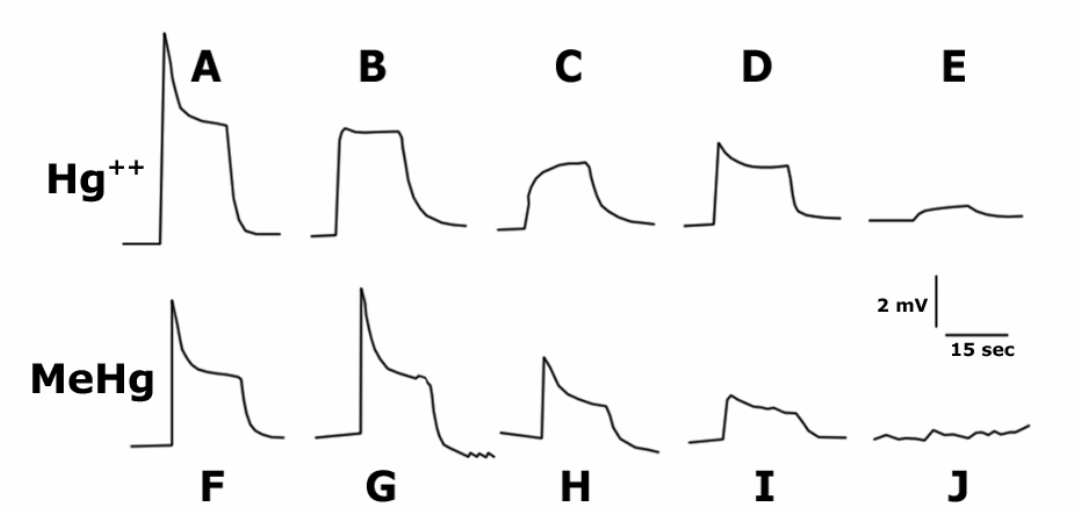

Figure 3. Examples of the effect of mercuric chloride**.** The appearance of the receptor potential, EOG, evoked by stimulating the salmon olfactory epithelium with 340 μM L-alanine, (A and F) during treatment of the sensory epithelium with ($HgCl_2$, A-E) and methyl mercuric chloride (CH_3HgCl, F-J). Before Hg treatment: (B and G) after exposure to mercury for one minute; *(C* and H) after exposure to mercury for four minutes; (D and I) after a subsequent 30-minute rinse with fresh water; (E and J) after further 15-minute exposure to mercury.

Deposition of organic and inorganic mercury compounds in the olfactory epithelium of Atlantic salmon was studied by histochemical methods (Baatrup and Døving 1990). One group of salmon was given fodder pellets containing methylmercuric chloride (CH_3HgCl, 99 micrograms $Hg \cdot g^{-1}$) for four weeks. Other groups of fish were exposed to dissolved mercuric chloride ($HgCl_2$, 270 micrograms $Hg \cdot L^{-1}$) for two, six, and 12 hours, respectively. In both series of experiments, the radioisotope ^{203}Hg was included to determine the accumulation of mercury in the olfactory system. Gamma-spectrometry showed both mercury compounds accumulated in the olfactory rosettes and their nerves. Microscopic analysis demonstrated an intense and comprehensive Hg deposition in the axons and Schwann cells of both methyl mercury- and inorganic mercury-exposed fish. On the other hand, the two mercury compounds showed

different staining patterns in the sensory epithelium. Silver grains evoked by methyl mercury were localized predominantly in lysosome-like inclusions within receptor cells, while those produced by mercuric chloride exposure were situated mainly along the borders of neighbouring cells. The present findings that organic and inorganic mercury compounds were deposited in the olfactory system along its whole length, from the receptor cell apices to the brain, support the electrophysiological results reported previously.

Effect of Cupric Ions

The effect of inorganic copper species has been studied by recording the EOG from the olfactory epithelium of Atlantic salmon (Winberg *et al.* 1992). The olfactory organ was irrigated with aqueous copper solutions with concentrations of the free cupric ion (Cu^{2+}) ranging from 0.2 to 9.7 μM. Diverse copper species were created by varying the amount of bicarbonate ($NaHCO_3$) in artificial fresh water solutions containing equal concentrations of copper. In general, these copper solutions induced a slow depolarization of the baseline followed by hyperpolarization. The amplitudes of these variations in baseline potentials increased with increasing concentrations of Cu^{2+} ion, *i.e.*, decreasing concentrations of $NaHCO_3$. Stimulating the olfactory epithelium with L-alanine during copper exposure evoked atypical EOG responses. The amplitudes and form of the EOGs changed drastically with increasing Cu^{2+} concentrations, with significant correlation between the reduction in EGG amplitudes and the Cu^{2+} concentration (Figure 4). It was concluded that among the copper species tested, the toxic effect was caused mainly by the dissolved Cu^{2+} ion. Results suggested that the Cu^{2+} ion exerts its toxic effects on transduction mechanisms of the olfactory receptor cells.

These studies demonstrated that the EOG of salmon is affected by inorganic, monomeric copper and that the effect depends primarily on the activity of Cu^{2+}, not on the total copper concentration in the solution. The effect of copper on the EOG profile indicates that Cu^{2+} ions affected different stages of the transduction mechanism in the olfactory receptor cells. The effect of copper ions in solution is different from the effect when the olfactory epithelium is exposed to mercury compounds (Baatrup *et al.* 1990).

ODOR INDUCED BEHAVIOR

It is believed that behavioral changes are the most sensitive measures of neurotoxicity. Behavioral bioassays alone are probably not the best method of evaluating tolerance limits for ecological impact. This should not prevent us from using behavioral studies in toxicology, however, since they are the most relevant approach to the ecological impact of toxic agents. The objective of behavioral toxicology must be to acquire reliable measures of sublethal doses of toxic events. These sublethal doses should be compared with threshold concentrations of toxicants obtained by other methods; *e.g.*, neurophysiological, histological, biochemical, and/or acute toxicological tests. These composite measures from different disciplines should be taken into account when formulating the tolerance limits which induce ecologically significant changes in behavior, *i.e.* that decrease the ability of an animal to adapt or survive in its environment.

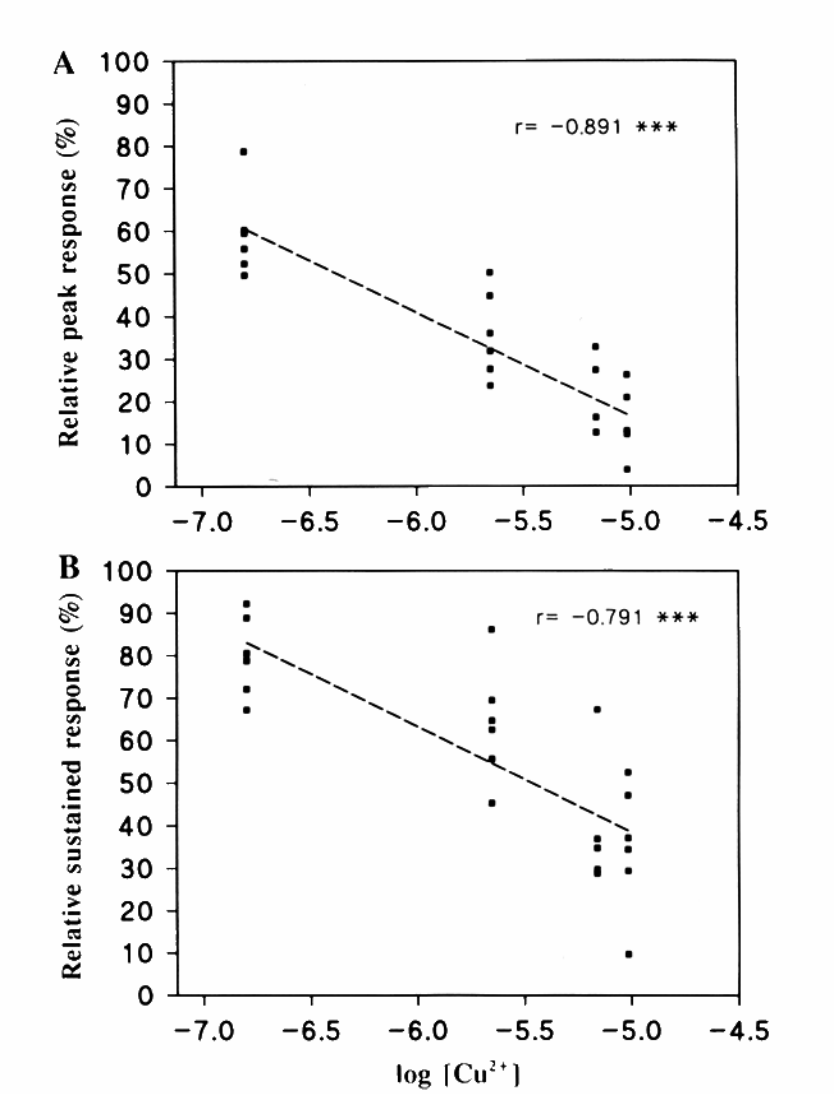

Figure 4. The relationship between Cu^{2+} concentration and EOG response. The EOG responses to 790 µM L-alanine after stimulation with various Cu^{2+} solutions for five minutes. The peak amplitude (A) and the sustained component (B) of the EOG responses after five minute recovery time following exposure to Cu^{2+} solutions.

A common belief is that when a fish is exposed to waste effluents, toxicants, or pollutants, it will adapt to the situation and perform the maneuvers most advantageous for its survival and the benefit of the species. This is not the situation in every case. For example, juvenile salmon have been shown not to avoid water in which crude oil had been dispersed, but to actually swim in the oil film (Bean *et al.* 1974, Morrow *et al.* 1975). These examples, and similar results of other experiments, indicate that fish are not equipped with the sensory apparatus necessary to make adjustments needed for appropriate reaction to the toxicant. When the signal that induces a specific behavior or part of a behavioral sequence is known, it is possible to use the behavior as a tool in behavioral toxicology.

Homing

The olfactory system is mandatory for salmon to return to their correct home river. The means for finding the correct tributary from open water was discussed in a recent review (Døving and Stabell 2003). Even though the urge of fish is great, homing behavior is not an easily accessible behavior for toxicological studies, since it is performed in open water and in relation to the layers of the ocean.

Feeding behavior

Cod *Gadus morhua* L., like many other species of fish, spend most of their time searching for food. Cod are attracted by the odors of their prey. One of the first signs of search or exploratory behavior is seen when these fish move up and down in the water mass. In the later phases of food search, cod search the bottom trailing their pelvic fins and barbel along the bottom. If it smells a particular substance, it stops, swims backward, and takes a position with its tail up in the water. In this position, it will snap with its jaws at prey (Døving and Selset 1980, Ellingsen and Døving 1986). It is known that in cod the amino acids alanine and glycine induce bottom food search via the olfactory organs (Ellingsen and Døving 1986).

Lemly and Smith (1987) demonstrated the effect of acidified water on the feeding behavior of fathead minnows *Pimephales promelas*. Feeding response was completely eliminated at pH = 6 and lower. These results corresponded well with environmental studies that showed fathead minnows were eliminated from natural waters when pH levels were lowered to 5.8-6.0.

This type of behavior is easily evoked by several chemicals with known composition; the behavior is conspicuous and can be quantified. Thus, the conditions are favorable for studies of the effect of toxic substances on the olfactory system.

Reproductive behavior

Many behaviors made by fish in conjunction with reproduction are induced via the olfactory system. In crucian carp, one particular behavior called ‘short following gut’ involves the male following the female and pushing her with the nose close to the anal papilla. This behavior depends on the lateral part of the lateral olfactory tract being intact (Weltzien *et al.* 2003).

Spawning behavior of females can be induced by intramuscular injections of prostaglandin, $PGF_{2\alpha}$, and a mature male will demonstrate this behavior. Impairment of this behavior by substances suspected to induce aberrant behavior might be a useful tool in toxicological studies.

Alarm Reaction

The last type of behavior considered to be induced by olfactory stimuli is the alarm reaction known in carp fishes, first described by von Frisch (1941). This behavior in crucian carp is mediated by the medial bundle of the medial olfactory tract that receives information from ciliated cells with long dendrites (Hamdani *et al.* 2000, Hamdani and Døving 2002).

Although the alarm substance found in the skin of conspecifics is easily degraded, the reaction is conspicuous and quantifiable. However, crucian carp do not return to normal behavior until 30 minutes to one hour after exposure, which prevents repetitive trials.

Avoidance Reaction

For reasons not clearly stated, the avoidance reaction has been a popular tool in behavioral toxicology. The reason may be the ease by which such experiments can be performed and the reactions monitored. The idea is that solutions of toxic substances are avoided by the fish. The sensory organ that provides the animal with the necessary information is not stated. In my opinion, olfactory sense is a clear candidate for the input channel. Avoidance reactions have been used in several studies (Hose *et al.* 1984, Hidaka and Tatsukawa 1986, Rehnberg and Schreck 1986, Hartwell *et al.* 1989)

Let us consider what happens when a fish is in a flow channel, a trough, or Y-maze where the fish can select its position. The fish is exposed to the toxicant. There is no evidence that it will perceive the toxicant as a forthcoming danger. Most probably it is unknown to the fish and, therefore, has no biological meaning, and it is unlikely that the olfactory system is equipped with receptor sites for the toxicant. Most probably, the olfactory receptors will ingest the toxicant; the olfactory receptors may cease to function and the fish will be rendered anosmic without, of course, knowing that it is so. If the fish is programmed to avoid regions with no odor it will do so, but by then it has already been exposed to the extent that the toxic substances have already exerted their influence. On the other hand, if the fish is programmed to remain stationary when anosmic, the observer will not detect any significant movements by the fish.

REFERENCES

Bean, R.M., J.R. Vanderhorst, and P. Wilkinson. 1974. Interdisciplinary study of the toxicity of petroleum to marine organisms. Battelle Pacific Northwest Laboratories, W.A. Richland (ed.). 31 pp.

Baatrup, E. and K.B. Døving. 1990. Histochemical demonstration of mercury in the olfactory system of salmon (*Salmo salar* L.) following treatments with dietary methylmercuric chloride and dissolved mercuric chloride. Ecotoxicology and Environmental Safety 20**:**277-289.

Baatrup, E., K.B. Døving, and S. Winber. 1990. Differential effects of mercurial compounds on the electroolfactogram (EOG) of salmon (*Salmo salar* L.). Ecotoxicology and Environmental Safety 20:269-276.

Døving, K.B. 1986. Functional properites of the fish olfactory system. Progress in Sensory Physiology 6:39-104.

Døving, K.B. 1991. Assessment of animal behavior as a method to indicate environmental toxicity. Comparative Biochemistry and Physiology C 100:247-252.

Døving, K.B. and K. Holmbert. 1974. A note on the function of the olfactory organ of the hagfish *Myxine glutinosa*. Acta Physiologica Scandinavica 91:430-432.

Døving, K.B. and R. Selset. 1980. Behavior patterns in cod released by electrical stimulation of olfactory tract bundlets. Science 207:559-560.

Døving, K.B. and O.B. Stabell. 2003. Trails in open waters: Sensory cues in salmon migration. *In*: Sensory processing in the aquatic environment, S.P. Collin and N.J. Marshall (eds.). Springer-Verlag, New York. Pp 39-52.

Ellingsen, O.F. and K.B. Døving. 1986. Chemical fractionation of shrimp extracts inducing bottom food search behavior in cod (*Gadus morhua* L.). Journal of Chemical Ecology 12:155-168.

Hamdani, E.H., G. Alexander, and K.B. Døving. 2001a. Projection of sensory neurones with microvilli to the lateral olfactory tract indicates their participation in feeding behavior in crucian carp. Chemical Senses 26:1139-1144.

Hamdani, E.H. and K.B. Døving. 2002. The alarm reaction in crucian carp is mediated by olfactory neurones with long dendrites. Chemical Senses 27:395-398.

Hamdani, E.H., A. Kasumyan, and K.B. Døving. 2001b. Is feeding behavior in the crucian carp mediated by the lateral olfactory tract? Chemical Senses 26:1133-1138.

Hamdani, E.H., O.B. Stabell, G. Alexander, and K.B. Døving. 2000. Alarm reaction in the crucian carp is mediated by the medial bundle of the medial olfactory tract. Chemical Senses 25:103-109.

Hansen, A., P. Eller, T.E. Finger, and E. Zeiske. 1997. The crypt cell; A microvillous ciliated olfactory receptor cell in teleost fishes. Chemical Senses 22:694-695.

Hartwell, S.I., J.H. Jin, D.S. Cherry. 1989. Toxicity versus avoidance response of golden shiner, *Notemigonus crysoleucas*, to five metals. Fish Biology 35:447-456.

Hidaka, H. and R. Tatsukawa. 1986. Variations by sex and body size in the avoidance tests of sodium linear laurylbenzene sulfonate and fenitrothion using a fish, *Medaka oryzias latipes*. Bulletin of the Japanese Society for Scientific Fisheries 52:1753-1757.

Hose, J.E., T.D. King, and J.S. Stephens. 1984. Effects of dechlorinated seawater on fish behavior. Marine Environmental Research 11:67-76.

Kasumyan, A. and K.B. Døving. 2003. Taste preferences in fish. Fish and Fisheries 4, accepted. In Press.

Kotrschal, K. 1996. Solitary chemosensory cells: Why do primary aquatic vertebrates need another taste system? Trends in Ecology & Evolution 11:110-114.

Lemly, A.D. and R.I.F. Smith. 1987. Effects of chronic exposure to acidified water on chemoreception of feeding stimuli in fathead minnows (*Pimephales promelas*): mechanisms and ecological implications. Environmental Toxicology and Chemistry 6:225-238.

Morrow, I.E., R.L. Gritz, and M.P. Kirton. 1975. Effects of some components of crude oil on young Coho salmon. Copeia 2:326-331.

Ottoson, D. 1956. Analysis of the electrical activity of the olfactory epithelium. Acta Physiologica Scandinavica 35:1-83.

Rehnberg, B.C. and C.B. Schreck. 1986. Acute metal toxicology of olfaction in coho salmon: behavior, receptors, and odor-metal complexation. Bulletin of Environmental Contamination and Toxicology 36:579-586.

Thommesen, G. 1982. Specificity and distribution of receptor cells in the olfactory mucosa of char (*Salmo alpinus* L.). Acta Physiology Scandinavica 115:47-56.

von Frisch, K. 1941. Über einen Schreckstoff der Fischhaut und seine biologische Beteutung. Zeitschrift fur verglechende Physiologie 29**:**46-145.

Weltzien, F.-A., E. Höglund, E.H. Hamdani, and K.B. Døving. 2003. Does the lateral bundle of the medial olfactory tract mediate reproductive behavior in male crucian carp? Chemical Senses 28:293-300.

Winberg, S., R. Bjerselius, E. Baatrup, and K.B. Døving. 1992. The effect of Cu (II) on the electro-olfactogram (EOG) of the Atlantic salmon (*Salmo salar* L) in artificial freshwater of varying inorganic carbon concentrations. Ecotoxicology and Environmental Safety 24:167-178.

TOXIC EFFECTS OF AMMONIA ON RAINBOW TROUT *ONCORHYNCHUS MYKISS* IN ALL STAGES OF DEVELOPMENT

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ABSTRACT

The effects of ammonia on the rainbow trout *Oncorhynchus mykiss* in all stages of development (embryos, larvae, adults) were investigated. The most sensitive to the acute toxic effect of ammonia were found to be larvae, then followed embryos and adult fish (96-hour LC50 values were 0.20; 0.27 and 0.36 mg/L ammonia, respectively). Long-term exposure to sublethal concentrations of ammonia affected embryo development, growth of larvae and their cardio-respiratory responses, and induced significant changes in hematological parameters of adult fish. Parameters of red blood (erythrocyte count, hemoglobin concentration, hematocrit level) of fish were found to be the most sensitive to low concentrations of ammonia. The lowest "safe" or "predicted-no-effect" concentration values for ammonia obtained in long-term tests were 0.02 mg/L of ammonia for adult fish, which is very close to the 0.025 mg/L value proposed by EU and accepted in Lithuanian water quality norms for salmonid fish. Meanwhile, the Maximum Acceptable Concentration for ammonia to fish in early stages of development is lower. This concentration (0.014 mg N/L) is many times lower than the Maximum Permissible Concentration for ammonia (0.39 mg N/L) accepted for Lithuanian inland waters.

INTRODUCTION

Water pollution by ammonia (NH_3) can have inorganic (industrial wastewaters) and organic (municipal sewage, large-scale agricultural pollution, biochemical reduction of water nitrates and nitrites) origin. Ammonia can be produced naturally during biodegradation of organic substances and the eutrophication process is often accompanied by elevated ammonia levels in the water (Randall and Tsui 2002). In water or in biological liquids, ammonia is present either in its molecular (un-ionized form) (NH_3), or in the form of the ionized ammonium ion (NH_4^+). Living cells are not easily penetrated by the ammonium ion, but un-ionized NH_3 penetrates the tissue barriers very easily, so it is toxic to aquatic animals. Modifying factors altering the acute toxicity of ammonia include dissolved oxygen, carbon dioxide, salinity, temperature, pH, previous acclimation to ammonia, fluctuations in exposure and the presence of other toxicants (U.S. EPA 1985). Ammonium ion is not as toxic as un-ionized ammonia, but regardless of its lower toxicity, it can still be important because it is generally present in natural waters in much greater concentrations than un-ionized ammonia (U.S. EPA 1999).

Recently-measured ammonia concentrations in river water ranged very widely, from very low (at detection limit), up to 47.70 mg N/L in the Kulpe River mouth (Anonymous 2002). These data demonstrate that aquatic animals, especially fish in the early stages of development, can experience negative impacts from ammonia in Lithuanian rivers.

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Fish mortality caused by ammonia may be due to different effects in different cases, and it is likely that ammonia has different modes of action at high and low concentrations (Russo 1985, Twichen and Eddy 1992). Chronic exposure of fish to lower concentrations of ammonia induces a slowdown in growth and morphological development, increases in susceptibility to disease, and produces deleterious histological changes and a decrease in reproductive capacity (Thurston *et al.* 1984, 1986). Adult fish increased glutamine-glutamate production in the brain when they were exposed to higher concentrations of ammonia (Vedel *et al.* 1998, Iwata 1988). Current data suggest that elevated NH_4^+ displaces K^+ and depolarizes neurons, causing excessive activation of NMDA-type glutamate receptors, leading to influx of excessive Ca^+ and subsequent cell death in the central nervous system (Randall and Tsui 2002).

No experimental data on the sensitivity of Lithuanian fishes to ammonia have been generated, although differing sensitivities of the same fish species to the same pollutants in different regions have been considered. Specification (definition) of ammonia Maximum Permissible Concentrations for Lithuanian inland waters is needed.

The aim of the present research was: 1) by means of acute and chronic toxicity tests to estimate the sensitivity to ammonia of rainbow trout in all stages of development (embryos, larvae, adult fish); 2) to recommend Maximum Acceptable Concentrations of ammonia according to changes of morphological and physiological parameters of rainbow trout in all developmental stages; and 3) to specify and compare calculated concentrations with water quality norms for ammonia accepted for salmonid fish and for Lithuanian inland waters.

MATERIALS AND METHODS

Rainbow trout eggs and one-year-old adults were obtained from Žeimena hatchery and acclimated under laboratory conditions.

Deep well water of high quality was used for dilution. Average hardness of the dilution water was approximately 284 mg/L as $CaCO_3$, alkalinity was 244 mg/L as HCO_3^-, mean pH was 8.0, temperature was equal to 12 ± 0.5^o C, and oxygen concentration ranged from 8 to 10 mg/L.

Ammonium nitrate (NH_4NO_3) was used as the toxicant. This chemical is the most popular nitrogen fertilizer used in Lithuania. Since this chemical has a very high solubility in water, its probability of entering ambient waters is obviously considerable.

Studies on embryos and larvae: Acute toxicity tests (96-hour duration) were conducted on all test-specimens. LC50 values (24, 48, 72 and 96 hrs) and their 95% confidence intervals were estimated by the use of the trimmed Spearman-Karber method (Hamilton *et al.* 1977). Both acute and chronic toxicity tests were conducted under semi-static conditions.

The tests were started with “eyed-egg” stage embryos and ended prior to active feeding of larvae. Two hundred embryos were exposed to each concentration of ammonia with two replications each. A total of 4000 embryos were used for these tests and the impact

of eight concentrations (0.36, 0.18, 0.09, 0.044, 0.024, 0.012, 0.006 and 0.003 mg/L) of ammonia were evaluated. The ninety-six-hour LC50 for larvae was determined with hatched larvae (the effect of each concentration of ammonia was tested with different numbers of individuals and then the LC50 was calculated). During the tests, embryo survival and heart rate were evaluated, and morphological measures (total body mass in mg), physiological (cardio-respiratory) parameters such as heart rate (HR, count/min), gill ventilation frequency (GVF, count/min), and integrated parameters (total body mass increase and relative mass increase in percent) of the larvae also were recorded.

Adult fish: Acute toxicity tests of 96-hour duration were performed with 10 adult rainbow trout at each concentration studied, with two replications. Long-term tests on adult fish were performed for 14 days. Fish were exposed to four different concentrations of ammonia (0.09, 0.044, 0.024 and 0.012 mg/L) in two replicates (Table 1). Tested fish were fed every day. Fish body lengths and weights were measured at the beginning and end of exposure. The tissue weights were measured at the end of exposure and tissue-somatic indices for the test fish calculated according to the methods of Vosylienė and Svecevičius (1997): gill-somatic index (GSI), spleen-somatic index (SSI), and liver-somatic index (LSI). Gill ventilation frequency (GVF, count/min) and "coughing" rate, or gill-cleaning reflex (CR, count/min), were measured during 3-minute periods for each test fish individually and the mean value for 14 fish was calculated. Erythrocytes (RBC, $10^6 \cdot mm^{-3}$) and percent of their various forms, hemoglobin concentration (Hb, g/L), hematocrit level (Hct, L/L), leucocyte count (WBC, $10^3 \cdot mm^{-3}$), and lymphocyte and neutrophil percent were determined by the methods of Svobodova and Vykusova (1991).

Table 1. Concentrations of ammonium nitrate added to dilution water, measured total ammonia (Nessler's method), calculated total ammonia nitrogen, un-ionized ammonia and un-ionized ammonia nitrogen. Highest concentration used only for testing of larvae. (All concentrations in mg/L, mean±SD; n=2).

NH_4NO_3 Added	Measured	Estimated	Estimated	
	Total ammonia ($NH_4^+ + NH_3$)	Total ammonia nitrogen	Un-ionized ammonia	Un-ionized ammonia nitrogen
37.5	8.48±0.41	6.61±0.32	0.18±0.009	0.15±0.007
18.8	4.34±0.21	3.38±0.16	0.092±0.005	0.075±0.004
9.4	2.06±0.10	1.64±0.08	0.044±0.002	0.036±0.002
4.7	1.12±0.06	0.87±0.05	0.024±0.001	0.0197±0.0008
2.35	0.56±0.03	0.44±0.02	0.012±0.001	0.0098±0.0008

The Maximum Acceptable Toxicant Concentration (MATC) was calculated for each measured parameter as the geometric mean of the Lowest Observed Effect Concentration (LOEC) and the No Observed Effect Concentration (NOEC), the method suggested by Rand and Petrocelli (1985). The significance of all the data obtained was determined by use of Student's *t*-test with $p \leq 0.05$.

RESULTS AND DISCUSSION

Acute toxicity: Acute toxicity tests were conducted in order to determine the basic toxic characteristics of ammonia. The results of our acute toxicity tests are presented in Table 2. Larvae were found to be the most sensitive to the acute toxic effect of ammonia, followed in order by embryos and adult fish. The 96-hour LC50 for larvae was significantly lower than that of adult fish or embryos.

Table 2. Acute toxicity of ammonia (mg/L) to rainbow trout at all stages of development.

Stage of development	96-hour LC50 (mg/L)	95% confidence interval (mg/L)
Embryos (eyed-egg stage)	0.27	0.26 — 0.29
Larvae	0.20	0.18 — 0.21
Adult fish	0.36	0.34 — 0.38

Trout embryos have a remarkable ability to tolerate environmental ammonia. Our data confirmed that embryos are more resistant to the impact of toxicants than larvae. Probably the embryo's chorion acts as a barrier and saves the developing organism from the external harmful impact; meanwhile fish larvae, which have lost the chorion, are very sensitive to adverse external affects (Rice and Stokes 1975, Kazlauskienė and Stasiūnaitė 1999). Data of Steele *et al.* (2001) demonstrate that during acute and chronic hyper-ammonia stress, embryo-tissue ammonia levels remain constant and the time to hatching is not altered, although a considerable ammonia load is absorbed from the environment. Their data also indicate that trout embryos have an efficient system to prevent ammonia accumulation in embryonic tissue based on the conversion of ammonia to urea in the embryonic tissues, and through elevation of ammonia levels in the yolk. Most of the absorbed ammonia is found in the yolk, whereas a small percentage is converted to the relatively non-toxic nitrogen end product urea. The investigators hypothesized that these strategies might contribute to the relatively high tolerance of trout early life stages, compared to adult trout, to high environmental ammonia levels (Steele *et al*. 2001).

Studies of Thurston *et al.* (1978), Tomasso and Carmichael (1986), Svobodova *et al.* (1993) and Tilak *et al.* (2002) demonstrated that acute exposure of fish to high concentrations of ammonia causes an increase in gill ventilation and hyper-excitability. If the exposure continues, ventilation becomes irregular, fish lose balance, convulsions begin, and individuals die. These effects are most likely the result of a direct effect of ammonia on the central nervous system (Daost and Ferguson 1984, Russo 1985). In the studies of Thurston and Russo (1983), acute toxic ammonia concentrations to adult rainbow trout (*Oncorhynchus mykiss*) ranged from 0.694 to 0.758 NH_3 mg/L recalculated for pH equal to 8.0, and from 26.8 to 37.7 mg N/L total ammonia nitrogen (TAN). Our data were found to be lower: 0.36 mg/L of ammonia and 16.75 mg N/L TAN, respectively.

Chronic Toxicity: Long-term studies demonstrated that the sensitivity of heart rate, gill ventilation frequency, and total body mass to ammonia exposure did not depend on the age of the larvae. Probably this is a specific toxic effect of ammonia. The MATC value for

ammonia according to heart rate was the same for embryos and larvae, although it was lower for gill ventilation frequency in the larvae. The integrated growth parameters of total body mass and relative body mass increase were found to be the most sensitive indices of the toxic effect of ammonia. The MATC (mg/L) for ammonia relative to body mass increase was 0.004 mg/L ammonia (Figure 1).

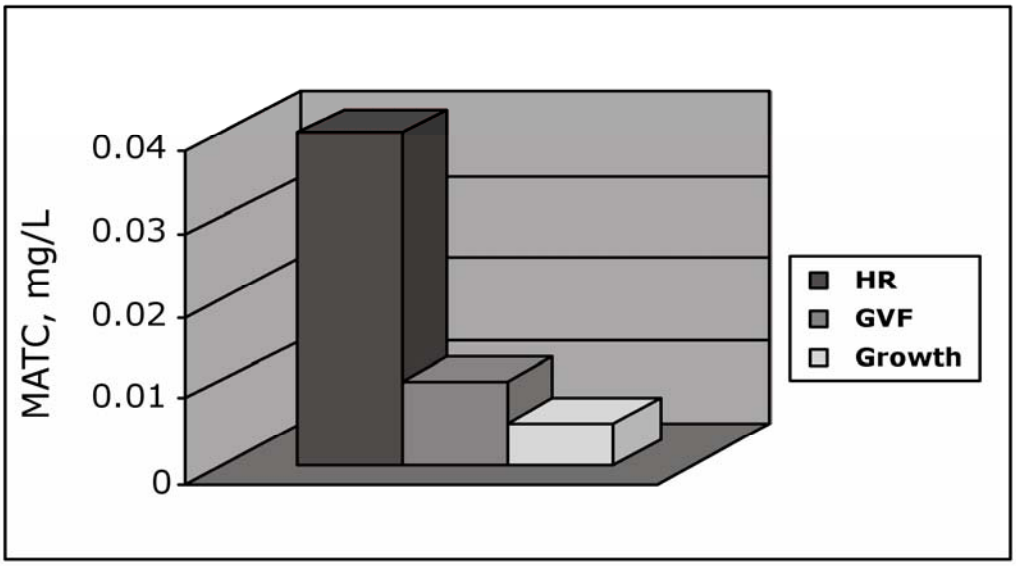

Figure 1. MATC (mg/L) of ammonia for heart rate (HR, counts/min), gill ventilation frequency (GVF, counts/min) and growth (relative body mass increase, %) for rainbow trout larvae.

Our data confirmed that integrated biological parameters like survival, growth rate, development and reproduction indices are the most sensitive to various environmental impacts (Sinderman 1984).

Ammonia exposure did not induce significant changes in the morphological parameters (fish length and weight) of adult rainbow trout. Absolute length of fish at the initiation of exposure ranged from 16.8±0.4 to 17.4±0.3 cm and weight ranged from 41.9±0.4 to 43.9±3.0 g. At the end of exposure the mean length of control fish was 17.6±0.3 cm while exposed fish ranged from 17.1±0.2 to 17.8±0.3 cm in length, respectively. No significant differences were found in the weights of the gills, liver and spleen of the exposed fish versus the controls, or their somatic indices. However, Thurston *et al*. (1984) after exposure of rainbow trout to five concentrations of un-ionized ammonia ranging from 0.008 to 0.06 mg NH_3-N/L at pH 7.7 found alterations of gill and kidney tissues that showed a positive correlation with un-ionized ammonia concentrations and histopathological alterations that increased in severity with increasing ammonia concentration. Gill lamellae of fish exposed to un-ionized ammonia concentrations of 0.02-0.05 mg NH_3-N/L for four months showed mild to moderate fusion, aneurysms, and separation of the epithelia from the underlying basement membrane (Thurston *et al.* 1984). These discrepancies between our data and those of Thurston *et al*. (1984) were probably the result of the different durations of exposure to ammonia.

The present study demonstrated diverse changes in respiratory parameters of adult rainbow trout: higher ammonia concentrations induced a slowing down of the GVF in fish, while the effect of lower concentrations tended to be the opposite – ventilation frequency of fish increased as compared to control individuals. However, these data were not significantly different in adult fish. According to Adams *et al*. (2001), significant increases in oxygen consumption rate and ventilation frequency occurred at 14.8 and 19.9 mg/L total ammonia nitrogen concentration, respectively, in juvenile big-bellied seahorse. In the present studies, statistically significant ($p<0.05$) changes in GVF induced by different concentrations of ammonia were found in 10-day and 20-day old larvae.

Although changes in respiratory parameters (GVF, coughing rate) of adult fish did not demonstrate adverse effects of ammonia in our studies, changes in red blood parameters (hemoglobin concentration, erythrocyte count, hematocrit level) revealed ammonia toxicity to the fish. Hemoglobin concentration and erythrocyte count significantly decreased ($p< 0.05$) in the blood of fish exposed to 0.09 – 0.024 mg/L of ammonia (Figure 2).

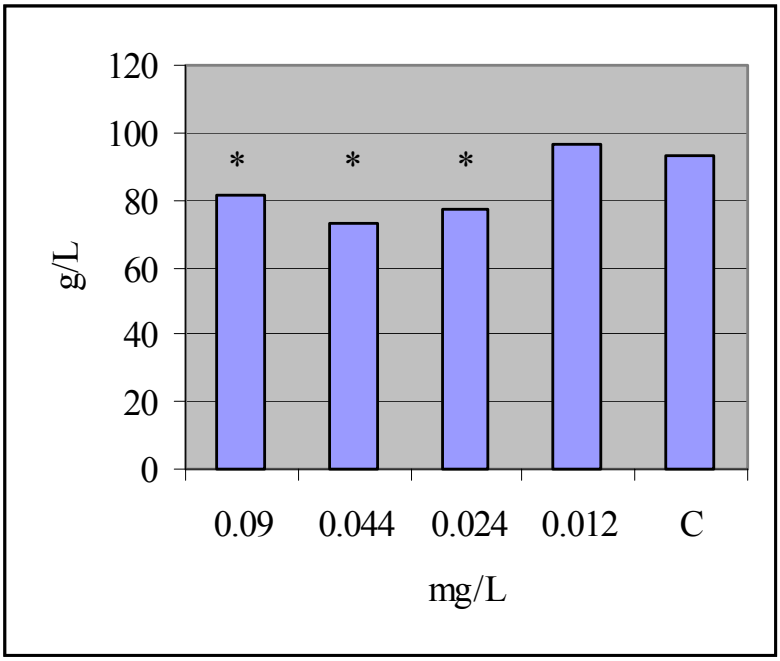

Figure 2. Changes in hemoglobin concentration in blood of fish exposed to ammonia. N=14; asterisks denote significant differences from the control.

Submicroscopic evaluation of erythrocytes did not demonstrate any considerable changes in normal erythrocyte count. A significantly elevated percent of juvenile erythrocytes was found in the blood of fish exposed to 0.024 mg/L of ammonia, and the percent of old, removal forms of erythrocytes was higher in the blood of all exposed fish. The most strongly-expressed changes were found in the percent of damaged erythrocytes: significantly increased amounts of these cells were seen in fish exposed to 0.09-0.024 mg/L of ammonia (Figures 3 and 4).

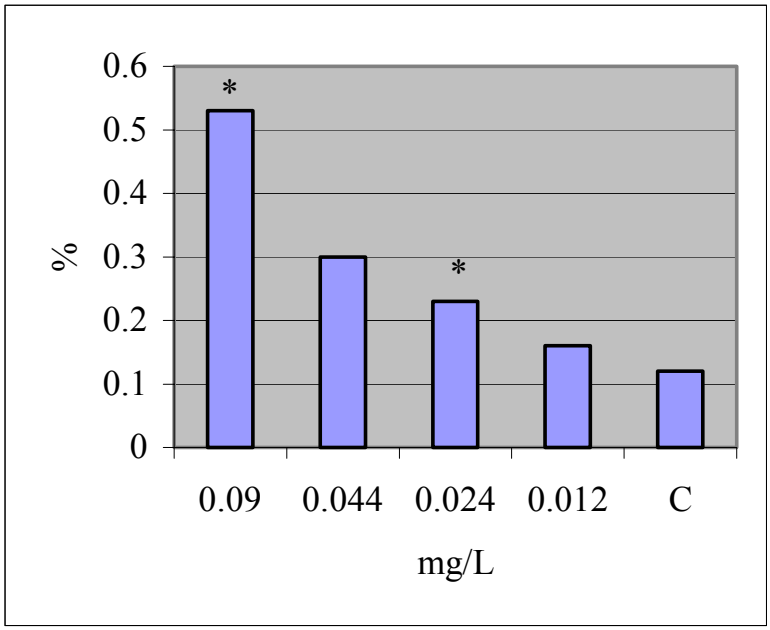

Figure 3. Increase in percent of damaged erythrocytes in blood of fish exposed to ammonia. N=14; asterisks denote significant differences from the control.

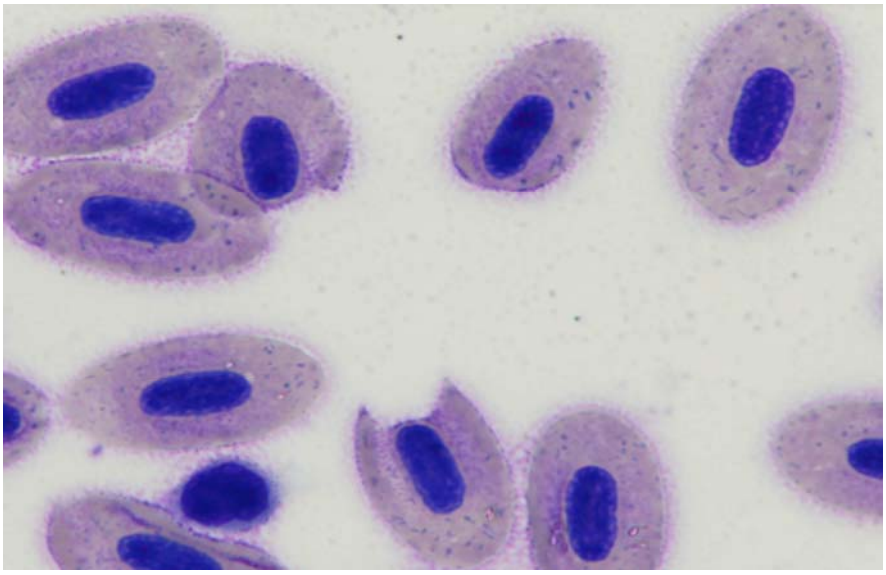

Figure 4. Damaged erythrocytes.

Leucocyte count decreased in the blood of fish exposed to 0.09-0.044 mg/L of ammonia. In all samples, lymphocytes predominated; their share ranged from 86.7 to 94.8 %, and were mainly small lymphocytes. Neutrophilic granulocytes were much less numerous than lymphocytes, and their share ranged from 5.2±0.6 to 13.3±1.5 % of the

leucocytes. The amount of neutrophils was significantly increased in fish exposed to 0.09-0.044 mg/L of ammonia, while in the blood of fish exposed to 0.024 mg/L it was significantly decreased as compared to the control value. The youngest neutrophils - myelocytes and metamyelocytes -predominated and their values did not significantly differ from the controls. Percent of older cells - lobed neutrophils - was increased in blood of fish exposed to 0.09-0.044 mg/L ammonia. Monocytes were found very occasionally.

The present study demonstrated obvious toxic effects of ammonia on the hematological parameters of rainbow trout. Erythrocyte count in blood of fish exposed to 0.09-0.024 mg/L ammonia was significantly lower as compared to controls, accompanied by a decrease in hemoglobin concentration. Probably these alterations were induced by the toxic impact of ammonia on the erythropoietic system of fish. The character of changes in the level of hematocrit was similar to the erythrocyte count: a more-pronounced decrease in erythrocyte count was accompanied by a decrease in hematocrit level. According to the data of Thurston *et al*. (1984), in rainbow trout exposed to concentrations of 0.05 mg NH_3-N/L and greater, hematocrit was reduced and, to a lesser extent, blood hemoglobin content decreased also. The present data demonstrated a slight decrease in the percent of juvenile erythrocytes in fish exposed to ammonia, while the percent of old, removal forms of erythrocytes increased. In our previous study, an increase in the share of old, removal forms of erythrocytes in blood of fish was induced by a three-month exposure of fish to model heavy metal mixtures (Vosylienė and Svecevičius 1997). Damaged red blood cells were observed in blood of fish exposed to 0.09-0.024 mg/L ammonia and the different shapes of damaged erythrocytes were observed on blood smears. These were probably caused by specific cytotoxic action of ammonia on erythrocytes.

Changes in leucocyte count of fish exposed to different ammonia concentrations demonstrated its adverse effect on the immune system of fish. The highest concentrations of ammonia studied (0.09-0.044 mg /L) induced leucocytopenia. Possible toxic effects of ammonia on kidney function can lead to the inhibition of leucocyte production. A decrease in percent of small lymphocytes also took place, accompanied by an increase in the proportion of neutrophils. Neutrophilia might be a response of the organism to chronic effects reflecting a modulation of the immune defense system. Analogous data have been obtained in fish exposed to higher concentrations of ammonia by other investigators (Soderberg *et al.* 1983, Dabrowska and Wlasow 1986). Pollutants affecting the fish may induce chronic stress that can modulate the responses of the cells of the immune system (Secombes *et al.* 1991). A significant decrease in the number of leucocytes (small lymphocytes) in fish can cause a decrease in antigen production and, thus, a reduced resistance to disease (Svobodova *et al.* 1991, Carballo and Munoz 1991). This same increased susceptibility to infectious agents may not be pathogenic in an unstressed environment (Anderson 1990).

The calculation of the Maximum Acceptable Toxicant Concentration for ammonia relative to the most sensitive parameters of rainbow trout (Table 3) revealed that the lowest MATC was 0.004 mg/L ammonia, for the integrated parameter relative body mass increase of larvae.

Table 3. Maximum Acceptable Concentrations of ammonia (MATC) to biological parameters of rainbow trout at all stages of development

Parameter studied	**MATC, mg/L NH_3**
Embryos	
Heart rate	0.034
Larvae	
Gill ventilation frequency	0.09
Relative body mass increase	0.004
Adult fish	
Parameters of RBC	0.02
Leucocyte count	0.03

GUIDELINE RECOMMENDATIONS

The MATC of 0.02 mg/L ammonia for adult fish is very close to the value 0.025 mg/L proposed by the European Union and accepted in Lithuanian water quality norms for salmonid fish. The lowest MATC for ammonia exposure to fish in the early stages of development is lower. The concentration calculated for ammonia nitrogen (0.014 mg N/L) is many times lower than the Maximum Permissible Concentration for ammonia (0.39 mg N/L) accepted for Lithuanian inland waters.

CONCLUSIONS

A number of morphological and physiological parameters of rainbow trout *Oncorhynchus mykiss* in all stages of development were investigated to evaluate their sensitivity to the effect of ammonia. The biological parameters investigated were found to be of different sensitivity, the most sensitive being integrated indices such as the growth of larvae (total body mass increase, relative body mass increase) in chronic bioassays.

Hematological parameters of adult rainbow trout were found to be sensitive to the effect of ammonia. Even low concentrations of ammonia (close to Lithuania's Maximum Permissible Concentration) induced significant changes in red blood cell (erythrocyte count, hemoglobin concentration, hematocrit level, percent of damaged erythrocytes) and white blood cell (leucocyte, lymphocyte count, neutrophil percent) parameters.

The Maximum Acceptable Concentration, 0.02 mg/L of ammonia to adult fish is very close to the 0.025 mg/L proposed by the EU and accepted in Lithuania as a water quality norm for salmonid fish. The Maximum Acceptable Concentration for ammonia to fish in earlier stages of development is lower. The calculated ammonia nitrogen concentration that corresponds to this Maximum Acceptable Concentration (0.014 mg N/L) is many times lower than the Maximum Permissible Concentration for ammonia (0.39 mg N/L) accepted for Lithuanian inland waters.

REFERENCES

Anderson, D.P. 1990. Immunological indicators: effects of environmental stress on immune protection and diseases outbreaks. American Fisheries Society Symposium 8: 38-50.

Adams, M.B., M.D. Powell, and G.J. Purser. 2001. Effect of acute and chronic ammonia and nitrite exposure on oxygen consumption and growth of juvenile big bellied seahorse. Journal of Fish Biology 58:848-860.

Anonymous. 2002. Annual Report of Water Quality of Rivers of Lithuania. Ministry of Environment of the Lithuanian Republic, Vilnius (in Lithuanian).

Carballo, M., and M.J. Munoz. 1991. Effect of sublethal concentrations of four chemicals on susceptibility of juvenile rainbow trout, *Oncorhynchus mykiss*, to saprolegniosis. Applied and Environmental Microbiology 57:1813-1816.

Dabrowska, H., and T. Wlasow. 1986. Sublethal effect of ammonia on certain biochemical and haematological indicators in common carp (*Cyprinus carpio* L.). Comparative Biochemistry and Physiology C 83:179-184.

Daost, P.Y., and H.W. Ferguson. 1984. The pathology of chronic ammonia toxicity in rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Diseases 7:199-205.

Hamilton, M.A., R.C. Russo, and R.V. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentration in toxicity bioassays. Environmental Science and Technology 11:714-719.

Iwata, K. 1988. Nitrogen metabolism in the mudskipper *Periophthalmus cantonens*: Changes in free amino acids and related compounds in various tissues under conditions of ammonia loading, with special reference to its high ammonia tolerance. Comparative Biochemistry and Physiology A 91:499-508.

Kazlauskienė, N., and P. Stasiūnaitė. 1999. The lethal and sublethal effect of heavy metal mixtures on rainbow trout *Oncorhynchus mykiss* in early stages of development. Acta Zoologica Lithuanica. Hydrobiologia, 9:47-55.

Rand, G.M., and S.R. Petrocelli, editors. 1985. Fundamentals of aquatic toxicology. Methods and Applications, New York, Washington, Philadelphia, London. Hemisphere Publishing Corporation.

Randall, D. J., and T.K.N. Tsui. 2002. Ammonia toxicity in fish. Marine Pollution Bulletin 45:17-23.

Russo, R.C. 1985. Ammonia, nitrite, and nitrate. *In:* Fundamentals of Aquatic Toxicology. Methods and Applications. G.M. Rand and S.R.. Petrocelli, (eds.), New York, Washington, Philadelphia, London: Hemisphere Publishing Corporation. pp. 455-471.

Secombes, C.J., T.C. Fletcher, J.A. O'Flynn, M.J. Costello, R. Stagg, and D.F. Houlihan. 1991. Immunocompetence as a measure of the biological effects of sewage sludge pollution in fish. Comparative Biochemstry and Physiology C 100:133-136.

Sinderman, C.J. 1984. Fish and environmental impact. Archiv FischerieWissenschaft 35 (1): 125-160.

Soderberg, R.W., J.B. Flynn, and H.R. Schmittow, 1983. Effects of ammonia on growth and survival of rainbow trout in intensive static-water culture. Transactions of the American Fisheries Society 112:448-451.

Steele, S.L., T.D. Chadwick, and P.A. Wright. 2001. Ammonia detoxification and localization of urea cycle enzyme activity in embryos of the rainbow trout *(Oncorhynchus mykiss)* in relation to early tolerance to high environmental ammonia levels. Journal of Experimental Biology 204:2145-2154.

Svobodova, Z., and B. Vykusova, editors. 1991. Diagnostics, prevention and therapy of fish diseases and intoxications. Vodnany, Chechoslovakia, pp. 270.

Svobodova, Z., R. Lloyd, J. Machova, and B. Vykusova. 1993. Water quality and fish health. EIFAC Technical Paper. No 54. United Nations FAO, Rome. pp. 59.

Thurston, R.V., R.C. Russo, and C.E. Smith. 1978. Acute toxicity of ammonia and nitrite to cutthroat trout fry. Transactions of the American Fisheries Society 107:361-368.

Thurston, R.V., and R.C. Russo. 1983. Acute toxicity of ammonia to rainbow trout. Transactions of the American Fisheries Society 112:696-704.

Thurston, R.V., R.C. Russo, R.J. Luedtke, C.E. Smith, E.L. Meyn, C. Chakoumakos, K.C. Wang, and C.J.D. Brown. 1984. Chronic toxicity of ammonia to rainbow trout. Transactions of the American Fisheries Society 113:56-73.

Thurston, R.V., R.C. Russo, E.L. Meyn, R.K. Zajdel, and C.E. Smith. 1986. Chronic toxicity of ammonia to fathead minnows. Transactions of the American Fisheries Society 115:196-207.

Tilak, K.S., Lakshmi, S.J., and T.A. Susan. 2002. The toxicity of ammonia, nitrite and nitrate to the fish *Catla catla* (Hamilton). Journal of Environmental Biology 23 (2): 147-149.

Tomasso, J.R., and G.J. Carmichael. 1986. Acute toxicity of ammonia, nitrite and nitrate to the Guadalupe bass, *Micropterus treculi*. Bulletin of Environmental Contamination and Toxicology 36:866-870.

Twitchen, I.D. and F.B. Eddy. 1992. Sublethal effects of ammonia on freshwater fish. EIFAC/XVII/92/Symposium 4. E14. United Nations FAO, Rome.

U.S. Environmental Protection Agency. 1985. Ambient water quality criteria for ammonia. EPA-440/5-85-001. National Technical Information Service, Springfield, VA.

U.S. Environmental Protection Agency. 1999. Update of ambient water quality criteria for ammonia. EPA-823-F-99-024. EPA Office of Water, Washington, DC.

Vedel, N.E., B. Korsgaard, and F.B. Jensen. 1998. Isolated and combined exposure to ammonia and nitrite in rainbow trout (*Oncorhynchus mykiss*): effects on electrolyte status, blood respiratory properties and brain glutamine/glutamate concentrations. Aquatic Toxicology 41:325-342.

Vosylienė, M.Z., and G. Svecevičius. 1997. Sublethal effects on rainbow trout of chronic exposures to mixtures of heavy metals. *In:* Fish Physiology, Toxicology, and Water Quality. Proceedings of the Fourth International Symposium, Bozeman, Montana, USA, September 19-21, 1995. EPA/600/R-97/098, pp. 141-151.

THE EFFECT OF TEMPERATURE ON THE DISTRIBUTION OF ATLANTIC COD (*GADUS MORHUA*)

Maria Faldborg Petersen[1]

ABSTRACT

Different values for the preferred temperature have been reported for Atlantic cod, ranging from approximately 8° to 14°C. Furthermore, the cod can be subdivided with regard to hemoglobin genotype. The hemoglobin components can be separated into two homozygous types, HbI-1 and HbI-2, and one heterozygous type, HbI-1/2. The geographical distribution of Atlantic cod with the different hemoglobin genotypes is heterogeneous, with a high frequency of the HbI-2 allele in the waters off Greenland, Iceland, the Faeroe Islands, northern Norway and the northern part of the Baltic Sea, while the HbI-1 allele is dominant in warmer areas. An analysis of existing data indicates that the great difference in the reported temperature preferences of cod is correlated with the different hemoglobin genotypes. Cod possessing the different genotypes have very different preferred temperatures: HbI-2 cod prefer 8.2° ± 1.5°C while HbI-1 cod prefer 15.4 ° ± 1.1°C. This paper also discusses the theory of optimized physiology at the preferred temperature for the different genotypes of cod, and the potential effect of rising water temperature on the distribution of cod.

INTRODUCTION

Abiotic parameters, such as temperature, are of considerable importance when studying the distribution patterns of temperate fish. Atlantic cod are found at temperatures ranging from – 1 ° to 24°C (Jobling 1988). Since cod are distributed within this wide range of temperatures, they can obviously tolerate them. Within these limits, however, the physiology of the cod is optimized in a narrower temperature range. This zone is termed the preference zone or more specifically the preferred temperature.

For several years, the generally-accepted value for the preferred temperature of Atlantic cod has been approximately 14°C; however values as low as 6°C have been reported from experiments conducted in Canada (Clark and Green 1991, Despatie *et al*. 2001). In this communication, I present theoretical considerations about the preferred temperature and empirical evidence to show that Atlantic cod can be subdivided into three major types depending on their hemoglobin type, each having its own preferred temperature. Whether the difference in the preferred temperature of Atlantic cod also is correlated with physiological differences will be discussed. Potential changes in the Atlantic cod stocks with a rise in seawater temperature as a consequence of global warming will also be discussed.

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THEORETICAL CONSIDERATIONS

Behavioral Thermoregulation

Most species of fish show behavioral temperature regulation and thereby tend to be found within a specific temperature interval. This behavior is advantageous for the fish since several processes, such as acid-base balance, metabolism, blood flow, locomotion, ion and water flux, as well as enzymes and the nervous system are influenced by temperature (Crawshaw 1979). The mechanism behind behavioral thermoregulation is controlled by the central nervous system and is comparable to similar mechanisms in higher vertebrates. Fish can register even small temperature changes. As small a difference as 0.03-0.1°C has been reported as whole-body sensitivity (Crawshaw 1979).

The temperature interval a fish is limited to can be subdivided into the resistance, tolerance and preference zones (Figure 1). In the resistance zone, the fish meets extreme temperatures and its survival time is strongly dependent on the exposure time (Jobling 1994). In the tolerance zone, the fish survives without temperature-associated problems, while the preference zone, centered on the preferred temperature, is thought to be the zone where physiological processes are optimized (Jobling 1994). Physiological processes that have been shown to be optimized at the preferred temperature are appetite, growth rate, active metabolic rate, the scope between standard and active metabolic rate, and sustained swimming speed (Figure 2) (Brett 1971, Beitinger and Fitzpatrick 1979, Jobling 1981).

Definitions for the Preferred Temperature

The preferred temperature of ectothermic animals has been measured over time using different methods and without being explicitly defined. However, in 1947 Fry defined a theory in two parts for preferred temperature. First, preferred temperature was defined as *"That temperature at which the preferred temperature is equal to the acclimation temperature."* The first part of the theory is based on determining acute preferred temperature, which is the temperature preferred by the fish within the first 1-2 hours after being placed in a gradient. Fish acclimated to different temperatures have different acute preferred temperatures. The first definition of the preferred temperature of a species is where the line of the acute preferred temperature and equality line cross (Figure 1). The second part of Fry's definition is: *"That temperature around which all individuals will ultimately congregate, regardless of their thermal experience before being placed in the gradient."* This definition is based on measurement over longer periods of time until the fish has reached its preferred temperature and thereafter stays within a narrow temperature range.

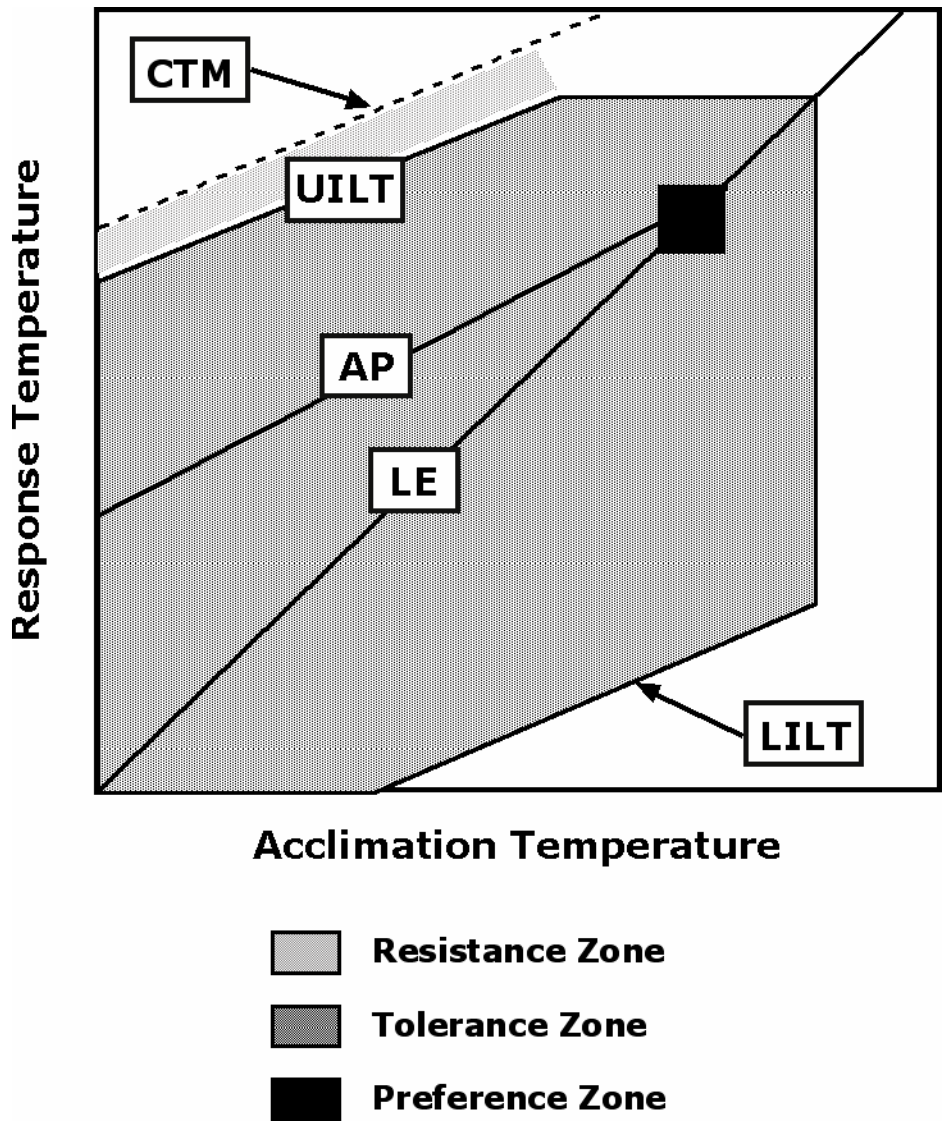

Figure 1. Temperature polygon showing the different temperature zones. The resistance zone is between the Critical Thermal Maximum (CTM) and the Upper Incipient Lethal Temperature (UILT). LILT = Lower Incipient Lethal Temperature. The preferred temperature, surrounded by a narrow preference zone, can be found at the intersection of the Acute Preference Line (AP) and the Line of Equality (LE) (from Jobling 1981).

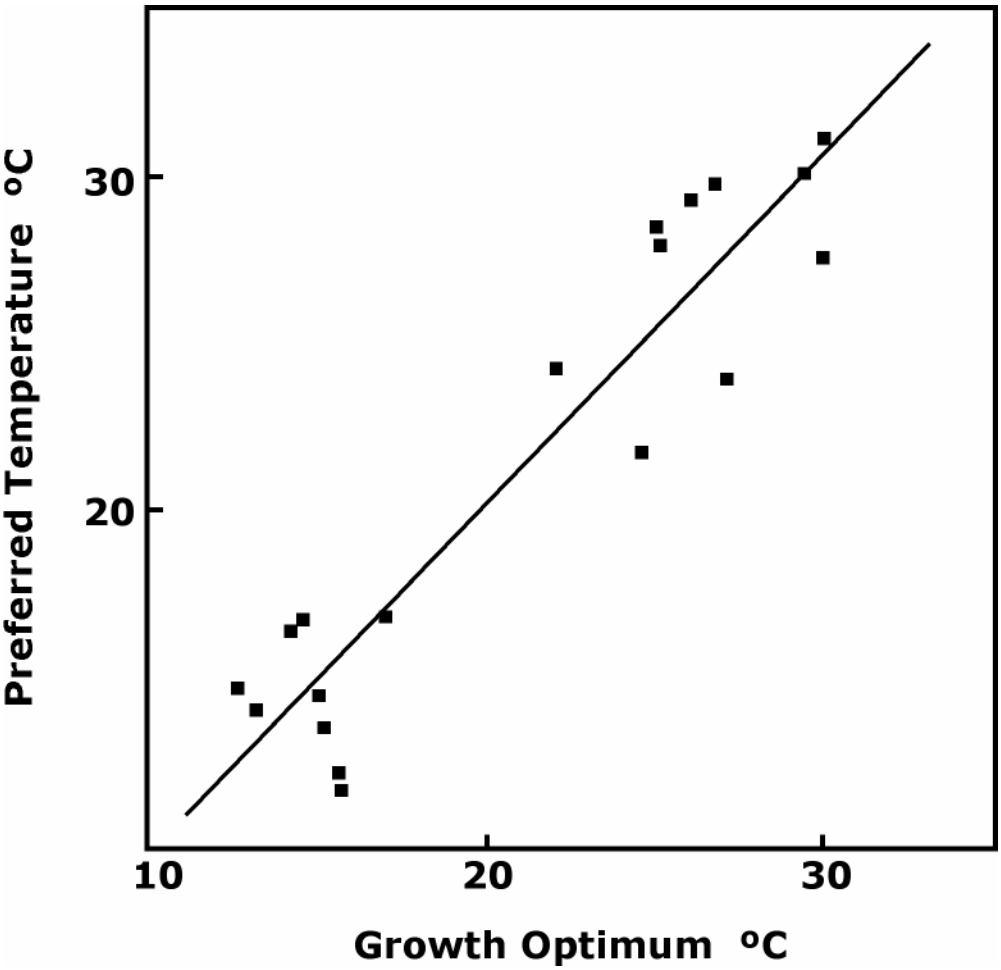

Figure 2. The preferred temperature as a function of growth optimum for various fish species, confirming the theory of optimized physiology at the preferred temperature (from Jobling 1988).

Reynolds and Casterlin (1979) compared the two methods generally used to measure preferred temperature. In experiments with the cichlid *Tilapia mossambica* and the bluegill *Lepomis macrochirus* no significant difference in preferred temperature based on measurement method was found. The preferred temperature of the Atlantic cod has been measured by both methods. Jobling (1988) used data from experiments where the acute preferred temperature had been measured, and the intersection between this line and the line of equality was approximately 14°C. A similar value (13.9±2.7°C) was measure by Schurmann and Steffensen (1992) using a shuttle box system, where the cod was allowed to control its own body temperature by shuttling between a warm and a cold chamber.

Factors Influencing the Preferred Temperature

The preferred temperature for a species of fish is not a fixed value, but changes with circumstance. Ontogeny, season, reproductive status, toxicity, health, diel rhythms, food ration, salinity, biological interactions and hypoxia are some of the factors influencing the preferred temperature (Reynolds and Casterlin 1980). For Atlantic cod, the effect of season, food ration and hypoxia on the preferred temperature have been examined. Clark and Green (1991) studied the effect of season and concluded that the preferred temperature changes significantly during the year. An average temperature of approximately 8°C was preferred during the summer months, whereas lower temperatures were preferred in winter. The amount of food consumed by the cod also influences the preferred temperature. Fish fed an intermediate food ration preferred the highest temperature of around 6°C (Despatie *et al*. 2001). Hypoxia results in a decrease of the preferred temperature. Experiments by Schurmann and Steffensen (1992) showed that cod prefer around 14°C at normoxia and 9°C at 15% oxygen saturation.

Besides showing that the preferred temperature of cod is not a fixed value, these studies also demonstrated great variation among the values of preferred temperature of cod under "normal" conditions. While Clark and Green (1991), as well as Despatie *et al*. (2001), found a preferred temperature of cod lower than 10°C, Schurmann and Steffensen (1992) and Jobling (1988) all measured values of approximately 14°C. Why the preferred temperature of cod, according to these literature values, seems to fall into two groups has been an unanswered question. We (Petersen and Steffensen 2003) found a reasonable answer to this when looking thoroughly into the different hemoglobin genotypes that exist among Atlantic cod.

Polymorphic Hemoglobin of Atlantic Cod

The different types of Atlantic cod hemoglobin have been known since 1961 (Sick 1961). Experiments with agar gel electrophoresis revealed that Atlantic cod hemoglobin consists of two homozygous genotypes (HbI-1 and HbI-2) and one heterozygous genotype (HbI-1/2) (Figure 3). These genotypes are controlled by two alleles referred to as HbI^1 and HbI^2. The different hemoglobin genotypes are heterogeneously distributed throughout the Atlantic cod's range. The frequency of the HbI^1 allele is low in the northern Baltic Sea (Sick 1965a), and off North America, the Faeroe Islands, Iceland and Greenland (Sick 1965b). It increases to the south and reaches a maximum of 0.72 off the coast of the Netherlands (Figure 4). Such polymorphism of fish hemoglobin is prevalent (Kirpichnikov 1981) and is often essential if fish change habitat during their development, or if they shift between different environments where changes in respiration media (water, air), salinity, or other factors occur (Jensen *et al*. 1998). However, for many species of fish the reason for polymorphic hemoglobin is not well understood, as was the case with the Atlantic cod.

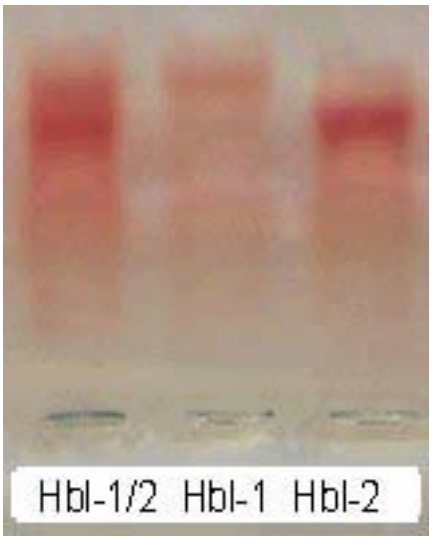

Figure 3. Agar gel of the three different hemoglobin genotypes. HbI-1 and HbI-2 can be separated by a fast and a slow moving single band, respectively (homozygous), and HbI-1/2 contains a double band (heterozygous).

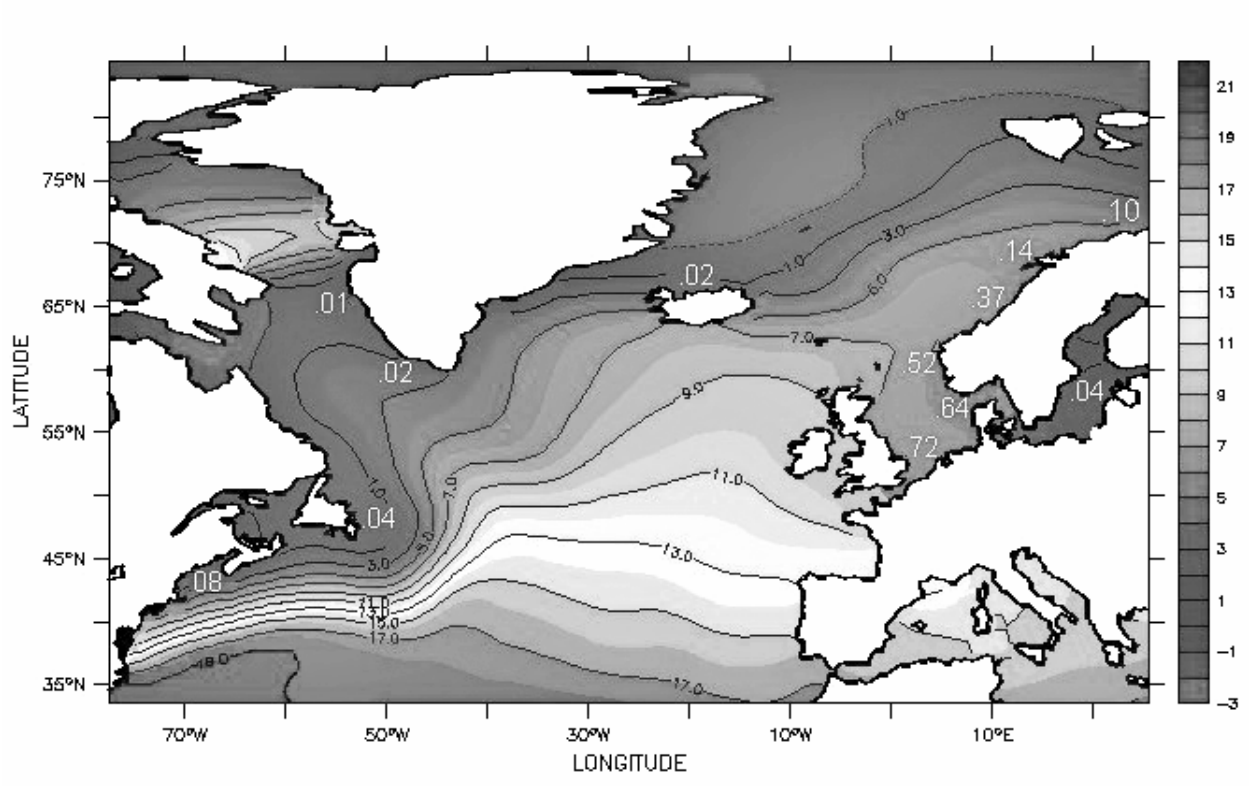

Figure 4. Sea-surface temperature (^{o}C) and the frequency distribution of the HbI^{1} allele (0-1) throughout the range of the Atlantic cod (from Petersen and Steffensen 2003).

EMPIRICAL EVIDENCE

Preferred Temperature of the Different Hemoglobin Genotypes

When searching for a reason why Atlantic cod blood is polymorphic, the heterogeneous distribution of the two alleles coding for the different genotypes and the great variety in preferred temperature measured for the Atlantic cod suggest that temperature could be a selective parameter. We (Petersen and Steffensen 2003) decided to measure the preferred temperature of Atlantic cod homozygous for HbI-1 and HbI-2 to test whether temperature is the selective parameter in the distribution of Atlantic cod. We determined the genotypes with agar gel electrophoresis and used a shuttle box system to measure the preferred temperature of the fish. After 24 hours of shuttling between a cold and a warm chamber, the temperature interval preferred by the cod was stabilized within a few degrees. A very large difference was found between the preferred temperatures of HbI-1 and HbI-2 cod. HbI-1 fish preferred a temperature of 15.4±1.1°C, while HbI-2 fish preferred a temperature of 8.2±1.5°C. Thus, we were able to conclude that the heterogeneous distribution of the hemoglobin genotypes of Atlantic cod is due to temperature. The high frequency of the HbI-2 allele where the ambient water temperature is low is due to the low preferred temperature of HbI-2 cod. Furthermore, the difference in the preferred temperature between the different genotypes can also explain why Clark and Green (1991) and Despatie *et al*. (2001) found a preferred temperature of Atlantic cod lower than 10°C. This is due to the fact that these experiments were conducted in Canada, where the HbI-2 allele is dominant. In contrast, a preferred temperature of approximately 14°C was measured by Schurmann and Steffensen (1992) with cod caught in waters around Denmark, where the frequency of the HbI-1 allele is high.

How the Hemoglobin Genotypes can Control the Preferred Temperature

The difference between the HbI-1 and HbI-2 genotypes is limited to an extra histidine-containing peptide in the HbI-1 type, and therefore the structural differences are minimal (Rattazzi and Pik 1965). However, differences in the biochemical properties have been described for the genotypes. The oxygen affinity of hemoglobin is higher for HbI-2 cod at low temperatures (<10°C), and for HbI-1 cod, at some blood pH values, at high temperatures (>14°C) (Karpov and Novikov 1981, Brix *et al*. 1998, McFarland 1998). This explains why HbI-2 cod prefer lower temperatures than HbI-1 cod.

Optimized Physiology at the Preferred Temperature

A few physiological studies have distinguished between the genotypes of cod. Data from these studies can be analyzed with respect to the known difference in the preferred temperature to test whether the theory of optimized physiology at the preferred temperature is valid. Differences in respiration parameters were analyzed by McFarland (1998). A significantly lower standard metabolic rate (SMR) for HbI-2 cod at 4°C compared with HbI-1 cod was found. However, a lower SMR for HbI-1 cod at high water temperatures was absent. Critical oxygen

partial pressure (P_{crit}) was also measured. HbI-2 cod had an advantageously lower P_{crit} value at 4°C and when the temperature was decreased rapidly from 10°C to 4°C. For HbI-1 cod, the P_{crit} value was lower compared to HbI-2 cod upon an acute temperature increase (10°C to16°C). Feeding behavior has also been examined, and a higher competitive performance was found for HbI-2 cod measured at 6°C (Salvanes and Hart 2000). Unfortunately, no such experiment has been made at higher temperatures. Growth rate is another parameter that has often been found to be optimized at the preferred temperature. Some studies have attempted to define the growth rates of the two hemoglobin genotypes (Nævdal *et al*. 1992, Jørstad and Nævdal 1994, Mork *et al*. 1983, 1984). In these experiments, however, the different genotypes must be separated to distinguish between exact growth rate and behavioral differences in competitive performance, and no such study has yet been performed. So far, it appears that the theory of optimized physiology at the preferred temperature fits HbI-2 cod perfectly, whereas the advantages of HbI-1 cod at high water temperatures are less well known.

Preferred Temperature of the Different Hemoglobin Genotypes during Hypoxia

Another question to ask, with regard to the difference in the preferred temperature between hemoglobin genotypes of cod, is whether the two types respond similarly to parameters affecting the preferred temperature. Hypoxia is a common phenomenon in several habitats where Atlantic cod reside, especially in coastal regions such as the Gulf of St Lawrence (Chabot and Dutil 1999) and the Baltic Sea (Nielsen and Gargas 1984). We (Petersen and Steffensen 2003) analyzed the effect of hypoxia on the preferred temperature. The oxygen saturation was decreased to 35% and the preferred temperature was measured for eight cod with the HbI-1 genotype and eight individuals of the HbI-2 genotype. Figure 5 shows that the preferred temperature decreased from 15.4±1.1°C to 9.8±1.8°C in HbI-1 cod exposed to hypoxia, but that this tendency was absent in HbI-2 cod. There are several physiological advantages in lowering the body temperature during hypoxia, including lower metabolic rate, higher oxygen affinity for hemoglobin and higher oxygen solubility in the water (Jobling 1994). The disadvantages of preferring lower water temperatures during hypoxia are a reduction in swimming speed as well as a reduction in food intake and digestion rate, that result in decreased growth (Brett 1971).

Previous studies where fish were exposed to hypoxia have shown a decrease in the preferred temperature; this was, however, dependent on the level of hypoxia (Schurmann *et al*. 1991, Schurmann and Steffensen 1992). The observation that the preferred temperature of HbI-2 cod did not decrease as a consequence of hypoxia indicates that the energy-saving advantage of an even lower temperature was not necessary for the HbI-2 cod to survive. However, if the HbI-2 cod had been exposed to a lower level of hypoxia, possibly the preferred temperature would have decreased.

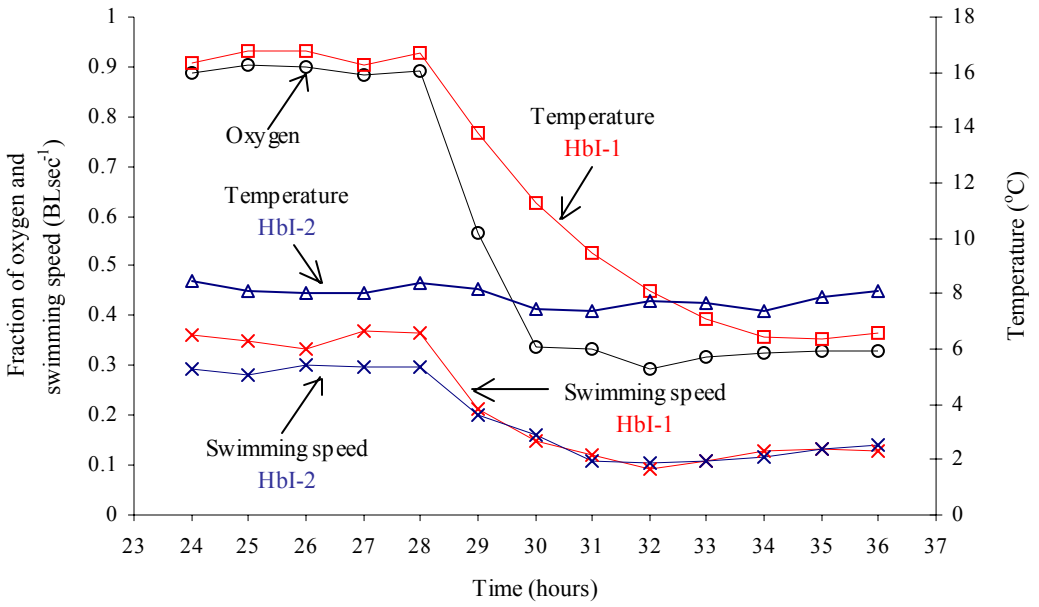

Figure 5. Oxygen saturation, swimming speed and water temperature measured for cod with hemoglobin types HbI-1 and HbI-2. When the oxygen saturation is lowered to 35%, the preferred temperature of HbI-1 cod decreases, while this tendency is absent for HbI-2 cod (from Petersen and Steffensen 2003). Swimming speed is expressed in body lengths/sec.

Distribution of Atlantic Cod with Temperature

When trying to answer the question of how Atlantic cod are distributed with respect to temperature, it is of primary importance to consider the different hemoglobin genotypes of cod. HbI-1 and HbI-2 cod prefer very different temperatures, and from the frequency distribution of occurrence for the different types, it is clear that they distribute themselves with regard to their preferred temperature. Whether this is true on a small scale, for instance in a temperature-stratified water column, is still a question of interest.

The information about the preferred temperatures of HbI-1 and HbI-2 cod also indicates that increasing water temperatures, for instance because of global warming, will result in an increased frequency of HbI-1 cod, because this hemoglobin type prefers a higher temperature. If a combination of increased water temperatures and hypoxia should occur, the predicted superiority of HbI-1 as a consequence of increased water temperatures, and the fact that HbI-1 cod prefer a lower temperature during hypoxia, will cause an unfavorable situation for the HbI-1 cod. This situation is especially relevant in coastal regions where hypoxia is common, and could cause extensive damage, for example, to the Baltic cod stock.

REFERENCES

Beitinger, T.L. and L.C. Fitzpatrick. 1979. Physiological and ecological correlates of preferred temperature in fish. American Zoologist 19:319-329.

Brett, J.R. 1971. Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of Sockeye Salmon (*Oncorhynchus nerka*). American Zoologist 11:99-113.

Brix, O., E. Forås, and I. Strand. 1998. Genetic variation and functional properties of Atlantic cod hemoglobins: Introducing a modified tonometric method for studying fragile hemoglobins. Comparative Biochemistry and Physiology 119A(2):55-583.

Chabot, D. and J.-D Dutil. 1999. Reduced growth of Atlantic cod in non-lethal hypoxic conditions. Journal of Fish Biology 55:472-491.

Clark, D.S. and J.M. Green. 1991. Seasonal variation in temperature preference of juvenile Atlantic cod (*Gadus morhua*), with evidence supporting an energetic basis for their diel vertical migration. Canadian Journal of Zoology 69:1302-1307.

Crawshaw, L.I. 1979. Responses to rapid temperature change in vertebrate ectotherms. American Zoologist 19:225-237.

Despatie, S.-P., M. Castonguay, D. Chabot, and C. Audet. 2001. Final thermal preferendum of Atlantic cod: Effect of food ration. Transactions of the American Fisheries Society 130:263-275.

Fry, F.E.J. 1947. Effects of the environment on animal activity. University of Toronto Study of Biology Series 55, Publisher. Ontario Fisheries Research Laboratory 68:1-62.

Jensen, F.B., A. Fago, and R.E. Weber. 1998. Hemoglobin structure and function. *In:* Fish Respiration. S.F. Perry and B.L. Tufts (eds.). London, New York: Academic Press. Pp. 1-40.

Jobling, M. 1981. Temperature tolerance and the final preferendum -rapid methods for the assessment of optimum growth temperatures. Journal of Fish Biology 19:439-355.

Jobling, M. 1988. A review of the physiological and nutritional energetics of cod, *Gadus morhua* L., with particular reference to growth under farmed conditions. Aquaculture 70:1-19.

Jobling, M. 1994. Fish Bioenergetics. Fish and Fisheries Series 13. London: Chapman and Hall.

Jørstad, E.J. and G. Nævdal. 1994. Studies on associations between genotypes and growth rate in juvenile cod. ICES Marine Science Symposium 198:671-675.

Karpov, A.K. and G.G. Novikov. 1981. Hemoglobin alloforms in cod, *Gadus morhua* (Gadiformes, Gadidae): Their functional characteristics and occurrence in populations. Journal of Ichthyology 6(6):45-49.

Kirpichnikov,V.S. 1981. Genetic bases of fish selection. Springer-Verlag.

McFarland, S. 1998. Biochemical and physiological adaptations of haemoglobin-I genotypes of Atlantic cod, *Gadus morhua* L. Ph.D. University of Birmingham.

Mork, J., R. Giskeødegård, and G. Sundnes. 1983. Haemoglobin polymorphism in *Gadus morhua*: Genotypic differences in maturing age and within-season gonad maturation. Helgoländer Meeresuntersuchungen 36:313-322.

Mork, J., R. Giskeødegård, and G. Sundnes. 1984. The hemoglobin polymorphism in Atlantic cod (*Gadus morhua* L.): Genotypic differences in somatic growth and in maturing age in natural populations. Flødevigen rapportser. 1. The propagation of cod *Gadus morhua* L. 721-732.

Nævdal, G., A. Folkvord, E. Otterlei, and S. Thorkildsen. 1992. Growth rate related to genotype of 0-group cod at three environmental temperatures. Sarsia 77:71-73.

Nielsen, G. and E. Gargas. 1984. Oxygen, nutrients and primary production in the open Danish waters. Limnologica (Berlin) 15:303-310.

Petersen, M.F. and J.F. Steffensen. 2003. Preferred temperature of juvenile Atlantic cod Gadus morhua with different haemoglobin genotypes at normoxia and moderate hypoxia. Journal of Experimental Biology 206:359-364.

Rattazzi, M.C. and C. Pik. 1965. Haemoglobin polymorphism in the cod (*Gadus morhua*): a single peptide difference. Nature 208:489-491.

Reynolds, W.W. and M.E. Casterlin. 1979. Behavioral thermoregulation and the "final preferendum" paradigm. American Zoologist 19:211-224.

Reynolds, W.W. and M.E. Casterlin. 1980. The Role of Temperature in the Environmental Physiology of Fishes. *In:* Environmental Physiology of Fishes, M.A. Ali (ed.). New York: Plenum Press. Pp. 497-518.

Salvanes, A.G.V. and P.J.B. Hart. 2000. Is individual variation in competitive performance of reared juvenile cod influenced by hemoglobin genotype? Sarsia 85:265-274.

Schurmann, H. and J.F. Steffensen. 1992. Lethal oxygen levels at different temperatures and the preferred temperature during hypoxia of the Atlantic cod, *Gadus morhua* L. Journal of Fish Biology 41:927-934.

Schurmann, H., J.F. Steffensen, and J.P. Lomholt. 1991. The influence of hypoxia on the preferred temperature of rainbow trout *Oncorhynchus mykiss*. Journal of Experimental Biology 157:75-86.

Sick, K. 1961. Hemoglobin polymorphism in fishes. Nature 192:894-896.

Sick, K. 1965a. Hemoglobin polymorphism of cod in the Baltic and the Danish Belt Sea. Hereditas 54:19-48.

Sick, K. 1965b. Hemoglobin polymorphism of cod in the North Sea and the North Atlantic Ocean. Hereditas 54:49-69.

CHRONIC NON-LETHAL LEVELS OF HYPOXIA LIMIT DISTRIBUTION AND GROWTH OF ATLANTIC COD (*GADUS MORHUA*) IN THE NORTHERN GULF OF ST. LAWRENCE, CANADA

Denis Chabot[1]

ABSTRACT

In the St. Lawrence estuary and Gulf of St. Lawrence, waters deeper than ≈150 m do not mix with surface waters. As a result, the deep channels that characterize this ecosystem are hypoxic. Oxygen levels vary from ≈50 to 60% saturation in Cabot Strait, where oceanic waters enter the Laurentian Channel, to ≈20-30% saturation in the estuary, approximately 1000 km upstream. Such low values of dissolved oxygen were expected to play a role in the survival, distribution, and growth of Atlantic cod.

Laboratory experiments were conducted to determine the lethal (LC_{50} 96 h) and incipient lethal (LC_5 96 h) hypoxia thresholds for cod. Two size classes (small cod, 45.2 cm ± 4.2 [mean ± SD] and large cod, 57.5 cm ± 3.8) and two water temperatures (2 and 6°C) were tested. We found no size or temperature effect on tolerance of hypoxia. The lethal threshold was 21.0% O_2 saturation (95% CI: 18.8-23.2% O_2), while the incipient lethal threshold was 28.1% O_2 saturation (95% CI: 23.4-32.8% O_2). No fish died when O_2 saturation was ≥34%.

To measure the impact of non-lethal hypoxia on growth, cod were raised for 84 days at 10°C and fed capelin *ad lib* for one hour, three times a week, while exposed to one of six oxygen treatments ranging from 45 to 93% saturation. Significant increases in length, mass, and condition were observed in all treatments. However, these increases were significantly less at 44 and 54% O_2 saturation than in the other treatments. Regression analysis showed that growth was limited by oxygen below 70% saturation.

We also observed that food ingestion, which was inversely related to dissolved O_2, explained almost all variability in growth between treatments. Hypoxia could limit maximum meal size, which would have resulted in a reduction in food ingestion because of the fixed feeding frequency. Hypoxia could also slow down digestion and hence food ingestion. A second growth experiment was undertaken to discriminate between these two alternatives. Cod were again raised at 10°C, and offered one, three, or seven meals per week, either at 45 or 90% O_2 saturation. The experiments lasted 56 days in 1999 (two replicate tanks for each treatment) and 62 days in 2000 (one tank per treatment). In normoxia, changes in mass and condition were significantly reduced at one meal per week compared to three and seven meals. In hypoxia, however, growth and condition were reduced at one compared to three meals per week and it was the only significant difference. At one meal per week, growth and condition were equally

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poor at both oxygen levels. At three and seven meals per week, growth was greater in normoxia than in hypoxia. Furthermore, cod in hypoxia ate the same size meals as cod in normoxia at the beginning of the experiment, and during the experiment for the one-meal treatment. Together, these results suggest that hypoxia does not limit the size of meals cod can eat, but slows digestion and therefore food ingestion.

Field observations of dissolved oxygen and cod distribution were used to estimate the proportion of fish from the northern Gulf of St. Lawrence stock that was exposed to each of six classes of dissolved oxygen during the 1995 feeding season. Relationships between oxygen and food ingestion, and between food ingestion and growth were then used to estimate growth production for the stock. Cod density was inversely related to dissolved oxygen. Nevertheless, close to 50 and 25% of the fish were found in hypoxic waters during the summer and fall surveys, respectively. Overall, this simple model estimated a decrease in growth production of about 18% in summer, and 9% in the fall for this particular stock.

INTRODUCTION

In most hypoxic coastal marine ecosystems such as the Black Sea, the Kattegat, the Adriatic Sea, the northern Gulf of Mexico, and others, the ultimate cause of hypoxia appears to be eutrophication due to excessive nutrient loading (Diaz and Solow 1999). Hypoxia follows a seasonal cycle in these ecosystems (Pihl 1989, Diaz and Solow 1999, Craig *et al.* 2001). The deep waters of the Baltic Sea are also periodically hypoxic, although not on a seasonal basis, but rather between storm events that replenish the deep basins with saline, oxygen-rich water from the North Sea (Nissling and Westin 1991, Tomkiewicz *et al.* 1998). However, the hypoxia also seems to have been aggravated by eutrophication (Diaz and Solow 1999) that accelerates oxygen utilization between storm events. Biological consequences of hypoxia include mass mortality of benthos or demersal fish, displacement (migration) of demersal fish to adjacent or shallower waters that can be suboptimal habitats, recruitment failure in fish stocks, declines in commercial fisheries, changes in diet of demersal fish, and community changes favoring hypoxia tolerant species (Seliger *et al.* 1985, Rosenberg and Loo 1988, Pihl 1989, Nissling *et al.* 1994, Pihl 1994, Wieland *et al.* 1994, Breitburg *et al.* 1997, Tomkiewicz *et al.* 1998, Diaz and Solow 1999, Craig *et al.* 2001). Even air-breathing species, such as sea turtles and marine mammals, can be forced to change their distribution in response to changes in the distribution of their prey (Craig *et al.* 2001).

The Gulf of St. Lawrence, a semi-enclosed sea of approximately 200,000 km^2 in eastern Canada (Figure 1), is a lesser-known example of a coastal system characterized by hypoxia. In this case, however, low levels of dissolved oxygen occur naturally at depths ≥200 m year-round, and a large proportion of the water volume is hypoxic. At such depths, levels of dissolved oxygen vary from about 25-70% saturation. For temperatures of 3-6°C and salinity of about 34, this results in a range of ≈2.7-7.0 mg · L^{-1} dissolved oxygen (DO) concentration (Benson and Krause 1984).

Most studies have focused on severe levels of hypoxia, typically ≤2 mg · L^{-1} DO, when assessing the impact of hypoxia on living organisms. However, organisms vary in their

susceptibility to hypoxia (Davis 1975), so a single threshold value does not represent the same severity of stressor for all species. Furthermore, responses to hypoxia are not limited to all-or-nothing types, and less severe hypoxia can still produce biologically significant responses (Davis 1975, Craig *et al.* 2001, Dutil and Chabot 2001). Given that energy requirements of organisms are not constant, but depend on many factors including ontogeny, ration, reproductive status, and activity levels, the response of different organisms to the same level of dissolved oxygen is not identical (Dutil and Chabot 2001). Therefore, we were interested in examining responses of what was once a major demersal predator in this ecosystem, the Atlantic cod (*Gadus morhua*), to the range of dissolved oxygen values found in the St. Lawrence Estuary and Gulf of St. Lawrence.

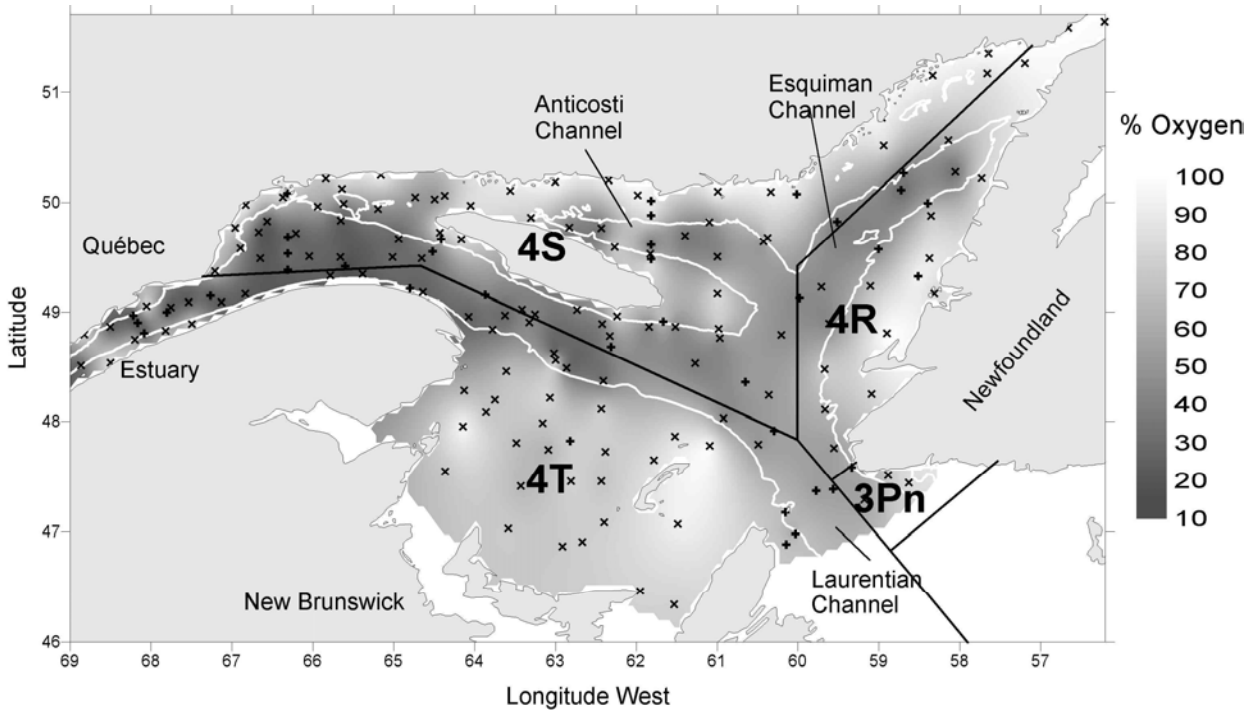

Figure 1. Level of dissolved oxygen (% saturation, Winkler titration) on the bottom of the Gulf of St. Lawrence. × : stations sampled between 11 Aug 1995 and 15 Sep 1995; + : stations sampled between 15 Oct 1995 and 17 Nov 1995. The 200 m isobath is shown as a white line. NAFO divisions borders are solid black lines.

After a brief description of the main features of the study area, this paper reviews some laboratory experiments undertaken at the Maurice-Lamontagne Institute to assess the impact of hypoxia on the survival and growth of cod. These findings were then combined with field measurements of dissolved oxygen, cod distribution, and cod stomach content data to assess the possible impact of hypoxia on growth production of one cod stock in the Gulf of St. Lawrence.

THE STUDY AREA

The Gulf of St. Lawrence and the St. Lawrence Estuary are characterized by deep troughs varying in depth from 300-500 m near Cabot Strait to 250-300 m in the estuary. The Laurentian Channel (Figure 1) that begins at the oceanic shelf margin south of Newfoundland runs approximately 1200 km from east to west through the entire Gulf of St. Lawrence, ending in the estuary. The Esquiman Channel runs approximately south to north along the west coast of Newfoundland, whereas the Anticosti Channel begins in the Esquiman Channels and runs westward along the north shore of Anticosti Island.

A second important characteristic of the Gulf of St. Lawrence, as it affects hypoxia, is stratification. A cold, intermediate layer (CIL, temperature <0°C and average salinity = 32.4) (Lauzier and Trites 1958, Gilbert and Pettigrew 1997) is present year-round. In winter, the water column is a two-layer system, with the cold layer mixing with the surface layer via storm events and density changes of surface waters caused by cold, winter air temperatures; (Koutitonsky and Bugden 1991). Starting in April, the water column becomes a three-layer system as surface temperature increases and salinity decreases (Koutitonsky and Bugden 1991, Gilbert and Pettigrew 1997). In summer, the CIL is found from ≈50 to 100 m deep, although this depends on one's definition of cold. Both surface waters and the CIL are rich in oxygen.

Deep waters at 3-6°C do not mix with the CIL or surface waters. This prevents them from being replenished in oxygen. These deep waters originate at the mouth of the Laurentian Channel and flow landward. Oxygen levels vary between 50 and 70% saturation in Cabot Strait (Bugden 1991, Gilbert and Pettigrew 1997). As these waters progress toward the head of the Laurentian and other channels, they become progressively more depleted in oxygen, probably due in part to metabolism by demersal and benthic fish and invertebrates, but mostly due to nutrient recycling from dead plankton sinking to the deep layer (Coote and Yeats 1979). Dissolved oxygen levels of 20-30% saturation are typical of the western end of the Gulf of St. Lawrence and the St. Lawrence Estuary (Figure 1). Recent evidence suggests that anthropogenic causes have worsened the situation in the estuary (D. Gilbert, Maurice-Lamontagne Institute, pers. comm.), but deep waters in the troughs of the Gulf of St. Lawrence are hypoxic because of normal hydrographic and biological processes. As a result, there are only minor seasonal fluctuations in dissolved oxygen levels of the deep waters of the Gulf of St. Lawrence with fluctuations over many years due to varying proportions of Labrador and western North Atlantic water entering at the mouth of the Laurentian Channel (Bugden 1991).

Two cod stocks inhabit the Gulf of St. Lawrence in summer. The first stock is found in the estuary and southern part of the gulf (Northwest Atlantic Fisheries Organization, NAFO, division 4T). Although oxygen levels are very low (20-30%) in the estuary, the southern gulf is too shallow for hypoxia to be a problem (Figure 1). The second stock lives in the northern part of the Gulf of St. Lawrence (NAFO divisions 4RS3Pn). In spring, cod from this stock disperse into the northern gulf for spawning and the post-spawning feeding period. In late autumn, cod older than two years migrate to deep waters in the Esquiman and Laurentian Channels (Chouinard and Fréchet 1994, Castonguay *et al.* 1999), and in winter many fish migrate out of the area altogether (east of 3Pn) (Castonguay *et al.* 1999).

SURVIVAL AND GROWTH OF COD UNDER HYPOXIA

Tolerance Experiments

As early as 1991, it was apparent that cod were not distributed randomly relative to dissolved oxygen. Cod were absent in trawl sets where there was less than about 30% oxygen saturation, which suggested that 30% was close to the lethal level of hypoxia (D'Amours 1993). The available literature gave a rather wide interval of lethal dissolved oxygen thresholds for cod. Scholz and Waller (1992) observed 50% mortality within 24 h when cod were exposed to 40% oxygen saturation at 8°C, and that no cod survived when exposed to 20%, showing intolerance to even mild hypoxia. On the contrary, Sundnes (1957) and Schurmann and Steffensen (1992) concluded that cod were very tolerant to hypoxia because loss of equilibrium only occurred below 15% saturation at 10°C, and at 5% at 5°C. Differences in methodology and definitions made it difficult to compare these studies.

To determine the lethal hypoxic threshold, we conducted LC_{50} 96h (lethal concentration for 50% of the fish within 96 h) toxicity experiments (Plante *et al.* 1998). This definition of lethal threshold is widely accepted in the toxicity literature, and future comparisons with new studies will be easier. However, the biological interpretation of a LC_{50} value in relation to distribution of wild cod is not ideal, because fish are known to detect small differences in dissolved oxygen (Claireaux *et al.* 1995), and would leave an area long before such high toxicity was reached unless there was nowhere else to go. Therefore, we also calculated the LC_5 96h, *i.e.* the level at which mortalities first begin to be statistically significant, and used it as a measure of the incipient lethal threshold for dissolved oxygen.

These experiments were conducted at 2^oC and 6^oC, temperatures that encompass the range of possible hypoxic waters in the Gulf of St. Lawrence. Two size-classes were tested: small cod (mean ± SD; 45.2 cm ± 4.2) and large cod (57.5 cm ± 3.8). Cod were transferred directly from normoxic to hypoxic conditions and mortalities assessed periodically (1, 3, 6, 12 h, and then every 12 h) over a period of 96 hours. For each size and temperature combination, the experiment was repeated twice, each time with 10 fish in each of six tanks, with six levels of dissolved oxygen ranging from 14 to 42% saturation. Using PROBIT analyses (Stephan 1977) on values of total mortality after 96 h and log-transformed oxygen saturations, we calculated the LC_{50} and LC_5.

No cod survived more than a few hours at 13.8% of oxygen saturation, and only a few survived 96 h at 17.8% of oxygen saturation. Mortality decreased quickly at 23.7% O_2 saturation, and all fish survived 96 h at 36.5 and 42.5%. Overall, LC_{50} and LC_5 were 21.0% saturation (95% confidence interval 19.9-22.1) and 28.1% (25.8-30.5), respectively (Plante *et al.* 1998). We found no difference between hypoxia tolerance at 2°C and 6°C, even though we expected a lower tolerance at 6°C than at 2°C due to increasing fish metabolic rate and oxygen consumption with increasing temperature (Brett and Groves 1979). The temperature range may have been too small in this study, because Schurmann and Steffensen (1992), working with 80-200 g cod, did find better hypoxia tolerance at lower temperatures. There was also no difference

in hypoxia tolerance between the two size classes, even though large fish have smaller mass-specific metabolic requirements than do small fish. Again, this was likely due to the small difference between the two size-classes in this study. Our values of lethal hypoxic thresholds fell within the range of published values, but they differed from the results of any single study, mostly because of methodological differences (for details, see Plante *et al.* 1998). Our results support the suggestion of D'Amours (1993): potentially lethal oxygen levels occur in the deep channels of the Gulf of St. Lawrence, particularly in the estuary and west of Anticosti.

Hypoxia as a Limiting Factor for Cod Growth

Figure 1 shows that most of the deep channels have dissolved oxygen levels between the 30% lethal threshold and what can be considered saturated (*e.g.* >80% saturation). There were no published data on the effects of such non-lethal levels of hypoxia on cod. We conducted an experiment to find out if growth rate was limited by dissolved oxygen, and if so, the relationship between dissolved oxygen and growth rate.

Using the same experimental setup as for the tolerance experiment, food ingestion, gross conversion efficiency, and growth in length and mass of cod averaging 44.2 ± 3.1 cm in length and 715.3 ± 188.2 g in weight (somatic condition factor 0.81 ± 0.10) were determined for 120 cod exposed to hypoxic waters ranging from 45 to 93% saturation over a period of 84 days, at 10°C. There were six levels of saturation, and 20 fish per tank. The fish were individually tagged to determine individual growth. Cod were fed three times a week with frozen capelin, *Mallotus villosus*, a natural prey item in the cod diet. The quantity of capelin eaten was measured for each tank.

Significant growth occurred in all treatments, even at 45% saturation (Chabot and Dutil 1999). However, growth in length and in mass, as well as change in condition, were significantly reduced at 45 and 56% saturation than at higher levels of dissolved oxygen (Figure 2). At 45% saturation, growth in length and mass was reduced by 35 and 56%, respectively, and change in condition was reduced by 64%. The incipient level of dissolved oxygen for growth was defined as the intersection of the regression line fitted to the data for the three lowest levels of dissolved oxygen, and the line representative of growth in normoxia (84 and 93% saturation). The incipient level was 65% saturation for growth in length, and 73% for growth in mass and change in condition (Figure 2). Cod inhabiting waters deeper than roughly 150-200 m in the Gulf of St. Lawrence are thus expected to grow more slowly than cod living in shallower waters under similar food and temperature conditions. A large proportion of the cod stock in the northern Gulf of St. Lawrence appears to live in growth-limiting, low oxygen conditions during the late summer feeding period (D'Amours 1993).

During this experiment, food ingestion rates were largely determined by oxygen availability (Chabot and Dutil 1999, Figure 4a):

$$(1)\ \text{Ingestion} = -74.22 + 54.51 \cdot \log(\text{dissolved oxygen, \% sat.}) \quad (r^2 = 0.93).$$

This variation in appetite explained almost all the variance in growth observed during our study (Chabot and Dutil 1999, Figure 4a):

(2) Growth $= -0.283 + 0.285 \cdot$ Ingestion ($r^2 = 0.93$).

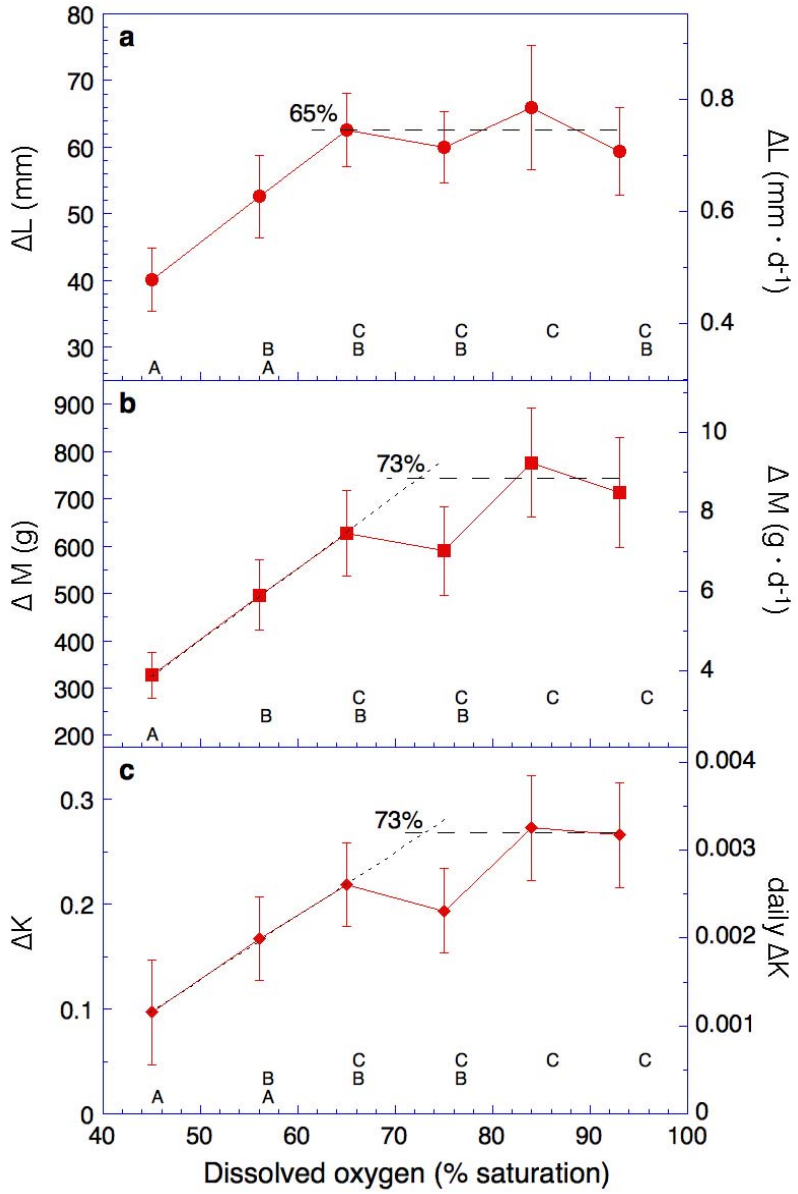

Figure 2. Growth (mean and 95% CI) in length and mass and change in condition factor in Atlantic cod during an 84 day growing period at different levels of dissolved oxygen. Means with the same letters were not different in post-hoc comparisons. Long-dash line = mean value in normoxia (84 and 93% O_2); short-dash line = regression fitted to data for the three lowest levels of dissolved oxygen. The intersection of these two lines is the critical level of dissolved oxygen. From Chabot and Dutil (1999).

Effect of Hypoxia on Meal Size

Our first growth experiment showed that cod eat less in hypoxia, and in turn grow less. High energy demands associated with post-prandial mechanisms (Soofiani and Hawkins 1982) can trigger a negative feedback on physiological mechanisms controlling appetite or behavioral processes associated with feeding activities. If peak oxygen consumption after a meal of a given size is related to meal size, hypoxia may force cod to ingest small quantities of food, or else the energy demand becomes too high and they are forced to regurgitate (regurgitation is often observed if cod eat in normoxia and are then placed in hypoxia). Because only three meals were offered each week, it is possible that the impact of hypoxia on growth was exaggerated compared to a more natural situation in which cod could eat more frequently and compensate somewhat for limited meal size. Alternatively, it is possible that reduced oxygen availability during hypoxia simply slows down digestion, in which case our experiment correctly describes the impact of hypoxia on growth.

To discriminate between these two hypotheses, a two-factor growth experiment was conducted at 10°C, using 9-12 individually tagged cod per tank (total n = 183, length = 46.5 cm ± 3.4, mass = 960.4 g ± 284.0 and condition = 0.93 ± 0.14). The first factor, oxygen, had two levels: normoxia (>90% saturation) and hypoxia (45% saturation). The second factor, meal frequency, had three levels: one, three, or seven times a week. One meal consisted of as much capelin as cod could eat in one hour. Two replicates were carried out in autumn 1999 (56 days), and a third one in autumn 2000 (62 days).

Unfortunately, growth was much slower in 2000 than in 1999, and the results could not be analysed as planned. We found a negative relationship between growth and initial condition (see Figure 7 in Chabot *et al.* 2001), probably as a result of compensatory growth mechanisms in lean cod (Dutil *et al.* 2001). Therefore, initial condition was included as a covariate.

Because of significant interactions, we looked at the effect of meal frequency separately for fish raised in normoxia and in hypoxia, and also compared growth between normoxic and hypoxic fish for each feeding frequency. The effect of meal frequency was clear in normoxia: growth increased significantly from one to three meals per week. Increasing meal frequency further resulted in a non-significant increase in growth (Figure 3). In hypoxia, growth was significantly faster at three than at one meal per week, but growth at seven meals per week did not differ from growth at the other feeding frequencies.

Comparisons between oxygen levels showed no difference in growth rate between cod raised in normoxia or hypoxia offered a single meal per week. However, growth was faster in normoxic than hypoxic cod at three and seven meals per week (Figure 3).

The large changes in growth (and food ingestion) between 1999 and 2000 rendered meaningless the analysis of the impact of oxygen and meal frequency on meal size, especially since only one measure of meal size was available for each tank (the individual meal size measurements were not independent, so the mean was used). The trends are quite informative, however (Figure 4). Regardless of oxygen treatment, meal size tended to decrease as meal frequency increased. Most importantly for the two investigated hypotheses, cod in normoxia that

were fed one meal per week should have had empty stomachs before each meal according to gastric evacuation models. Therefore, they should have eaten as much as stomach volume permitted. Figure 4 shows that cod raised in hypoxia and fed once a week were able to eat as large a meal as cod in normoxia. This eliminated the hypothesis that hypoxia limits meal size in cod.

In addition, cod raised in normoxia grew faster when feeding frequency was >1 meal per week (Figure 3). Added support to the suggestion that cod fed one meal per week were limited in their food intake by maximum stomach volume. The lack of a significant increase in growth when normoxic cod were offered seven meals per week instead of three suggested that food intake for those fish was limited by digestion rate. Conversely, the small increase in growth when hypoxic cod were offered more than one meal per week suggested that hypoxia slows down digestion rate, and that at 45% saturation and 10°C, cod cannot digest much more than the equivalent of a full stomach of capelin during one week.

Therefore, we think that wild cod facing hypoxic conditions will also suffer from a reduction in digestion rate, and, therefore, a reduction in ingestion and growth rate.

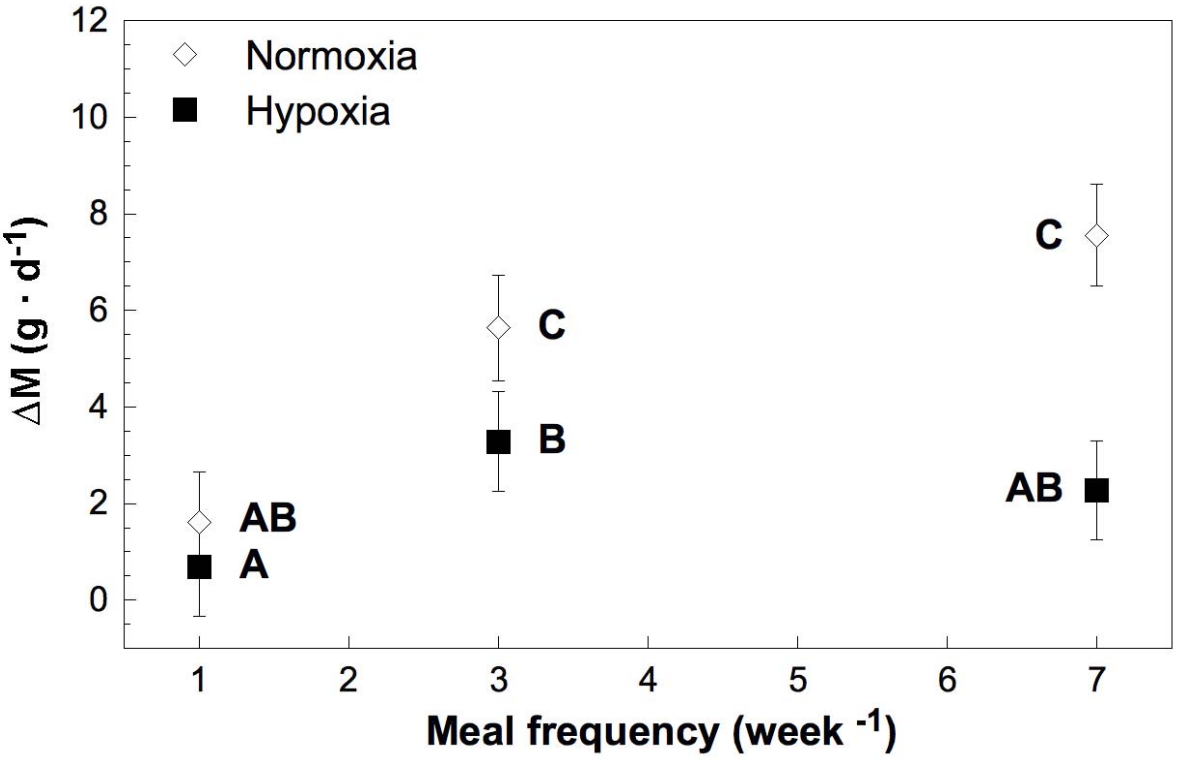

Figure 3. Growth in mass (adjusted for initial condition (Fulton K) of fish) of cod raised in hypoxia (45% oxygen saturation) or normoxia (>90%) and fed one, three or seven meals per week. Error bars = 95% CI. A posteriori, growth was compared between meal frequencies for hypoxic and then normoxic fish. Means with the same letter did not differ significantly.

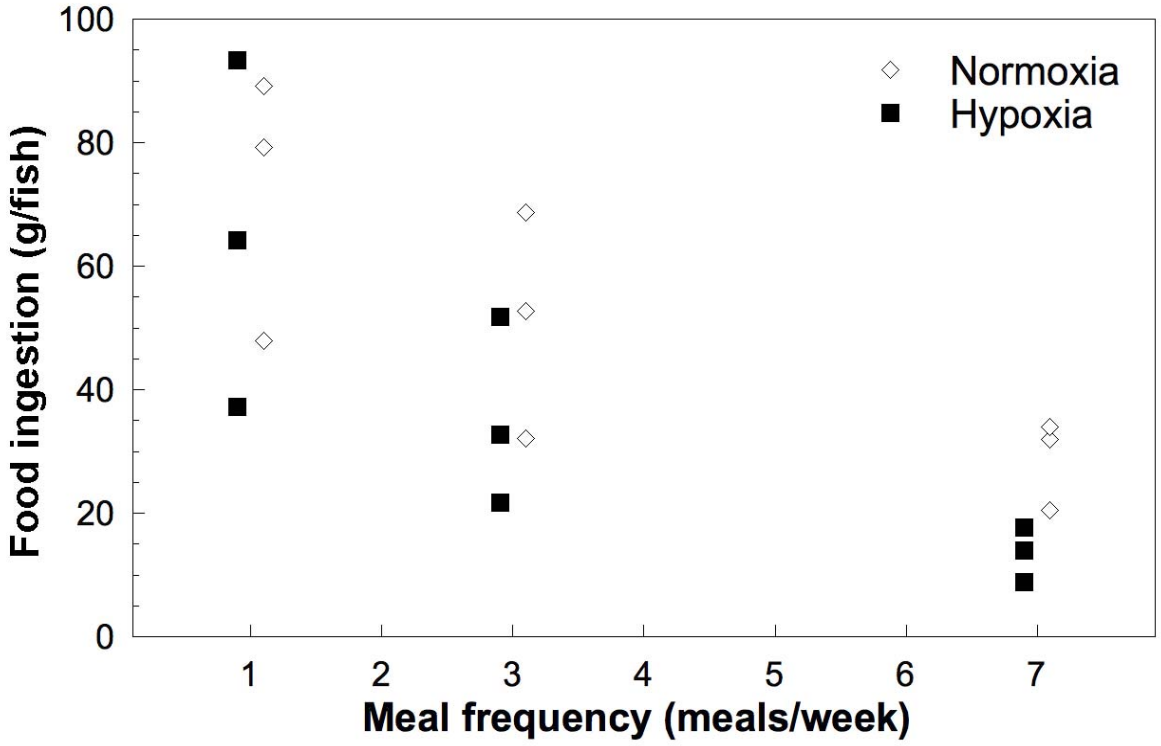

Figure 4. Average meal size eaten by cod in each tank.

MODELING THE IMPACT OF HYPOXIA ON WILD COD

Taken together, our laboratory results show that cod risk death if they remain in waters with less than 30% saturation in oxygen, and face reduced growth rates if they stay in waters with 30-70% saturation in oxygen. To assess the impact of hypoxia on the growth of wild cod, we needed to determine the levels of dissolved oxygen cod are actually exposed to in the field, and assess the likely growth rate of cod under those conditions. Chabot *et al.* (2001) made a first attempt to estimate the impact of hypoxia on growth production for this stock using field measurements of bottom dissolved oxygen for the summer and fall of 1995 and cod distribution data for July 1995 in conjunction with the general conclusions on growth under hypoxic conditions obtained from the laboratory studies described herein. Two more cod surveys were added in Chabot and Couturier (2002), so that cod distribution in relation to level of dissolved oxygen was known for most of the feeding season. However, a few survey strata from the adjacent NAFO division 4T were accidentally included in that study. Herein, the model was reconstructed for NAFO divisions 4RS3Pn only, and cod stomach content data are examined to verify that food ingestion in the field was influenced by oxygen level, as it was in the laboratory.

Dissolved Oxygen

Details of the data (Winkler titrations) used to describe bottom dissolved-oxygen levels are given in Chabot and Couturier (2002). Briefly, water samples were taken 5-10 m from the bottom at 141 stations visited between 11 Aug 1995 and 17 Nov 1995 (Figure 1).

Six classes of dissolved oxygen levels were defined according to the results of the laboratory experiments: <30% saturation, where survival is compromised (Plante *et al.* 1998), 30-39.9, 40-49.9, 50-59.9, and 60-69.9% saturation, where growth is limited by oxygen availability, and ≥70% saturation where growth is independent of oxygen availability (Chabot and Dutil 1999). To calculate the areas covered by these oxygen classes, coordinates of the oxygen stations and of the cod survey stratum boundaries were transformed to km (relative to 62°W and 49°N, near the centre of the study area) from decimal degrees according to Rivoirard *et al.* (2000).

There were no data on the interaction between oxygen, temperature, cod size, and cod growth. Therefore, for each oxygen class, a typical rate of food ingestion as a function of dissolved oxygen, and a typical growth rate as a function of ingestion rate were calculated for a fish the size of our laboratory fish (44 cm, 700 g, Fulton K = 0.8) using equations 1 and 2. We then assumed that these typical growth rates (expressed as the proportion of the growth achievable in normoxia) can be applied to cod of other sizes, at temperatures of 3-6° C. To extrapolate to the stock level, a weighted average of the six growth rates was calculated, using the number of fish living in each oxygen class as the weighting factor.

Table 1 shows the extent of the six classes of dissolved oxygen in NAFO division 4RS3Pn and the expected rate of food ingestion and growth for cod found in each class. Overall, 6.4% of the area was characterized by levels of dissolved oxygen low enough to jeopardize cod survival. No growth was expected in those conditions. Another 64.3% of the area was characterized by levels of hypoxia that limit growth potential. Only 29.3% of the area was normoxic.

Table 1. Main characteristics of the six oxygen classes used in this study: surface area in 4RS3Pn, rate of food ingestion and growth expected for a 44 cm, 700 g cod feeding in each oxygen class. For the <30 and ≥70% saturation classes, oxygen values of 25 and 85% saturation were used, respectively, to calculate the rate of food ingestion.

Dissolved Oxygen (% sat)	Area (km^2)	Area (% of total)	Food ingestion ($g \cdot d^{-1}$)	Growth ($g \cdot d^{-1}$)	Growth (% relative to normoxia)
< 30	5 784	6.4	2.0	0.3	3.3
30-40	7 756	8.5	9.9	2.6	29.9
40-50	20 695	22.8	15.9	4.2	49.7
50-60	18 619	20.5	20.6	5.6	65.6
60-70	11 317	12.5	24.6	6.7	78.8
≥70	26 648	29.3	31.0	8.5	100.0
Total	90 819	100.0			

Cod Abundance and Distribution

Cod distribution and abundance in relation to oxygen levels were estimated from three, random, stratified, bottom-trawl surveys: Sentinel Fishery surveys no. 3 (PS03, 311 sets between 25 Jul 1995 and 15 Aug 1995) and 4 (PS04, 322 sets between 06 Oct 1995 and 04 Nov 1995), and the Fisheries and Oceans shrimp and demersal fish survey no. 6 (AN06, 194 sets between 11 Aug 1995 and 04 Sep 1995). Details on ship and gear types can be found in Chabot and Couturier (2002).

Only the sets located in NAFO division 4RS3Pn were included in the analyses. However, part of 4RS3Pn was not fished because of bottom conditions or depth stratification, and strata 825, 826, 839, and 841 were excluded (depths <50 m and some coastal zones at depths >50 m along the northern shore of the Gulf of St. Lawrence or the northwest tip of Anticosti Island). Historical assessments of cod distribution at this time of year suggested that this stock is distributed almost entirely within the remaining 40 strata that cover 90,819 km^2. The proportion of each stratum associated with the six classes of dissolved oxygen was determined (Table 2) according to Chabot and Couturier (2002). The number of cod estimated for each stratum (Table 3) was distributed among the six oxygen classes in proportion to their respective area. Results were summed across all strata for each survey to obtain the proportion of the total number of cod that inhabited each oxygen class (Table 4).

RESULTS OF THE MODEL

Figure 5 shows the distribution of cod catches in the study area during the PS03 survey. Results were very similar for the AN06 survey, but fewer cod were caught in hypoxic waters during the PS04 survey. Few cod were caught in parts of the study area characterized by the most severe hypoxia level (<30% saturation). However, cod avoided all hypoxic zones, not just the most severe one: cod density increased exponentially with the level of dissolved oxygen (Figure 6). Because such a large proportion of the study area was hypoxic, however, this still resulted in a significant proportion of the stock living in hypoxic conditions.

In late July and early August, 45.3% of the total number of cod estimated to reside in the study area, representing 44.3% of the cod biomass, lived in waters with levels of dissolved oxygen ≤70% (Table 4). We estimated that during this period, growth production was decreased by 18.7% as a result of hypoxia. In the second half of August and early September, 45.9% of the total number of cod, and 46.9% of the biomass were estimated to be in hypoxic waters, resulting in a decrease in growth of 16.7%. Finally, in October and early November, 19.9% of the total number of cod and 29.4% of the cod biomass were estimated to be in hypoxic waters with a growth reduction of 6.4%.

Table 2. Surface area and proportion of this surface area associated with each class of dissolved oxygen for the 40 strata of NAFO division 4RS3Pn.

		Class of dissolved oxygen (% saturation)					
Stratum	**Area (km2)**	**< 30**	**30-40**	**40-50**	**50-60**	**60-70**	**≥ 70**
302	888	0.00	0.00	0.00	0.85	11.08	88.07
303	1 560	0.00	0.00	0.00	3.12	3.12	93.76
304	404	0.00	0.00	0.00	24.52	22.87	52.62
305	2 394	0.00	0.00	0.00	24.94	29.99	45.07
801	1 080	0.00	73.35	25.35	1.30	0.00	0.00
802	1 405	0.00	0.00	0.00	93.36	6.64	0.00
803	6 846	0.00	12.45	48.64	38.05	0.87	0.00
804	1 850	3.61	51.57	44.82	0.00	0.00	0.00
805	5 547	86.05	10.96	2.54	0.46	0.00	0.00
806	2 070	3.61	39.52	41.20	15.68	0.00	0.00
807	2 171	0.00	15.68	67.41	16.91	0.00	0.00
808	2 329	0.00	0.00	39.43	57.12	3.45	0.00
809	1 456	0.00	0.00	61.96	19.74	18.31	0.00
810	680	0.00	0.00	0.00	32.63	66.64	0.73
811	1 377	0.00	0.00	18.93	26.22	42.26	12.59
812	4 654	0.00	3.92	74.10	19.80	2.19	0.00
813	4 970	0.00	26.50	33.01	20.91	10.68	8.90
814	858	0.00	1.89	76.37	21.74	0.00	0.00
815	4 292	0.00	0.00	26.30	58.11	14.11	1.47
816	4 729	0.00	15.95	39.30	26.50	15.56	2.69
817	3 225	24.01	18.77	21.23	27.64	8.16	0.19
818	1 942	3.11	5.83	46.86	23.48	19.56	1.18
819	1 335	0.00	0.00	14.15	75.45	10.39	0.02
820	1 210	0.00	0.00	0.00	6.30	27.62	66.08
821	1 179	0.00	0.00	0.00	10.47	32.30	57.23
822	3 089	0.00	0.00	0.28	8.09	39.49	52.13
823	406	0.00	0.00	0.00	0.00	11.27	88.73
824	684	0.00	1.24	20.96	31.49	18.76	27.55
827	2 886	0.00	0.00	1.43	19.79	20.75	58.03
828	2 325	0.00	0.00	2.89	8.62	19.65	68.83
829	2 502	0.00	0.01	4.16	3.40	26.13	66.30
830	1 719	0.00	0.00	13.63	13.38	37.73	35.26
831	1 277	2.69	29.85	40.76	24.86	1.66	0.17
832	3 700	0.00	0.35	2.44	16.93	30.62	49.65
833	489	0.00	0.00	0.00	8.82	43.51	47.68
835	2 185	0.00	0.00	0.00	1.30	2.94	95.76
836	2 662	0.00	0.00	0.00	0.00	0.24	99.76
837	2 302	0.00	0.00	0.00	0.09	7.13	92.78
838	3 378	0.00	0.00	0.00	0.00	0.00	100.00
840	765	0.00	0.00	0.00	0.00	0.00	100.00
All strata	**90 819**	**6.37**	**8.54**	**22.79**	**20.50**	**12.46**	**29.34**

Table 3. Number and biomass of Atlantic cod estimated for each of the 40 strata of NAFO division 4RS3Pn during three surveys conducted in summer and autumn of 1995. Empty cells indicate lack of data.

	Cod number			Cod biomass (kg)		
Stratum	**PS 3**	**AN 6**	**PS 4**	**PS 3**	**AN 6**	**PS 4**
302	1 245 585	829 149	1 723 535	827 848	617 144	2 266 978
303	393 649	38 052	492 320	394 698	3 805	261 740
304	0	0	7 529	0	0	16 092
305	5 972	0	0	4 778	0	0
801	121 615	0	5 337	22 489	0	13 343
802	0	0	5 013	0	0	9 525
803	12 944	0	13 940	3 236	0	13 940
804	0	25 707	10 946	0	32 390	27 365
805	0	0	0	0	0	0
806	0	0	15 782	0	0	27 619
807	13 023	0	0	7 814	0	0
808	0	0	0	0	0	0
809	0	0	11 333	0	0	10 766
810	0	0	0	0	0	0
811	527 406	84 199	354 584	372 735	47 993	442 521
812	817 185	195 913	575 153	200 040	187 517	663 353
813	9 695 808	2 524 659	430 835	7 576 146	2 284 696	420 478
814	0		75 386	0		22 616
815	85 051	36 969	204 506	54 306	30 684	134 543
816	1 354 507	244 572	66 355	664 759	195 527	46 448
817	1 205 847	21 777	348 753	1 230 651	17 406	297 259
818	30 485	57 275	926 742	22 864	74 744	353 148
819	102 902	46 237	227 666	40 017	17 724	135 695
820	1 157 958	2 982 816	572 179	1 328 351	2 879 633	487 596
821	2 842 427	259 447	1 319 163	2 432 395	256 086	1 594 183
822	3 197 709	2 183 366	3 498 697	2 399 845	1 807 270	4 634 598
823	4 389 543	595 412	122 040	2 287 950	449 473	200 954
824	1 716 560	384 194	481 828	881 120	387 055	705 114
827	169 249	400 764	94 685	103 088	258 394	10 415
828	344 933	0		178 116	0	
829	498 375	27 796	29 589	158 480	4 447	3 847
830	978 772	455 249	362 713	383 497	302 048	98 454
831	239 615	74 364	37 109	174 052	71 723	16 899
832	1 352 190	329 448	282 773	1 184 268	222 453	258 849
833	0	10 719	8 192	0	1 608	4 997
835	3 966 730	733 116	2 620 423	3 202 278	505 858	1 298 602
836	2 918 886	409 022	5 781 962	2 676 786	358 233	3 390 473
837	1 591 382	387 838	20 587 600	1 235 374	573 984	6 402 674
838	3 192 980	571 991	267 319	2 683 994	311 535	46 929
840	463 507		61 596	8 165		7 317
Total	**44 632 796**	**13 910 048**	**41 623 584**	**32 740 139**	**11 899 430**	**24 325 331**

Table 4. Estimation of the number and biomass of cod living in each of six classes of dissolved oxygen in NAFO division 4RS3Pn during three cod surveys carried-out in summer and autumn 1995.

Dissolved oxygen (%)	Cod number	(% of total)	Cod biomass (kg)	(% of total)	Growth x cod number
PS03, July-August 1995					
<30	296 970	0.8	300 925	0.9	83 682
30-40	3 236 050	7.0	2 439 063	7.4	8 258 197
40-50	5 457 832	11.9	3 642 511	11.1	23 182 333
50-60	4 909 342	11.0	3 517 154	10.7	27 499 404
60-70	6 341 636	14.6	4 617 038	14.1	42 669 976
>70	24 390 965	54.7	18 223 447	55.7	208 262 211
Total	**44 632 796**	**100.0**	**32 740 139**	**100.0**	**309 955 803**
			Average growth relative to normoxia (%):		**81.3**
			Decrease in growth due to hypoxia (%):		**18.7**
AN06, August-September 1995					
<30	9 939	0.1	9 603	0.1	2 801
30-40	764 535	5.5	695 305	5.8	1 951 046
40-50	1 343 973	9.7	1 208 927	10.2	5 708 571
50-60	1 476 593	10.6	1 268 923	10.7	8 271 052
60-70	2 792 007	20.1	2 400 119	20.2	18 786 144
>70	7 523 001	54.1	6 316 553	53.1	64 235 129
Total	**13 910 048**	**100.0**	**11 899 430**	**100.0**	**98 954 742**
			Average growth relative to normoxia (%):		**83.3**
			Decrease in growth due to hypoxia (%):		**16.7**
PS04, October-November 1995					
<30	114 510	0.3	84 797	0.3	32 267
30-40	303 721	0.7	272 840	1.1	775 077
40-50	1 524 989	3.7	1 261 153	5.2	6 477 444
50-60	1 770 612	4.3	1 631 679	6.7	9 917 985
60-70	4 575 242	11.0	3 905 647	16.1	30 784 717
>70	33 334 509	80.1	17 169 214	70.6	284 626 650
Total	**41 623 584**	**100.0**	**24 325 331**	**100.0**	**332 614 140**
			Average growth relative to normoxia (%):		**93.6**
			Decrease in growth due to hypoxia (%):		**6.4**

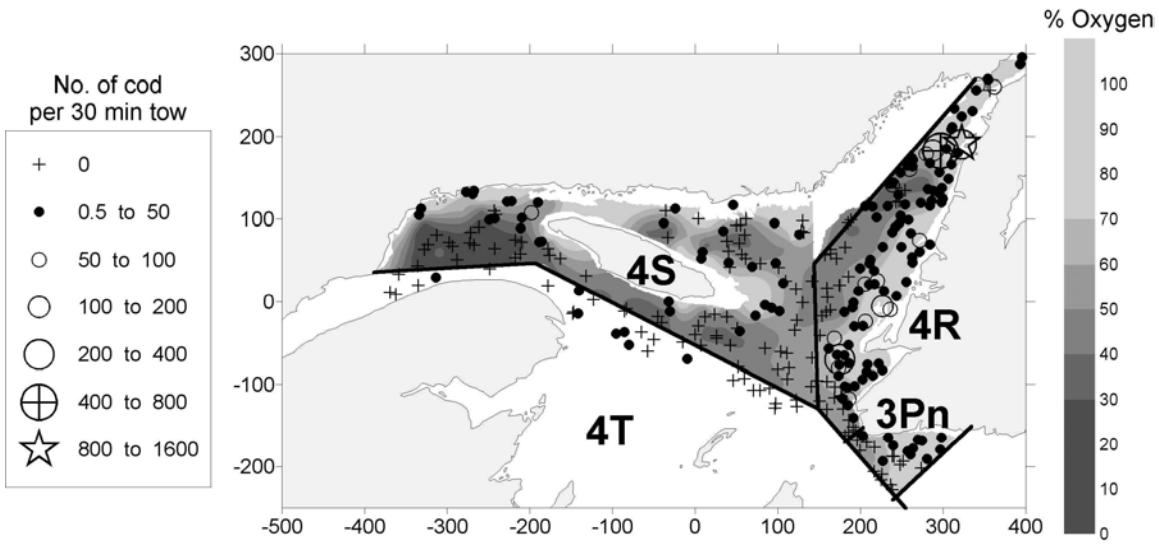

Figure 5. Number of cod caught in each 30 min tow during the Sentinel Fishery survey no. 3 (PS03) carried out between 25 Jul and 15 Aug 1995, in relation to the six classes of dissolved oxygen. Coordinates are in km relative to 49° N and 69° W.

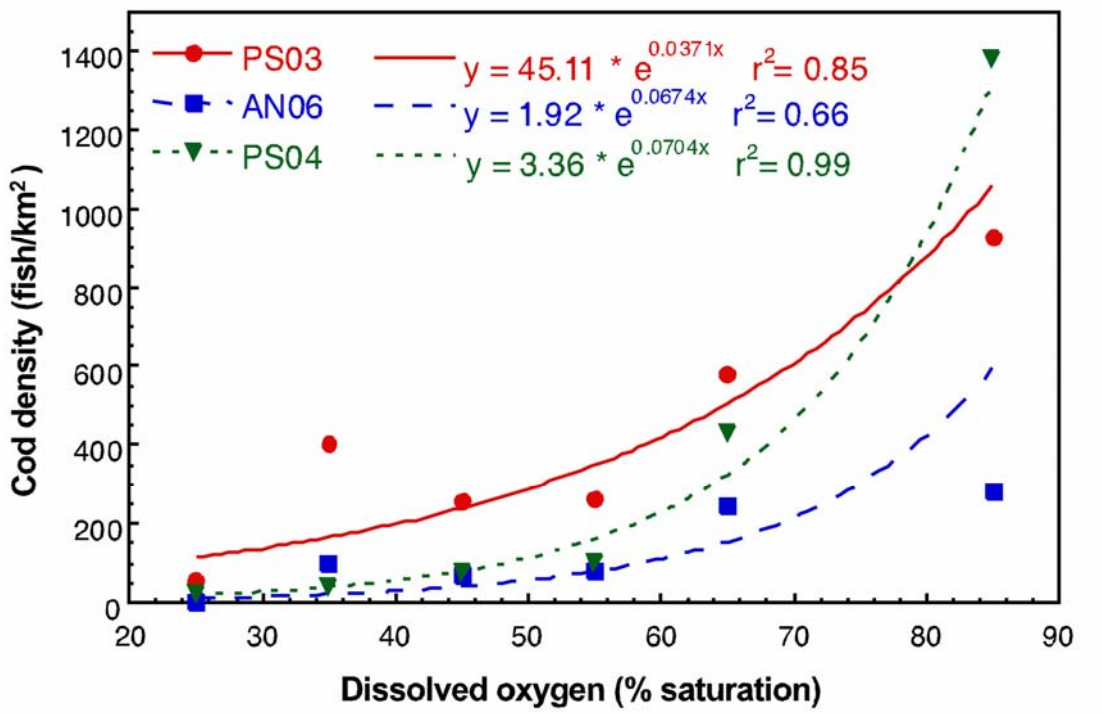

Figure 6. Relationship between cod density and level of dissolved oxygen on the bottom for three bottom-trawl surveys conducted in NAFO division 4RS3Pn between summer and autumn 1995. PS03 and PS04 are Sentinel Fishery mobile-gear surveys 3 and 4, respectively, and AN06 is the Needler shrimp and ground-fish survey no. 6.

Supporting Evidence from Stomach Content Data

For 111 of the sets fished during the three surveys used in this study, stomachs were collected from a length-stratified subsample of the cod that were captured. Stomach mass was transformed into a stomach fullness index to correct for allometric differences between fish:

$$\text{Fullness Index} = 10000 \cdot C \cdot FL^{-3}$$

where C is stomach content mass in g, and FL is fork length in cm.

Stomach content in wild cod tended to increase along with dissolved oxygen (Figure 7, Table 5). The distribution of the tow averages for each level of dissolved oxygen was not normal, but skewed to the right; however, whether or not one uses the mean or the median of these values, there is a clear tendency for fullness to positively correlate to dissolved oxygen (Figure 7, Table 5). This agrees with the hypothesis that wild cod reduce their food intake when exposed to hypoxia, as cod did in the laboratory.

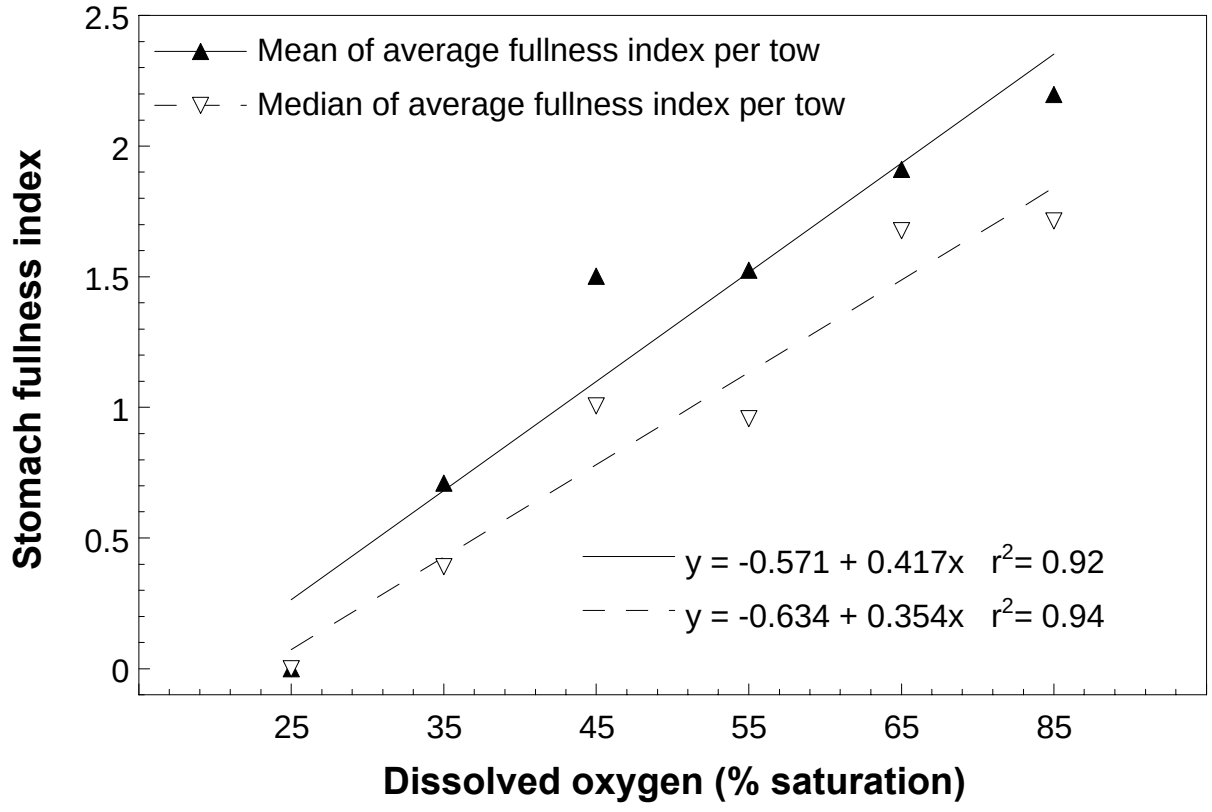

Figure 7. Relationship between stomach fullness and dissolved oxygen. Mean and median values of the average fullness per tow are shown.

Table 5. Average and median fullness index calculated from average fullness for each tow, as a function of dissolved oxygen. Data from all three surveys were pooled. Number of tows for which stomach contents were available, as well as total number of stomachs analyzed, are indicated.

Dissolved oxygen (%)	Number of tows	Total number of stomachs	Average fullness index	Median fullness index
< 30	1	1	0.00	0.00
35	3	4	0.71	0.39
45	15	73	1.50	1.01
55	23	271	1.53	0.96
65	17	139	1.91	1.68
≥ 70	52	451	2.20	1.71

IMPLICATIONS AND MODEL ASSESSMENT

A solid dataset was used to describe levels of dissolved oxygen in NAFO division 4RS3Pn. Furthermore, three extensive, stratified surveys provided a good description of the distribution of cod in the study area during what was believed to be the peak feeding season for cod from this stock. Spawning generally occurs from May to early July (Lambert and Dutil 1997), and in the laboratory, mature cod did not eat during spawning (Fordham and Trippel 1999). Condition was at a minimum during the spring, and increased rapidly in July and August (Lambert and Dutil 1997). A large sample collected in the northern gulf in January 1994 suggested that cod eat little in winter, since over 80% of the stomachs were empty, and average stomach fullness was very low (D. Chabot, unpublished data). In the neighboring 4T cod stock, the feeding cycle was better documented and agreed very well with this proposed cycle (Schwalme and Chouinard 1999).

As was observed by D'Amours (1993), there is no doubt that few cod were caught in the most severe hypoxia class (<30% saturation). Cod are very sensitive to small differences in dissolved oxygen (Claireaux *et al.* 1995), although the lowest levels observed in the study area (20-30%) do not kill cod quickly: 95% of fish survived 96 h in water 28% saturated in oxygen, and 50% of cod survived 96 h in water 21% saturated in oxygen (Plante *et al.* 1998). Therefore avoidance is the most probable reason for the low density of cod in waters with <30% oxygen.

The relationships observed between cod density and dissolved oxygen in each of the three surveys further demonstrate that cod also avoid non-lethal levels of dissolved oxygen. This is similar to the situation in the Kattegat (Denmark), where catches of demersal fish, mostly gadoids and pleuronectids, in standard trawl sets were directly related to oxygen concentration (Pihl 1989).

However, because too small a proportion of the study area was normoxic, and/or because cod distribution is a trade-off between oxygen and other characteristics of the habitat (temperature, food distribution, etc.), a significant proportion of cod, both in number and in biomass, were found to live in waters with levels of dissolved oxygen that are known to limit

growth in laboratory fish. Furthermore, this happens during the season of peak feeding, when any reduction in the amount of food eaten is bound to result in lower energy reserves for winter and spawning, and lower growth rates.

This simple model estimated that, for the stock as a whole, hypoxia curtailed growth production by 17-19% in summer, during the peak of the feeding season, and 6% in fall. Hypoxia appears to exert a significant constraint on growth production for this stock, especially considering its low productivity due to the cold temperatures of the Gulf of St. Lawrence (Dutil *et al.* 1999).

However, this first model is simplistic in terms of how the impact of hypoxia exposure on growth production is calculated, and makes assumptions that clearly are not correct. The most important omission is that temperature effects on the solubility of oxygen or on metabolic and digestion rates are not considered. Hypoxic waters in the St. Lawrence Estuary and Gulf of St. Lawrence are typically between 3 and 6°C, with a salinity of 34-35 psu. This results in an oxygen solubility of 10-11 mg $\cdot$ L^{-1} (Benson and Krause 1984). Oxygen solubility in the experiments of Chabot and Dutil (1999) was only 9.4 mg $\cdot$ L^{-1} (10°C and S = 28). Thus, at similar oxygen saturation, the concentration of oxygen is almost 10% greater in the field. This could lower the lethal threshold and the incipient threshold for growth relative to what was observed in the laboratory (28 and 73%, respectively, Chabot and Dutil 1999), as shown by Claireaux *et al.* (2000).

Wild fish need to expend more energy swimming to capture prey, avoid predators, and migrate, than do cod in the laboratory. Because hypoxia imposes a limit on maximum metabolism (Claireaux *et al.* 2000), it can limit swimming capacity, making cod more vulnerable to predation or fishing, and less able to hunt for food. But more importantly, increased swimming activity will further reduce the oxygen available for processing food, and wild fish may eat less food for the same level of hypoxia than laboratory fish. These opposing trends may minimize the impact of omitting the temperature effect from the model. But clearly, data on the interaction between temperature, oxygen, and growth are needed, and experiments in our laboratory were intended to fill this gap. In addition to improving the model, our data would allow calculation of the relationship between metabolic scope and growth, making it possible to use the model of Claireaux *et al.* (2000) to assess the impact of hypoxia and temperature on growth production.

The simple model also assumes that the impact of hypoxia on cod growth is the same for cod of all sizes and condition. There are no data on the interaction between cod size, oxygen, and growth, and new experiments are needed before the effect of cod size can be included in the model. Furthermore, Chabot *et al.* (2001) have shown a negative relationship between condition and food ingestion, regardless of oxygen level. The effect of cod condition that varies with the month of the year, should also be included to make the model more realistic.

Stomach fullness of wild cod was positively related to dissolved oxygen. Stomach fullness, averaged across many fish, is an indicator of average consumption rate (Bromley 1994, Temming and Andersen 1994, dos Santos and Jobling 1995). This confirmed that wild cod reduce food intake when exposed to hypoxia, as they do in the laboratory. Therefore, the

simplifications made in this first model affect the precision of the estimation of the quantitative impact of hypoxia on growth production of the stock, but all available evidence indicates that there is a negative impact on this stock due to hypoxia. In further analyses, it may be possible to estimate the reduction in food consumption of wild cod by using gastric evacuation models that will have been validated for hypoxic fish, along with field data on stomach fullness.

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REFERENCES

Benson, B.B. and D. Krause, Jr. 1984. The concentration and isotopic fractionation of oxygen dissolved in freshwater and seawater in equilibrium with the atmosphere. Limnology and Oceanography 29:620-632.

Breitburg, D.L., T. Loher, C.A. Pacey, and A. Gerstein. 1997. Varying effects of low dissolved oxygen on trophic interactions in an estuarine food web. Ecological Monogram 67:489–507.

Brett, J.R. and T.D.D. Groves. 1979. Physiological energetics. *In:* Bioenergetics and Growth. W.S. Hoar, D.J. Randall and J.R. Brett (eds.). Academic Press. New York. Vol. VIII pp. 279-352.

Bromley, P.J. 1994. The role of gastric evacuation experiments in quantifying the feeding rates of predatory fish. Reviews in Fish Biology and Fisheries 4:36-66.

Bugden, G.L. 1991. Changes in temperature-salinity characteristics of the deeper waters of the Gulf of St. Lawrence over the past several decades. Canadian Special Publication of Fisheries and Aquatic Science 113:139-147.

Castonguay, M., C. Rollet, A. Fréchet, P. Gagnon, D. Gilbert, and J.-C. Brêthes. 1999. Distribution changes of Atlantic cod (*Gadus morhua*) in the northern Gulf of St. Lawrence in relation to an oceanic cooling. ICES Journal of Marine Science 56:333-344.

Chabot, D. and C. Couturier. Estimating the impact of naturally occurring hypoxia on growth production of Atlantic cod (*Gadus morhua*) from the northern Gulf of St. Lawrence (Canada). *In:* Responses of Fish to Aquatic Hypoxia, 5th International Congress on the Biology of Fish, University of British Columbia, Vancouver, Canada. Physiology Section, American Fisheries Society pp. 35-53.

Chabot, D. and J.-D. Dutil. 1999. Reduced growth of Atlantic cod in non-lethal hypoxic conditions. Journal of Fisheries Biology 55:472-491.

Chabot, D., J.-D. Dutil, and C. Couturier. 2001. Impact of chronic hypoxia on food ingestion, growth and condition of Atlantic cod (*Gadus morhua*). *In:* ICES Annual Science Conference, 89th Statutory Meeting. Oslo, Norway. ICES CM 2001/V05:1-17.

Chouinard, G.A. and A. Fréchet. 1994. Fluctuations in the cod stocks of the Gulf of St. Lawrence. ICES Marine Science Symposium 198:121-139.

Claireaux, G., D.M. Webber, S.R. Kerr, and R.G. Boutilier. 1995. Physiology and behaviour of free-swimming Atlantic cod (*Gadus morhua*) facing fluctuating salinity and oxygenation conditions. Journal of Experimental Biology 198:61-69.

Claireaux, G., D.M. Webber, J.-P. Lagardère, and S.R.Kerr. 2000. Influence of water temperature and oxygenation on the aerobic metabolic scope of Atlantic cod (*Gadus morhua*). Journal of Sea Research 44:257-265.

Coote, A.R. and P.A. Yeats. 1979. Distribution of nutrients in the Gulf of St. Lawrence. Journal of Fisheries Research Board of Canada 36:122-131.

Craig, J.K., L.B. Crowder, C.D. Gray, C.J. McDaniel, T.A. Henwood, and J.G. Hanifen. 2001. Ecological effects of hypoxia on fish, sea turtles, and marine mammals in the northwestern Gulf of Mexico. *In*: Coastal and Estuarine Studies. Coastal hypoxia: consequences for living resources and ecosystems. Edited by American Geophysical Union. Pp. 269-292.

D'Amours, D. 1993. The distribution of cod (*Gadus morhua*) in relation to temperature and oxygen level in the Gulf of St. Lawrence. Fisheries Oceanography 2:24-29.

Davis, J.C. 1975. Minimal dissolved oxygen requirements of aquatic life with emphasis on Canadian species: a review. Journal of Fisheries Research Board of Canada 32:2295-2332.

Diaz, R.J. and A. Solow. 1999. Ecological and economic consequences of hypoxia: Topic 2 Report for the integrated assessment on hypoxia in the Gulf of Mexico. NOAA Coastal Ocean Program, Silver Spring, MD. Decision Analysis Series No. 16:45.

dos Santos, J. and M. Jobling. 1995. Test of a food consumption model for the Atlantic cod. ICES Journal of Marine Science 52:209-219.

Dutil, J.-D. and D. Chabot. 2002. Impact of hypoxia on Atlantic cod (*Gadus morhua*) in the northern Gulf of St. Lawrence. *In:* Proceedings of the 6th International Symposium on Fish Physiology, Toxicology, and Water Quality, La Paz, B.C.S. Mexico, January 22-26, 2001, R.V. Thurston (ed). U.S. Environmental Protection Agency, Ecosystems Research Division, Athens, Georgia, USA. EPA 600/R-02/097. pp 51-60.

Dutil, J.-D., G. Godbout, F. Belanger, and P. Blier. 2001. The effect of energetic condition on growth in cod (*Gadus morhua*). *In*: ICES Annual Science Conference, 89th Statutory Meeting, Oslo, Norway. ICES CM 2001/V11:1-17.

Dutil, J.D., M. Castonguay, D. Gilbert, and D. Gascon. 1999. Growth, condition, and environmental relationships in Atlantic cod (*Gadus morhua*) in the northern Gulf of St. Lawrence and implications for management strategies in the Northwest Atlantic. Canadian Journal of Fisheries and Aquatic Science 56:1818-1831.

Fordham, S.E. and E.A. Trippel. 1999. Feeding behaviour of cod (*Gadus morhua*) in relation to spawning. Journal of Applied Ichthyology. 15:1-9.

Gilbert, D. and B. Pettigrew. 1997. Interannual variability (1948-1994) in the CIL core temperature in the Gulf of St. Lawrence. Canadian Journal of Fisheries and Aquatic Science 54:57-67.

Koutitonsky, V.G. and G.L. Bugden. 1991. The physical oceanography of the Gulf of St. Lawrence: A review with emphasis on the synoptic variability of the motion. *In*: The Gulf of St. Lawrence: small ocean or big estuary? J.-C. Therriault (ed.). Vol. 113:57-90.

Lambert, Y. and J.-D. Dutil. 1997. Condition and energy reserves of Atlantic cod (*Gadus morhua*) during the collapse of the northern Gulf of St. Lawrence stock. Canadian Journal of Fisheries Aquatic Science 54:2388-2400.

Lauzier, L.M. and R.W. Trites. 1958. The deep waters in the Laurentian Channel. Journal of Fisheries Research Board of Canada 15:1247-1257.

Nissling, A., H. Kryvi, and L. Vallin. 1994. Variation in egg buoyancy of Baltic cod *Gadus morhua* and its implications for egg survival in prevailing conditions in the Baltic Sea. Marine Ecology Program Series 110:67-74.

Nissling, A. and L. Westin. 1991. Egg buoyancy of Baltic cod (*Gadus morhua*) and its implications for cod stock fluctuations in the Baltic. Marine Biology 111:33-35.

Pihl, L. 1989. Effects of oxygen depletion on demersal fish in coastal areas of the south-east Kattegat. *In:* 23rd European Marine Biology Symposium — Reproduction, genetics and distributions of marine organisms. University of Wales, Swansea. Olsen & Olsen. Pp. 431-439.

Pihl, L. 1994. Changes in the diet of demersal fish due to eutrophication-induced hypoxia in the Kattegat, Sweden. Canadian Journal of Fisheries and Aquatic Science 51:321-336.

Plante, S., D. Chabot, and J.-D. Dutil. 1998. Hypoxia tolerance in Atlantic cod. Journal of Fisheries Biology 53:1342-1356.

Rivoirard, J., J. Simmonds, K.G. Foote, P. Fernandes, and N. Bez. 2000. Geostatistics for estimating fish abundance. Blackwell Science Ltd, Oxford, UK.

Rosenberg, R. and L.O. Loo. 1988. Marine eutrophication induced oxygen deficiency: effects on soft bottom fauna, western Sweden. Ophelia 29:213-225.

Scholz, U. and U. Waller. 1992. The oxygen requirements of three fish species from the German Bight: cod Gadus morhua, plaice *Pleuronectes platessa*, and dab *Limanda limanda*. Journal of Applicable Ichthyology 8:72-76.

Schurmann, H. and J.F. Steffensen. 1992. Lethal oxygen levels at different temperatures and the preferred temperature during hypoxia of the Atlantic cod, *Gadus morhua* L. Journal of Fisheries Biology 41:927-934.

Schwalme, K. and G.A. Chouinard. 1999. Seasonal dynamics in feeding, organ weights, and reproductive maturation of Atlantic cod (*Gadus morhua*) in the southern Gulf of St Lawrence. ICES Journal of Marine Science 56:303-319.

Seliger, H.H., J.A. Boggs and J.A. Biggley. 1985. Catastrophic anoxia in the Chesapeake Bay in 1984. Science 228:70-73.

Soofiani, N.M. and A.D. Hawkins. 1982. Energetic costs at different levels of feeding in juvenile cod, *Gadus morhua* L. Journal of Fisheries Biology 21:577-592.

Stephan, C.E. 1977. Methods for calculating an LC_{50}. *In*: Aquatic Toxicology and Hazard Evaluation, ASTM STP 634. F.L. Mayer and J.L. Hamelink (eds.). American Society for Testing and Materials. Pp. 65-84.

Sundnes, G. 1957. On the transport of live cod and coalfish. Journal du Conseil International de l'Exploration de la Mer 22:191-196.

Temming, A. and N.G. Andersen. 1994. Modelling gastric evacuation without meal size as a variable. A model applicable for the estimation of daily ration of cod (*Gadus morhua* L) in the field. ICES Journal of Marine Science 51:429-438.

Tomkiewicz, J., K.M. Lehmann, and M.A. St. John. 1998. Oceanographic influences on the distribution of Baltic cod, *Gadus morhua*, during spawning in the Bornholm Basin of the Baltic Sea and Fisheries. Oceanography 7:48-62.

Wieland, K., U. Waller, and D. Schnack. 1994. Development of Baltic cod eggs at different levels of temperature and oxygen content. Dana 10:163-177.

RUNNING THE UNSEEN, LOWLAND GAUNTLET: COMPOUNDING EFFECTS OF TEMPERATURE, HYPOXIA AND EXERCISE FOR DIADROMOUS FISHES

Henry James Bannon and Nicholas Ling[1]

ABSTRACT

Sustained swimming abilities (U_{crit}) of rainbow trout parr (*Oncorhynchus mykiss*), and larval (whitebait) and postlarval inanga (*Galaxias maculatus*), were tested at temperatures from 5°C to 25°C. Acclimated fish were swum to exhaustion under normoxic conditions to determine optimal aerobic exercise temperature. The potential effect of mild environmental hypoxia (75% sat.) was examined in trout and whitebait at 10°C, 15°C and 20°C. Hematological responses of trout were examined by acute caudal venepuncture.

Under normoxic conditions, $U_{crit\ max}$ for trout parr was 5.8 body lengths per second (BL/s) at 15.1°C and 5.1 BL/s at 17.7°C for inanga whitebait, but decreased at lower and higher temperatures. Hypoxia caused a significant reduction in U_{crit} at 20°C, but had no effect on trout at 10°C or 15°C. $U_{crit\ max}$ in hypoxia was 6 BL/s at 14.1°C. Hypoxia significantly reduced U_{crit} at 15°C and 20 °C in whitebait, lowering the optimal aerobic temperature to 13.9°C and reducing $U_{crit\ max}$ to 4.2 BL/s. Postlarval inanga performed poorly at increased temperatures with a $U_{crit\ max}$ of 5.6 BL/s at 9.4°C in normoxia, indicating an ontogenetic change in swimming ability, possibly resulting from a developmental shift in red muscle kinetics or a greater dependence on anaerobic muscle.

Temperature acclimation significantly improved trout swimming ability. Fish acclimated to 20°C for two weeks performed better at 20°C than fish acclimated to 10°C. Trout acclimated at 20°C showed an adaptive elevation in oxygen carrying capacity due to an increase in mean erythrocyte volume and hemoglobin content. Following exercise, hematocrit was elevated under both normoxic and hypoxic conditions. However, the primary cause of this apparent increase in oxygen carrying capacity was splenic release of erythrocytes under normoxic conditions, whereas stress-induced erythrocytic swelling contributed to the observed increase in hypoxia. This contrasting response was most pronounced at 10°C.

These results demonstrate that even mild hypoxia can significantly reduce the swimming abilities of migratory fishes in warm water, although acclimation can assist performance. The implications for the management of lowland rivers are clear: elevated water temperatures and hypoxia could significantly limit fish migration.

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INTRODUCTION

Diadromous fishes use lowland riverine systems as migratory pathways when moving between freshwater and marine habitats. Successful migration and colonization of suitable adult habitats by diadromous fishes require sustained swimming over many kilometers in these lowland riverine systems. Globally, these migratory pathways are becoming increasingly degraded by anthropogenic impacts including chemical, thermal and nutrient pollution, canalization, abstraction, loss of riparian shading, construction of physical barriers, and global climate change. Proximate consequences include eutrophication and hypoxia, loss of habitat complexity, alterations to water flow, and elevated temperatures. Migratory fishes, therefore, run a gauntlet of diverse stressors to reach adult or reproductive habitats. While some impacts, such as physical barriers and chemical pollution, represent obvious threats to migration, hypoxia and elevated temperatures represent an invisible challenge.

The combined effects of elevated temperatures and hypoxia create compounding bioenergetic problems for sustained swimming in fish. Elevated temperatures increase metabolic rate, resulting in increased oxygen demand, whilst decreasing oxygen solubility and eutrophic hypoxia reduce environmental oxygen availability. However, these negative impacts could be offset by reduced water viscosity, increased diffusion rates, and a temperature dependent increase in muscle performance so that the effect on swimming ability is difficult to predict. Taylor *et al*. (1997) concluded that a small acute temperature increase is unlikely to be a problem for sustained swimming unless the ambient temperature is close to the upper thermal limit of the species. However, sustained swimming ability is temperature dependent. In eurythermal fishes, the critical swimming velocity (U_{crit}; Brett 1964, 1967) increases with temperature to a maximum several degrees below the upper thermal limit; performance then declines markedly as the upper thermal limit is approached (Brett 1971).

The compounding effects of increased temperature and decreased oxygen availability on sustained swimming have not been investigated. Most fishes function as oxygen regulators, increasing gill ventilation as ambient oxygen decreases until a critical oxygen saturation (S_{crit}) is reached (Randall 1982). Although S_{crit} increases with temperature in resting fish (Schurmann and Steffensen 1997), there is little information available on the combined effects of decreased oxygen and increased temperature on active swimming. Brett (1964) concluded that any reduction in saturation is likely to reduce activity above the optimum temperature. However, it is widely assumed that the metabolic performance of fish decreases only at ambient oxygen concentrations below 70% saturation (Hammer 1995). Although Bushnell *et al*. (1984) observed a significant decline in U_{crit} of rainbow trout at an ambient oxygen concentration of 27.5% saturation and an optimal performance temperature of 15^{o}C, the metabolic responses of fish to hypoxia in the upper thermal range have not been studied.

The aim of this study was to investigate possible anthropogenic impacts on fish migration in lowland rivers by examining the effect of mild hypoxia (75% saturation) on the sustained swimming abilities of diadromous fishes over a wide thermal range. *Galaxias maculatus* and *Oncorhynchus mykiss* were used to assess the compounding effects of temperature, hypoxia, and exercise. Catadromous *Galaxias maculatus*, or inanga, migrate downstream to spawn in estuarine and tidal reaches from late winter to early summer (McDowall 1990). Following a

period of marine dispersal, inanga larvae migrate upriver as whitebait to settle in lowland adult habitats. Rainbow trout (*Oncorhynchus mykiss*), native to North America, have diadromous populations only in the northern, colder regions of their natural range. In New Zealand, rainbow trout are potamodromous, with lake and lowland river fish migrating upstream to spawn in colder upland tributaries.

MATERIALS AND METHODS

Experimental animals

Rainbow trout parr, *Oncorhynchus mykiss* (Walbaum), 7.0 ± 0.5 cm fork length, were obtained from the Ngongataha Trout Hatchery and Forest Research, Rotorua, New Zealand. Fish were held at the University of Waikato in fiberglass aquaria supplied with dechlorinated tap water (17^{o}C).

Inanga whitebait larvae, *Galaxias maculatus* (Jenyns), 5 ± 0.2 cm total length (TL), netted in the Waikato River near Port Waikato, New Zealand, were purchased from commercial whitebait fishermen. Inanga were kept in glass aquaria in dechlorinated tap water (17^{o}C) containing 0.35% NaCl.

Postlarval inanga were grown in the laboratory from whitebait larvae. Postlarval individuals showed a well-developed spleen and a marked pink coloration due to the presence of red blood cells in circulation. The body was typically more opaque than the clear glass-like appearance of the larvae.

Acclimation of fish

Trout parr were randomly allocated to treatment groups and acclimated to 5^{o}C, 10^{o}C, 15^{o}C, 20^{o}C or 25^{o}C (± 0.5^{o}C) for at least two weeks prior to swimming tests. Acclimation temperatures were achieved by increasing or decreasing water temperature by 1^{o}C per day. Fish were fed every day to satiation.

Migrating whitebait metamorphosed into the post-larval form within two to three weeks of capture. Fish were therefore randomly allocated to treatment groups as soon as they were obtained and were acclimated to the experimental temperatures of 5^{o}C, 10^{o}C, 15^{o}C, 20^{o}C or 25^{o}C (± 0.5^{o}C) over the course of three days. Temperatures were adjusted in three even increments over the three day acclimation period. Whitebait were fed live *Daphnia* daily.

To obtain post-larval inanga, whitebait were grown in the laboratory until pigmented and flushed with red blood. Because inanga shrink during metamorphosis, post-larval fish were 4.5 ± 0.2 cm TL. Fish were fed live *Daphnia*, and after three days in the laboratory were also fed blood worms (chironomid larvae) once a day to satiation. Fish were acclimated to test temperatures as described above for trout. All fish were maintained on a 12:12 photoperiod during acclimation.

Effects of temperature and hypoxia on U_{crit}

Critical swimming speeds (U_{crit}) were measured in an enclosed, 2 meter, 230 liter variable velocity recirculating flume. The flume allowed for precise temperature and oxygen control. The flume channel was 16 cm x 16 cm in cross-section. Hypoxia (75% sat.) was produced by nitrogen stripping in a bubble tower, and oxygen saturation and temperature were continuously recorded during U_{crit} trials.

For all experiments and at all temperatures, trout parr were swum individually, whereas whitebait and postlarval inanga were swum in groups of five. Fish were selected at random from the acclimated groups and their length was estimated visually to the nearest 0.5 cm. Handling was kept to a minimum to reduce stress; immediately after transfer to the flume, fish were swum for two hours at a low speed of 0.5 BL/s to aid recovery from handling and transfer stress (Milligan *et al*. 2000). During experiments, fish were swum to exhaustion (U_{crit}) at their acclimation temperatures under conditions of normoxia (>96% sat. at 5°C, 10°C, 15°C, 20°C or 25°C) or mild hypoxia (75% sat. at 10°C, 15°C or 20°C) by increasing water velocity in 0.5 BL/s increments every 15 minutes. Exhaustion was determined when the fish was forced on to, and remained on, an electrified rear grill. After exhaustion, all fish were weighed to the nearest 0.01g, and length was measured to ± 0.1 cm.

U_{crit} was determined for each fish using the equation from Brett (1964):

$$U_{crit} = U_i + U_{ii} \, T_i / T_{ii}$$

where U_i is the highest velocity maintained for a complete time interval, U_{ii} is the velocity increment (0.5 BL/s), T_i is the interval time elapsed at fatigue velocity, and T_{ii} is the interval time (15 min). U_{crit} values were corrected for the measured lengths of individual fish. Swimming velocities were not corrected for the solid blocking effect of the fish because the cross-sectional area of the fish was not greater than 10% of the cross-sectional area of the flume (Brett 1964).

Hematological acclimation in rainbow trout

To determine the effects of thermal acclimation on rainbow trout, five individuals from normoxic and hypoxic U_{crit} trials, as well as non-swum, rested fish removed from the acclimation aquaria at 10°C, 15°C and 20°C, were sampled by acute caudal venepuncture. Blood samples (~50 µl) were drawn into pre-heparinized syringes and analysed for hematocrit (Hct), whole blood hemoglobin (Hb), red blood cell count (RBCC), mean cell hemoglobin concentration (MCHC), mean cell hemoglobin (MCH), and mean cell volume (MCV) according to standard methods (Dacie & Lewis 1991). A deproteinated extract was made by adding 25 µl of whole blood to 50 µl of perchloric acid (8% solution). Whole blood lactate, glucose, and triglyceride concentrations were measured from the supernatant of the deproteinated extract (following centrifugation) using micro methods adapted from Sigma methods 826-UV, 18-UV, and 334-UV, respectively.

To determine whether acclimation affected swimming ability in trout, five fish acclimated to either 10°C or 20°C were swum at 20°C and 10°C, respectively. Their performances were then compared with fish swum at the respective acclimation temperatures.

RESULTS

Swimming performance

The critical swimming speeds for trout parr in normoxia are shown in Figure 1. $U_{crit\ max}$ for trout parr was 5.8 BL/s at 15.1°C, but decreased at lower and higher temperatures. This result implied that swimming performance was limited by temperature below 15°C, whereas performance at higher temperatures was limited by oxygen availability. In support of this hypothesis, mild hypoxia caused a significant reduction in U_{crit} at 20°C, but had no effect at 10°C or 15°C (Figure 1). $U_{crit\ max}$ in hypoxia was 6 BL/s at 14.1°C.

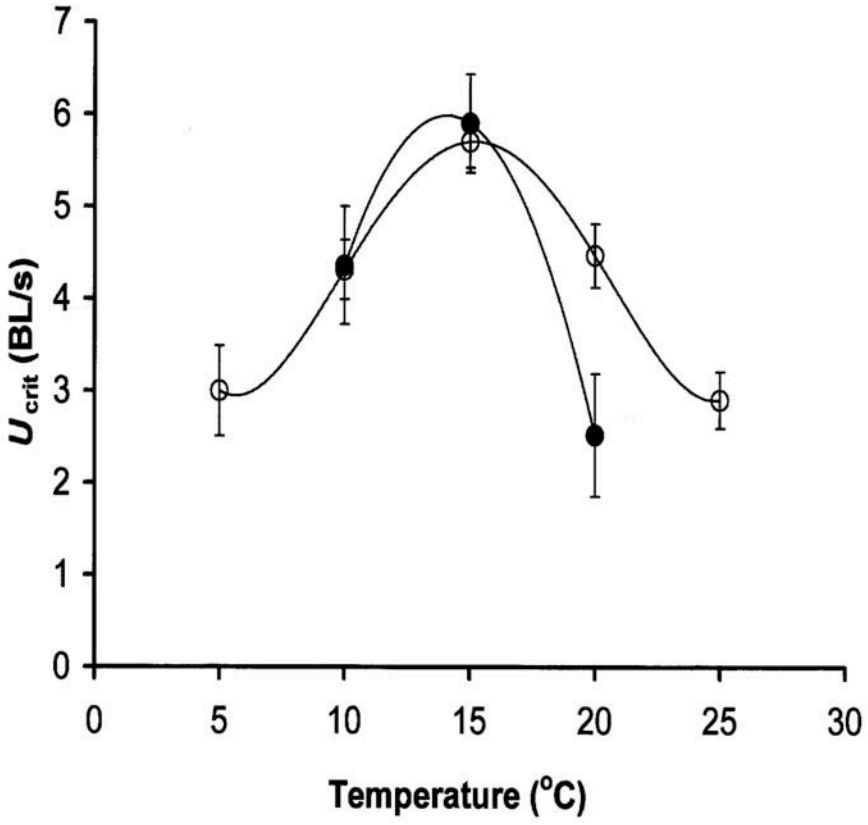

Figure 1. Sustained swimming speeds (U_{crit}) of rainbow trout parr at different temperatures in normoxia (>96% sat.;open circles, n = 15) and mild hypoxia (75% sat.; closed circles, n = 5). Means ± S.E.M.

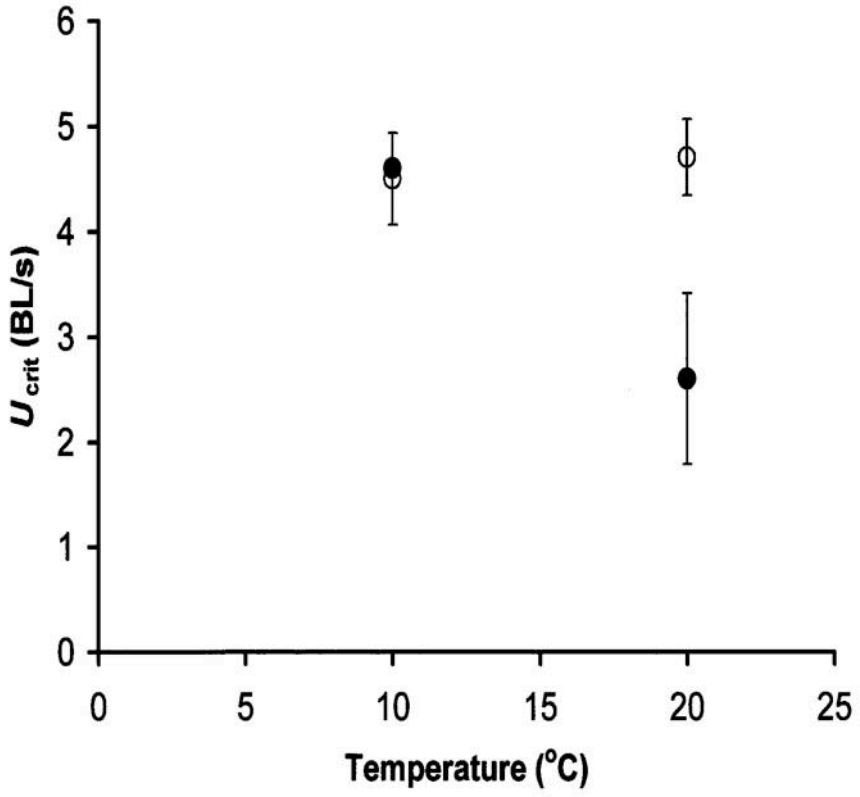

Figure 2. Sustained swimming speeds (U_{crit}) of rainbow trout parr acclimated for two weeks at 10°C (closed circles) or 20°C (open circles) and swum at 10°C and 20°C (n = 5 for each group). Means ± S.E.M.

Acclimation to 20°C improved warm water swimming performance. Parr acclimated to 10°C performed significantly worse than fish acclimated to 20°C when swum at 20°C (Figure 2). However, fish acclimated to 20°C performed as well as fish acclimated to 10°C when swum at 10°C.

Inanga whitebait also showed temperature dependence of sustained swimming ability with a $U_{crit\ max}$ of 5.1 BL/s at 17°C (Figure 3). Although mild hypoxia had no effect at 10°C, it significantly reduced U_{crit} at 15°C and 20°C, lowering the optimal aerobic temperature to 13.9°C and reducing $U_{crit\ max}$ to 4.2 BL/s. Mild hypoxia, therefore, had a more pronounced impact on inanga whitebait than trout.

Post-larval inanga performed poorly in normoxia at higher temperatures compared to whitebait; $U_{crit\ max}$ was 5.6 BL/s at 9.4°C (Figure 4). This indicated an ontogenetic shift in swimming ability, possibly resulting from a developmental change in red muscle kinetics or a greater reliance on anaerobic muscle. Not only did post-larval inanga swim poorly at warmer temperatures, but few individuals survived the two hour acclimation period in the flume prior to the start of the U_{crit} experiment (0.5 BL/s; 25°C).

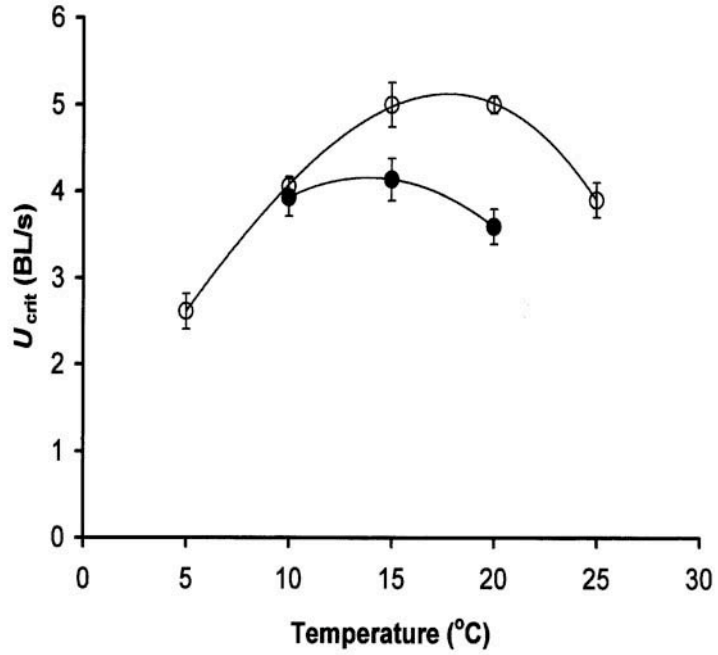

Figure 3. Sustained swimming speeds (U_{crit}) of inanga whitebait at different temperatures in normoxia (>96% sat.; open circles, n = 20 to 25 in each group) and hypoxia (75% sat.; closed circles, n = 16 to 18 in each group). Means ± S.E.M.

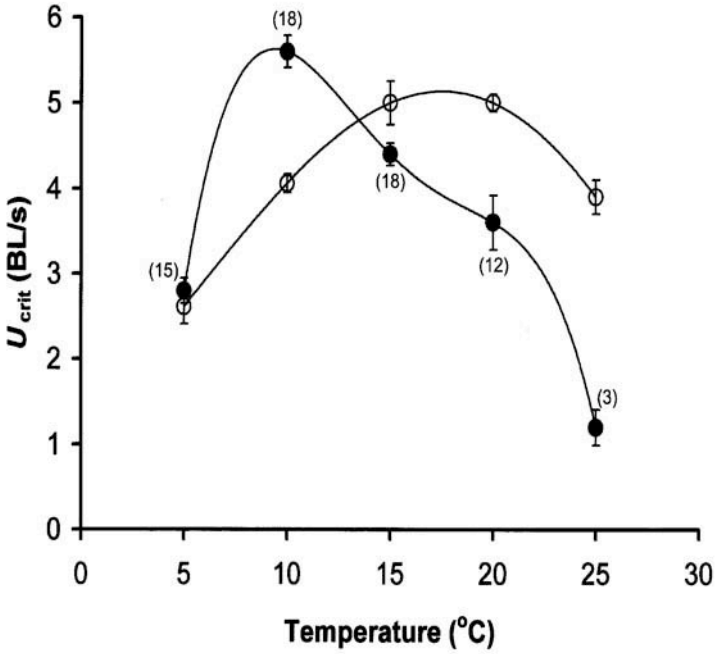

Figure 4. Sustained swimming speeds (U_{crit}) of inanga whitebait (open circles, n = 20 to 25 in each group) and postlarval inanga (closed circles, numbers of fish in each group in parentheses) at different temperatures under normoxic conditions (>96% sat.). Means ± S.E.M.

Hematological responses of rainbow trout

Trout acclimated at 20°C showed an adaptive elevation in oxygen carrying capacity due to a significant increase in red blood cell volume (MCV) and cell hemoglobin content (MCH), compared with fish acclimated at colder temperatures (Table 1). This accounted for the observed increase in packed cell volume and whole blood hemoglobin concentration in these fish. Packed cell volume was further elevated in all acclimated groups following exhaustive exercise under both normoxic and hypoxic conditions. However, the primary cause of this increase in oxygen carrying capacity appeared to be the splenic release of stored erythrocytes under normoxic conditions, indicated by unchanged MCHC and increased red cell numbers. Conversely, stress-induced, adrenergic erythrocytic swelling accounted for much of the observed increase in hypoxia as evidenced by the significant decline in mean cell hemoglobin concentration at all temperatures. This contrasting response was most pronounced at 10°C, whereas hypoxic fish at 20°C employed both strategies. Whole blood lactate and glucose were elevated in all exhausted fish indicating anaerobiosis and stress, respectively. Although triglycerides were slightly elevated in some exercised fish, differences between treatments were not significant.

DISCUSSION

The critical swimming speed of fish is dependent on many factors including size, temperature and ambient gas concentrations (Hammer 1995). Temperature dependence of U_{crit} is well established; the typical response is an increase to an optimum as temperature rises, followed by a subsequent decline at temperatures greater than optimal (Beamish 1978). Optimal temperature varies with species and often coincides with the preferred temperature of the fish (Brett 1971, Reynolds and Casterlin 1980, Gunderly and Blier 1988). However, significant and fundamental differences in the temperature dependence of critical swimming speed may occur between species that are anatomically or ecologically similar (Beamish 1980, Duthie 1981).

The establishment of optimum aerobic temperature and associated U_{crit} performance curves for fish species, measured in normoxic clean water, provides a reference for evaluating the effects of water quality parameters on sustained swimming of fish. Hammer (1995) concluded that critical swimming speed should provide a sensitive measure for environmental or physiological stress factors.

U_{crit} performance curves for rainbow trout parr, inanga whitebait, and juvenile inanga exhibited the characteristic temperature dependence, but inanga showed a significant developmental shift in optimum temperature. The reduced performance of juvenile inanga at warmer temperatures was somewhat surprising. The developmental transition of inanga from whitebait to the juvenile stage coincides with development and enlargement of the spleen; unlike clear, glass-like whitebait, juvenile fish become noticeably suffused with red blood. It was expected that the increased oxygen carrying capacity of juvenile inanga would improve aerobic performance. Although relative swimming performance is dependent on size and declines as fish increase in length (Bainbridge 1960, 1962), juvenile inanga are actually shorter than whitebait, so the decline in relative swimming speed of their juveniles is not related to growth. Developmental shifts in red muscle kinetics and swimming kinematics have been demonstrated in juvenile rainbow trout (Coughlin *et al*. 2001). Therefore, the observed ontogenetic shift in

Table 1. Hematological values of trout parr acclimated to 10°C, 15°C and 20°C and sampled at rest or following exhaustive exercise (U_{crit}) under normoxic (>96% sat.) or hypoxic (75% sat.) conditions. Values are means with S.E.M. in parentheses. Values with the same superscript are significantly different ($P < 0.05$). Superscripts a-i denote comparisons between treatments at each temperature. Superscripts r-z denote comparisons between acclimated groups within the same treatment. n = 5 for all groups.

Temp. (°C)	Variable	Control Resting	Normoxic Exercise	Hypoxic Exercise
10	PCV	25.0 (2.0)^{a,b}	32.8 (1.5)^{a}	30.6 (0.4)^{b}
15	(%)	24.0 (1.8)^{e,t}	26.0 (2.7)^{w}	32.4 (2.0)^{e}
20		29.6 (0.9)^{g,t}	38.0 (1.9)^{g,w}	34.8 (2.4)
10	[Hb]	63.7 (4.2)^{a,s}	81.8 (6.3)^{a}	68.0 (2.3)
15	(g/L)	60.6 (4.3)^{t}	64.2 (6.3)^{w}	75.1 (5.8)
20		75.2 (2.2)^{s,t}	101 (13)^{w}	75.9 (3.9)
10	MCHC	257 (6)^{b}	250 (13)	222 (6)^{b}
15	(g/L)	252 (4)^{e}	247 (10)	230 (6)^{e}
20		254 (7)^{h}	261 (26)	218 (4)^{h}
10	MCH	59.9 (3.5)	62.9 (3.9)	65.0 (1.9)
15	(pg)	70.5 (5.5)	66.3 (3.6)	73.4 (5.0)
20		74.3 (5.8)	86.1 (12.4)	62.5 (4.0)
10	MCV	233 (11)^{b,s}	251 (4)^{c,v}	294 (14)^{b,c}
15	(fL)	280 (20)	269 (9)^{f,w}	319 (17)^{f}
20		292 (17)^{s}	325 (23)^{v,w}	286 (17)
10	RBCC	1.08 (0.09)	1.30 (0.05)^{c}	1.05 (0.06)^{c}
15	(x 10^{12} cells/L)	0.90 (0.14)	0.99 (0.21)	1.04 (0.09)
20		1.03 (0.06)	1.19 (0.07)	1.23 (0.10)
10	Lactate	1.79 (0.31)^{a,b}	4.79 (1.14)^{a}	4.82 (0.42)^{b}
15	(mM)	1.21 (0.26)^{e}	4.47 (1.54)	4.62 (0.82)^{e}
20		1.74 (0.25)^{g,h}	5.46 (1.50)^{g}	7.20 (1.53)^{h}
10	Glucose	3.51 (0.06)^{a,b}	5.28 (0.56)^{a}	4.39 (0.15) ^{b,x,y}
15	(mM)	4.01 (0.64)	4.81 (0.35)	5.57 (0.35)^{x}
20		3.56 (0.22)^{h}	4.44 (0.33)	5.08 (0.18)^{h,y}
10	Triglycerides	222 (9.1)	230 (8.4)	252 (11.2)
15	(mg/dL)	214 (5.4)	212 (10.7)	210 (10.5)
20		240 (10.2)	258 (19.4)	228 (4.9)

swimming performance may have resulted from developmental changes in aerobic and anaerobic muscles. Assessments of potential impacts of environmental factors on sustained swimming in migratory species must therefore take into account the influence of ontogenetic shifts in swimming ability.

The effects of hypoxia on sustained swimming over a wide range of temperatures are unpredictable, given that most studies have exposed fish to hypoxia at temperatures at or below the optimum. The declining solubility of oxygen and the concomitant increase in metabolic oxygen requirements would be expected to constrain aerobic metabolism at higher temperatures. Graham (1949) reported the cruising speed of brook trout was noticeably reduced when oxygen concentrations were decreased to about 50% of the air-saturated level at 8^{o}C; however, this is below the optimum temperature for this species. Davis *et al*. (1963) reported that juvenile coho (*Oncorhynchus kisutch*) and chinook salmon (*O. tshawytscha*) usually showed some reduction in sustained swimming speeds when exposed to even a slight reduction in dissolved oxygen concentration from the air-saturated level. At the optimum temperature for rainbow trout (15^{o}C), Bushnell *et al*. (1984) observed a significant reduction in U_{crit} with severe hypoxia (27.5% sat.). Brett (1964) reported that for yearling sockeye salmon in air-saturated freshwater, oxygen could become a limiting factor for active metabolism above the optimum temperature. Our results show that even a small reduction in ambient oxygen (75% sat.) above the optimum temperature can result in a significant reduction in U_{crit}. These results confirm that the decline in swimming performance at temperatures above optimum is probably due to limited oxygen delivery to active muscles.

Further evidence for the dependence of swimming performance on oxygen transport at higher temperatures is the enhanced performance of warm-acclimated trout. Such adaptive acclimation may be a factor responsible for the observed dependence of swimming performance on season (Brett 1964). Trout parr acclimated to 20^{o}C for two weeks performed nearly twice as well at that temperature when compared with fish acclimated at 10^{o}C, due to an adaptive elevation in oxygen carrying capacity in the former. Moreover, mild hypoxia imparts some degree of physiological impact, even at colder temperatures; rainbow trout swum at 10^{o}C showed a significant decrease in MCHC, indicating adrenergic swelling of erythrocytes. This response increases hemoglobin oxygen affinity by a reversal of the Bohr and Root effects (Nikinmaa 1990) and would enhance oxygen uptake at the gills. Surprisingly, this effect did not occur in normoxic fish swum at 20^{o}C, where normoxic oxygen saturation is almost equivalent to the 75% saturated value at 10^{o}C.

Inanga whitebait were more sensitive to mild hypoxia than rainbow trout parr. The greater sensitivity of inanga whitebait may reflect their reduced acclimation times, necessitated by the need to test the fish soon after capture before development into the juvenile form. However, inanga whitebait do not possess significant numbers of red blood cells, so adaptive increase in oxygen carrying capacity is unlikely in these early stage fish.

The consequences of this study for lowland river water quality are clear: hypoxia resulting from eutrophication is unlikely to markedly affect swimming abilities of fish at

temperatures below optimum unless the reduction in ambient oxygen is very large. However, at temperatures above the species optimum, even minor reductions in oxygen will severely impede swimming ability and fish migration. Anthropogenic influences that collectively increase water temperatures and decrease oxygen availability will have the greatest impact on fish populations.

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REFERENCES

Bainbridge, R. 1960. Speed and stamina in three fish. Journal of Experimental Biology 37:129-153.

Bainbridge, R. 1962. Training, speed and stamina in trout. Journal of Experimental Biology 39:537-555.

Beamish, F.W.H. 1978. Swimming Capacity. *In*: Fish Physiology, W.H. Hoar and D.J. Randall (eds). Academic Press. New York. Pp. 101-187.

Beamish, F.W.H. 1980. Swimming Performance and Oxygen Consumption of the Charrs. *In*: Charrs. Salmonid Fishes of the Genus Salvelinus, E.K. Balon (ed). Dr W. Junk Publishing. The Hague. Pp. 739-748.

Brett, J.R. 1964. The respiratory metabolism and swimming performance of young sockeye salmon. Journal of Fisheries Research Board of Canada 21**:**1183-1226.

Brett, J.R. 1967. Swimming performance of sockeye salmon (*Oncorhynchus nerka*) in relation to fatigue time and temperature. Journal of Fisheries Research Board of Canada 24:1731-1741.

Brett, J.R. 1971. Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). American Zoology 11:99-113.

Bushnell, P.G., J.F. Steffensen , and K. Johansen. 1984. Oxygen consumption and swimming performance in hypoxia-acclimated rainbow trout *Salmo gairdneri*. Journal of Experimental Biology 113**:**25-235.

Coughlin, D.J., J. Burdick, K.A. Stauffer, and F.E. Weaver. 2001. Rainbow trout display a developmental shift in red muscle kinetics, swimming kinematics and myosin heavy chain isoform. Journal of Fisheries Biology 58**:**701-715.

Davis, G.E., J. Foster, C.E. Warren, and P. Doudoroff. 1963. The influence of oxygen concentration on the swimming performance of juvenile Pacific salmon at various temperatures. Transactions of the American Fisheries Society 92:111-124.

Duthie, G. 1981. The respiratory metabolism of temperature-adapted flatfish at rest and during swimming activity, and the use of anaerobic metabolism at moderate swimming speeds. Journal of Experimental Biology 97**:**359-373.

Graham, J.M. 1949. Some effects of temperature and oxygen pressure on the metabolism and activity of the speckled trout, *Salvelinus fontinalis.* Canadian Journal of Research D, 27:270-288.

Gunderly, H. and P. Blier. 1988. Thermal acclimation in fish: conservative and labile properties of swimming muscle. Canadian Journal of Zoology 66:1105-1115.

Hammer, C. 1995. Fatigue and exercise tests with fish. Computational Biochemistry Physiology 112A:1-20.

McDowall, R.M. 1990. New Zealand Freshwater Fishes. A Natural History and Guide. Heinemann Reed, Auckland.

Milligan, C.L., G.B. Hooke, and C. Johnson. 2000. Sustained swimming at low velocity following a bout of exhaustive exercise enhances metabolic recovery in rainbow trout. Journal of Experimental Biology 203**:**921-926.

Nikinmaa, M. 1990. Vertebrate Red Blood Cells: adaptations of function to respiratory requirements. Springer, Berlin.

Randall, D. J. 1982. The control of respiration and circulation in fish during exercise and hypoxia. Journal of Experimental Biology 100:275-288.

Reynolds, W.W. and M.E. Casterlin. 1980. The role of temperature in the environmental physiology of fishes. *In*: Environmental Physiology of Fishes, M.A. Ali (ed). Plenum Press. New York. Pp. 497-518.

Schurmann, H. and J.F. Steffensen. 1997. Effects of temperature, hypoxia and activity on the metabolism of juvenile Atlantic cod. Journal of Fisheries Biology 50:1166-1180.

Taylor, E.W., S. Egginton, S.E. Taylor, and P.J. Butler. 1997. Factors which may limit swimming performance at different temperatures. *In:* Global Warming: Implications for Freshwater and Marine Fish, C.M. Wood and D.G. McDonald (eds). SEB Seminar Series 61:105-134.

HYPOXIA ON CORAL REEFS AND HYPOXIA TOLERANCE IN CORAL REEF FISH

Göran E. Nilsson and Sara Östlund-Nilsson[1]

ABSTRACT

Coral reef fishes are not known for their hypoxia tolerance, and coral reefs are not generally thought of as hypoxic habitats. However, recent investigations on Australia's Great Barrier Reef paint a different picture. We will here discuss some examples of hypoxic coral reef habitats and hypoxia tolerant coral reef inhabitants, and we will even suggest that hypoxia tolerance is a widespread phenomenon among coral reef fishes.

THE EPAULETTE SHARK: A HYPOXIA TOLERANT TROPICAL ELASMOBRANCH

Our first, and best known, example of hypoxia and hypoxia tolerance on a coral reef is that of the epaulette shark *(Hemiscyllium ocellatum)* on Heron Island, close to the Southern edge of the Great Barrier Reef. In 1966, Kinsey and Kinsey reported measurements of water oxygen levels on the reef platform surrounding Heron Island. At low tide, they found that the water [O_2] could fall below 30% of air saturation (about 2.1 mg O_2 l^{-1}). At very low tides, the huge (ca 3 x 10 km) reef platform is cut off from the surrounding ocean, and essentially becomes a very large tide pool. Respiration of the coral, and all associated organisms, brings down the oxygen levels. On calm nights, with little convective water movements, we have measured water [O_2] levels around 18% of air saturation on the Heron Island reef platform (Routley *et al*. 2002).

During these hypoxic episodes, the epaulette shark stays on the reef platform. Wise *et al*. (1998) were the first to show that this shark was hypoxia tolerant, surviving in water with an [O_2] of 5% of air saturation for at least 3.5 hours without any impairment of neural functions like righting reflex, ventilation and rhythmic swimming. Subsequent studies have revealed that hypoxia is tolerated without any delayed phase of neuronal death (Renshaw and Dyson 1999), a common event in mammals subjected to hypoxia. Moreover, the epaulette shark also survives complete anoxia for about an hour, at temperatures close to 30 °C, without any considerable drop in brain [ATP] (Renshaw *et al*. 2002).

The critical oxygen concentration ($[O_2]_{crit}$) is the concentration below which the fish is unable to maintain a resting oxygen consumption rate (VO_2) that is independent of the ambient [O_2] (Beamish 1964), and is, therefore, a measure of the ability to tolerate hypoxia. Adult epaulette sharks, weighing about 600 g, have a mean VO_2 of 83.4 mg O_2 kg^{-1} h^{-1}, and a mean $[O_2]_{crit}$ of about 30% of air saturation (Routley *et al*. 2002). Such a low $[O_2]_{crit}$ indicates that they can cope with the hypoxia that regularly occurs on the reef quite well, and only have to resort to anaerobic metabolism when the oxygen level falls below 30% of air saturation.

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So far, experiments on this shark have yielded both interesting and surprising results. For example, the epaulette shark shows no change in the cerebral blood flow when exposed to severe hypoxia (5% of air saturation) for two hours (Söderström *et al*. 1999a). This result was unexpected, since hypoxia has a highly stimulatory effect on brain blood flow in other species, including mammals, crocodiles, turtles, and teleost fish (Söderström *et al*. 1999a-b). However, the brain blood flow in the epaulette shark was at least maintained during hypoxia, despite a fall in blood pressure, suggesting that hypoxia induces cerebral vasodilation (Söderström *et al*. 1999a).

In contrast to the hyperglycemic response of many vertebrates, including teleost fish, exposed to hypoxia, the blood glucose level remains constant during hypoxia in the epaulette shark (Figure 1b) (Routley *et al*. 2002). This is also the case in another shark, the dogfish, *Scyliorhinus canicula* (Butler *et al*. 1979), and may reflect an elasmobranch trait. Moreover, the erythrocyte content of the epaulette shark blood was found to be relatively low (a haematocrit of about 15%, Figure 1c) and did not increase, even after repeated hypoxic exposures (Routley *et al*. 2002).

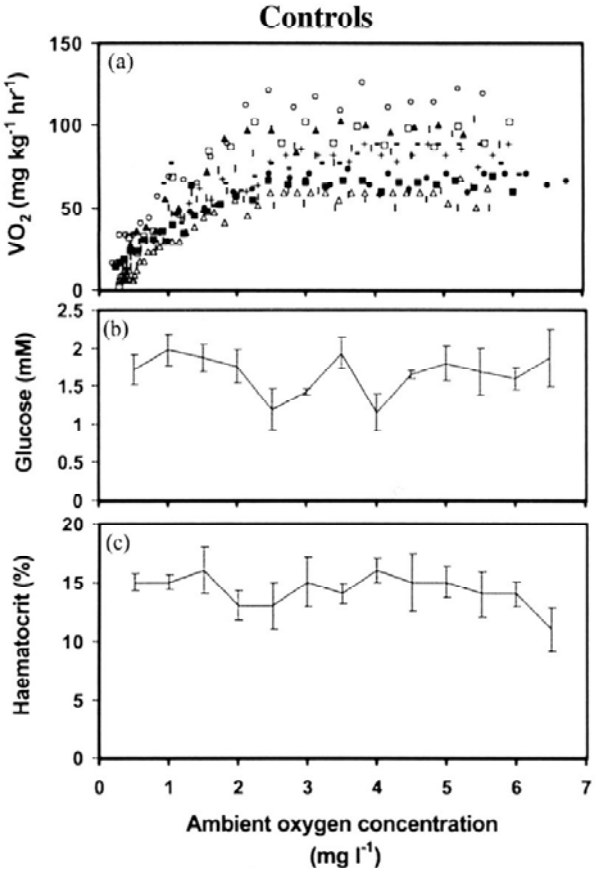

Figure 1. Effect of falling ambient [O_2] on (a) the rate of O_2 consumption VO_2, (b) blood glucose, and (c) hematocrit in epaulette sharks. The different symbols in (a) represent individual sharks while other values are means ± SEM from 10 animals. Data from Routley *et al*. (2002).

WIDESPREAD HYPOXIA TOLERANCE IN CORAL REEF FISHES

While studying the respiratory consequences of mouthbrooding in two species of cardinalfish (*Apogon leptacanthus* and *A. fragilis*) on Lizard Island on the northern portion of the Great Barrier Reef, we found that these fishes had an $[O_2]_{crit}$ just below 20% of air saturation (Nilsson and Östlund-Nilsson, in prep.). To us, this was an unexpectedly low $[O_2]_{crit}$ for fishes living in a tropical coral reef habitat. Firstly, to our knowledge, severe hypoxia had never been reported in this habitat. Secondly, the ability to maintain O_2 uptake in hypoxia is not uncomplicated in seawater at such a high temperature (30 °C) due to the combined effects of a low solubility of O_2 in warm seawater, and a high rate of oxygen consumption of a small fish at such a high temperature. In all animals, VO_2 decreases with body size and increases with body temperature.

The $[O_2]_{crit}$ of the cardinalfishes studied varied between 11% and 34% saturation. However, to our surprise all of the other fishes sampled from the same habitat, representing 25 species from six families, also showed strikingly low $[O_2]_{crit}$ values, varying from 14% to 32% saturation. These values are similar to those displayed by African freshwater cichlids, including tilapia, that are well known for their hypoxia tolerance (Verheyen *et al*. 1994).

Low $[O_2]_{crit}$ was, for example, displayed by several species of damselfishes (Pomacentridae), a large and well-known family of often colorful fishes, and one of the dominant fish groups on coral reefs around the world (Figure 2). Several of the coral reef fishes did not show any signs of distress or loss of coordination until the O_2 level fell below 5% of air saturation, indicating high anaerobic capacities. This hypoxia tolerance was combined with high metabolic rates. Most of the fishes studied weighed 1 – 20 g and had a VO_2 of about 300 – 700 mg O_2 kg^{-1} h^{-1}, several times higher than that of fishes in cold, temperate water.

Figure 2. Branching coral at the reef near Lizard Island Research Station – a habitat where all fish examined display a considerable hypoxia tolerance. Depth 3 m. (photo G.E. Nilsson).

But why do virtually all fishes in this habitat show a $[O_2]_{crit}$ that is much lower than the O_2 levels that they, at a first glance, can be expected to encounter? Possibly, this is related to the fact that the same species also occur on more shallow reefs that, like the reef platform around Heron Island further South on the Great Barrier Reef, become cut off from the surrounding ocean during low tides.

Indeed, we found that shallow parts of the reef around Lizard Island can get partly air-exposed, with the resultant formation of tidal pool, during exceptionally low tides (Figure 3). So far, we have not been able to explore the reef at such low tides. It would be particularly interesting to measure oxygen levels in the tidal pool during calm nights and examine the composition of the fish fauna that remain there.

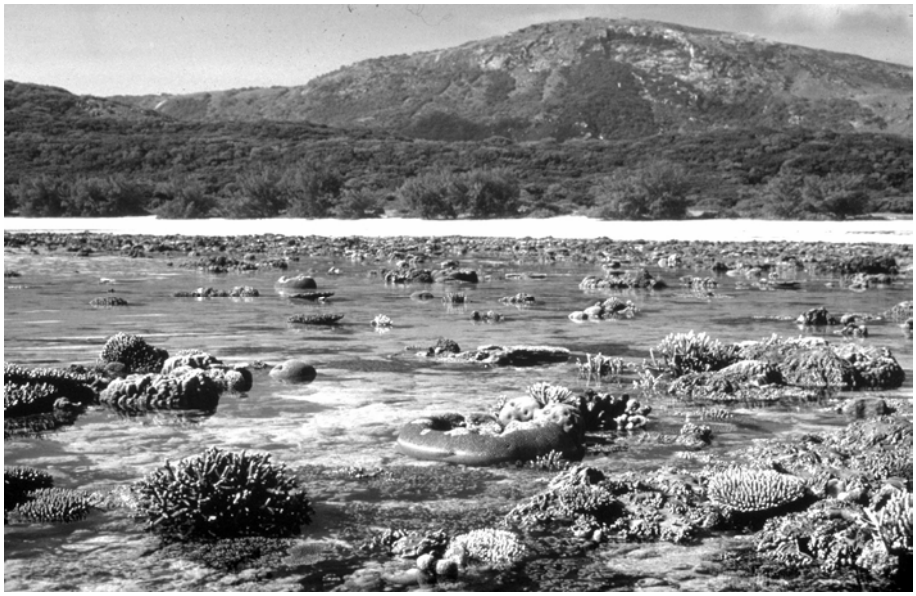

Figure 3. Extreme low tide at a shallow reef near Lizard Island Research Station. At night, this could be a hypoxic environment. (photo Lizard Island Research Station).

However, another possible explanation for the hypoxia tolerance of at least some of the fish species examined at Lizard Island is that they move more or less deep into the branching coral to feed or hide from predators. If they do this at night, as many night divers reportedly have seen, they enter a microhabitat that can become hypoxic due to coral respiration. To examine this hypothesis further, we decided to make a case study by taking a closer look at the respiratory characteristics of a true coral-dweller.

THE MOST COWARDLY FISH ON THE REEF: THE BROAD-BARRED GOBY

The broad-barred goby, *Gobiodon histrio* (Figure 4) is arguably an exceptionally cowardly fish. It secretes a poisonous mucus, and is therefore probably inedible to most predators. Its bright green color with red markings could possibly serve as a warning to predators, and fish fed with pieces of *Gobiodon* have been found to die within a few minutes (Schubert *et al*., in press). Nevertheless, it spends virtually its whole adult life in 5-10 mm wide spaces formed between the branches of *Acropora* corals (preferentially *A. nasuta*), a shelter that should make it inaccessible to most predators. Moreover, the need to leave the coral to find a sex partner is minimized by its ability to change sex. Thus, if two individuals of the same sex end up in the same coral, one of them will change its sex unless other suitable corals are very nearby (Munday *et al*. 1998).

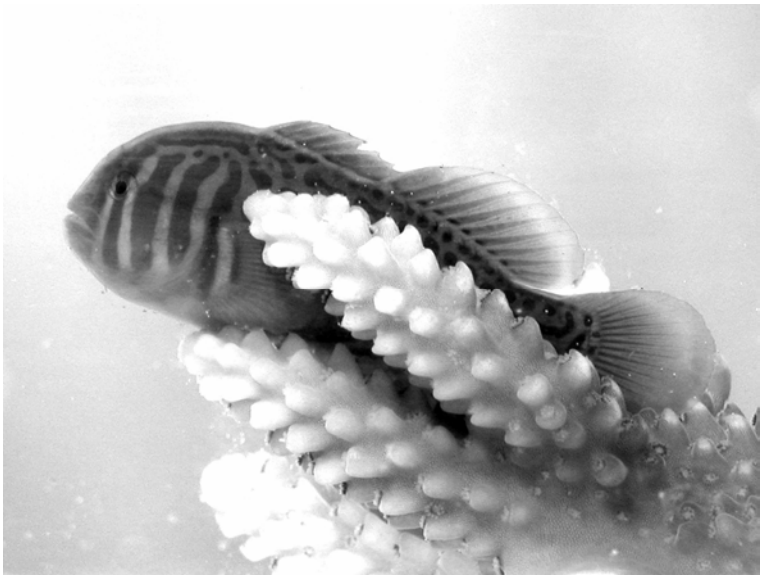

Figure 4. The broad-barred goby, *Gobiodon histrio* (photo G.E. Nilsson).

This extreme habitat fidelity makes the broad-barred goby ideal for studying whether a coral habitat demands hypoxia tolerance. In particular, we expected that the water between branches of coral becomes hypoxic during calm nights, due to the combined effects of the nocturnal cessation of photosynthesis, the continued respiration of the coral and associated organisms, and the lack of advective water movements. Low nocturnal oxygen levels were indicated by physiological studies revealing night-time hypoxia in coral tissue (Jones and Hoegh-Guldberg 2001).

Consequently, we set out to measure the water oxygen level in *A. nasuta* corals at night in calm water (simulated in a large outdoor tank at Lizard Island Research Station). We also used closed respirometry to examine the ability of *G. histrio* to tolerate hypoxia (Nilsson *et al*. in review).

The results indicated that the coral home of *G. histrio* can become severely hypoxic under calm conditions at night. The average $[O_2]$ minimum reached between branches in the coral (Figure 5) could be as low as 3% saturation for a short period of time in the early morning.

Our respirometric measurements showed that *G. histrio* has a $[O_2]_{crit}$ of 18% of air saturation, and can tolerate at least two hours at even lower O_2 levels, not losing its equilibrium until the water O_2 level falls below 3%.

Clearly, the ability of this goby to tolerate this severe hypoxia is likely to be a prerequisite for it to be able to remain in its coral shelter during hypoxic episodes that are most likely to occur during calm nights.

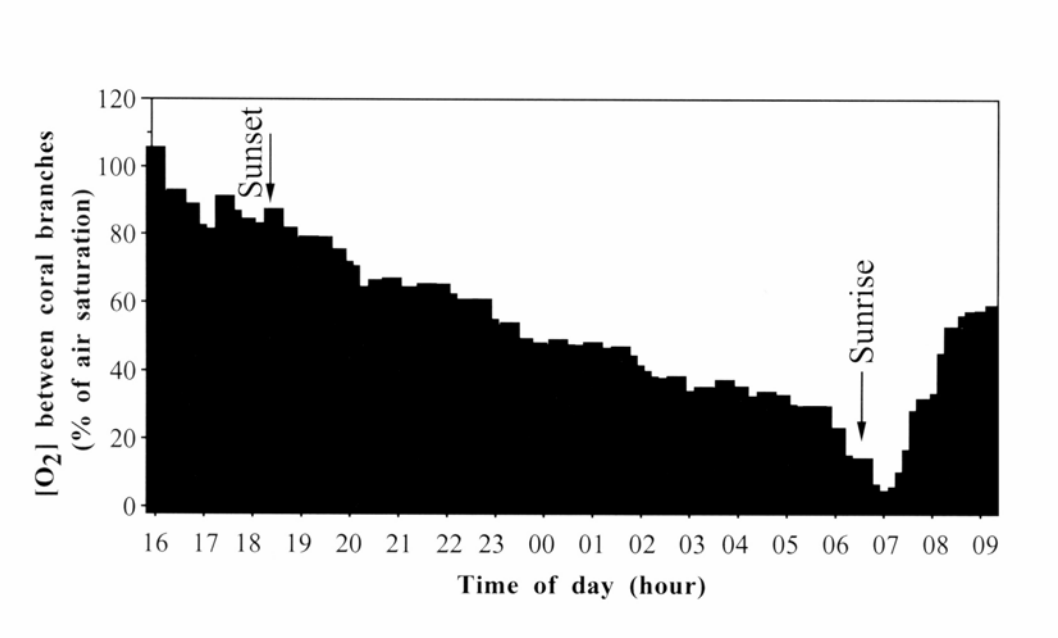

Figure 5. Tracing of the oxygen level inside the coral *Acropora nasuta*, the habitat where the broad-barred goby spends its entire adult life.

This is to our knowledge the first documented case of predator avoidance through hypoxia tolerance in a coral-dwelling fish. However, another goby, *Valenciennea longipinnis*, that lives in burrows in sandy areas near coral reefs, also appears to be quite tolerant of hypoxia (Takegaki and Nakazono 1995). This may be a prerequisite for it to stay in its burrow, that at times can become hypoxic.

CONCLUSIONS

Hypoxia in coral reef habitats, and hypoxia tolerance among coral reef fishes, are probably much more common phenomena than generally thought. Our studies indicate that hypoxia tolerance is widespread among, for example, damselfishes and cardinalfishes, and that it may be related to nocturnal hypoxia in their coral habitat or nocturnal hypoxia in tide pools. Our case study on a coral-dwelling goby indicated that hypoxia tolerance is a prerequisite for such fishes to stay in the shelter of their coral homes indefinitely.

REFERENCES

Beamish, F.W.H. 1964. Seasonal temperature changes in the rate of oxygen consumption of fishes. Canadian Journal of Zoology 42:189-194.

Butler, P.J., E.W. Taylor, and W. Davison. 1979. The effect of long term, moderate hypoxia on acid base balance, plasma catecholamines and possible anaerobic end products in the unrestrained dogfish *Scyliorhinus canicula*. Journal of Comparative Physiology B., 132:297-303.

Jones R.J. and O. Hoegh-Guldberg. 2001. Diurnal changes in the photochemical efficiency of the symbiotic dinoflagellates (Dinophyceae) of corals: photoreception, photoinactivation and the relationship to coral bleaching. Plant Cell Environment 24: 89-99.

Kinsey, D.W. and B.E. Kinsey. 1966. Diurnal changes in oxygen content of the water over the coral reef platform at Heron Island. Australian Journal of Marine and Freshwater Research 1:23-24.

Munday, P.L., M.J. Caley. and G.P. Jones. 1998. Bi-directional sex change in a coral-dwelling goby. Behavioral Ecology and Sociobiology 43:371-377.

Nilsson, G.E. and S. Öslund-Nilsson. 2003. A mouth full of eggs: respiratory consequences of mouthbrooding in cardinalfishes. In prep.

Nilsson, G.E., J.-P. Hobbs, P.L. Munday, and S. Östlund-Nilsson. 2003. Coward or braveheart: extreme habitat fidelity through hypoxia tolerance in a coral-dwelling goby. In review.

Renshaw, G.M.C. and S.E. Dyson. 1999. Increased nitric oxide synthase in the vasculature of the epaulette shark brain following hypoxia. NeuroReport 10:1707-1712.

Renshaw, G.M.C, C.B. Kerrisk, and G.E. Nilsson. 2002. The role of adenosine in the anoxic survival of the epaulette shark, *Hemiscyllium ocellatum*. Comparative Biochemistry and Physiology 131B:133-141.

Routley, M.H., G.E. Nilsson, and G.M.C. Renshaw. 2002. Exposure to hypoxia primes the respiratory and metabolic responses of the epaulette shark to progressive hypoxia. Comparative Biochemistry and Physiology 131A:313-321.

Schubert, M., P.L. Munday, M.J. Caley, G.P. Jones, and L.E. Llewellyn. 2003. The toxicity of skin secretions from coral-dwelling gobies and their potential role as a predator deterrent. Environmental Biology of Fishes. In press.

Söderström, V., G.M.C. Renshaw, and G.E. Nilsson. 1999a. Brain blood flow and blood pressure during hypoxia in the epaulette shark (*Hemiscyllium ocellatum*), a hypoxia tolerant elasmobranch. Journal of Experimental Biology 202:829-835.

Söderström, V., G.E. Nilsson, G.M.C. Renshaw, and C.E. Franklin. 1999b. Hypoxia stimulates cerebral blood flow in the estaurine crocodile (*Crocodylus porosus*). Neuroscience Letters 267:1-4.

Takegaki, T. and A. Nakazono. 1995. Responses of the egg-tending gobiid fish *Valenciennea longipinnis* to the fluctuation of dissolved oxygen in the burrow. Bulletin of Marine Science 65:815-823.

Verheyen, R., R. Blust, and W. Decleir. 1994. Metabolic rate, hypoxia tolerance and aquatic surface respiration of some lacustrine and riverine African cichlid fishes. Comparative Biochemistry and Physiology 107A:403-411.

Wise G., J.M. Mulvey, and G.M.C. Renshaw. 1998. Hypoxia tolerance in the epaulette shark *(Hemiscyllium ocellatum).* Journal of Experimental Zoology 281:1-5.

HYPOXIA AND PETROLEUM: EXTREME CHALLENGES FOR FISH OF THE AMAZON

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ABSTRACT

Natural changes in dissolved oxygen are related to changes in river water level and occur seasonally in the Amazon. Daily extreme variations in dissolved oxygen also occur. In *várzea* lakes, oxygen levels can drop to zero at night and reach saturation at noon the very next day. The fish of the Amazon have developed several adaptive strategies to deal with their complex environment, in particular with chronic hypoxia. These adaptive strategies include air-breathing, aquatic surface respiration, and adjustments of several physiological and biochemical parameters directed towards enhancing oxygen transfer to tissues. These adjustments can be considered as phylogenetic characteristics, developed during the evolutionary history of species and groups of species, or as adaptive characteristics, that have appeared in different groups during different times of evolutionary history as a response to the same environmental pressure, hypoxia for instance. Both air-breathing and aquatic surface respiration habits appeared several times during the evolution of fish, and can be considered as adaptations to the prevailing low oxygen conditions naturally occurring in water bodies of the Amazon. On the other hand, adjustments to chronically low oxygen tension have brought some fish groups of the Amazon to specializations in their metabolic characteristics and adjustments of physiological processes that can be considered as phylogenetic traits.

Currently, fish of some areas of the Amazon are exposed to a new challenge – petroleum. Near the Urucu River, in the central Amazon, crude oil is produced and transported in tankers down the Solimões River to Manaus to be refined. Strict safety protocols are observed, but nothing is failsafe. Many of the strategies the fish of the Amazon use to breath under hypoxia are useless when fish come in contact with petroleum. The first effect is the reduction of the ability of the animals to leave the polluted place, followed by an impairment of oxygen transfer to tissues caused mainly by the water soluble fraction of the petroleum. Internal hypoxia then takes place and elicits a series of adjustments such as air-breathing and aquatic surface respiration. The presence of an oil slick on the top of the water column reduces even further water oxygenation and makes these behavioral adjustments to improve oxygen transfer ineffective. This paper discusses the effectiveness of the strategies the fish of the Amazon have developed during their evolution as they face natural hypoxia and hypoxia caused by crude oil pollution.

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INTRODUCTION

Episodes of hypoxia and even anoxia occur naturally in aquatic environments of the Amazon basin. Excluding the main river courses, all other water bodies present both daily and seasonal changes in dissolved oxygen. Seasonal changes in dissolved oxygen have been related to river water level oscillations, the main environmental driving force in the region. A crest of 10 meters, occasionally more, occurs every year on the main channel in front of Manaus (Junk *et al.* 1989). The flooding pulse is not regular – its intensity and period of occurrence vary locally; for example, the flood pulse occurs in May with an amplitude of five meters in the Amazon River at Iquitos, in June/July with a crest of 10 meters in the Amazon River at the port of Manaus, and in August with a crest of 7.5 meters on average in the Orinoco River at Musinacio. This means that a significant part of the local basin is flooded during high water. This is the time of an intense propagation of water macrophytes in the *várzea* (floodplain areas of white water rivers) and oversized *igapós* (flooded forest of black water rivers). These habitats experience a significant oxygen deficit at the bottom of the water column during this time. During the low water period, the receding water leaves behind very poor conditions as regards dissolved oxygen, in addition to the presence of other dissolved gases produced by the decomposition of water plants, such as methane and hydrogen sulfide. Indeed, water level oscillations affect several other limnological parameters, such as water chemical composition, light penetration, photosynthesis, water body shape and depth, and temperature. All of these affect the amount of dissolved oxygen in one way or another.

In parallel to the seasonal changes in dissolved oxygen, extreme variations tend to occur in a very short period of time in the *várzea* lakes and *igapó* formations. Because these areas become rich in nutrients during the low water season, there are favorable conditions for photosynthesis during the flooded season, and so supersaturated levels of oxygen are often observed during the day and very low levels, or even anoxia, occur at night (Junk *et al*. 1983, Val 1996). During the low water season, a similar situation is observed, although decomposition consumes oxygen continuously and there is an increase in the levels of other dissolved gases.

Vertical micro-stratification of oxygen near the water surface takes place in many water bodies. In Amazonian waters, oxygen is concentrated in the top few millimeters of the water column; below 10 centimeters, in general, the oxygen levels are close to zero (Junk *et al.* 1983). For many aquatic animals, this is the sole source of oxygen. This makes the aquatic animals highly vulnerable since this stratification depends on diffusion of aerial oxygen and can be easily disrupted, particularly by anthropogenic activities. The mixed patterns of dissolved oxygen associated with temperatures ranging from 20^0 to 40^0C impose several challenges for fish living in these environments.

In contrast to naturally-occurring hypoxia, there are concerns about the effects of several human activities in the region (and in other parts of the world) that can make oxygen availability even worst. According to Diaz and Rosenberg (1995), hypoxia has increased in severity, frequency and number of affected areas, and is now considered among the most pressing water pollution problem in the world (Wu 1999). In predicting that hypoxia will get worse in the coming years, Wu (2002) pointed to three factors: a) the increase in world population will result in further increased loadings of nutrients into coastal waters; b) the use of fertilizers,

deforestation, and the creation of nitrogen oxides are likely to increase; and c) the increase in global temperature caused by greenhouse gases will warm surface waters. There are no reasons to exempt the Amazon basin from the effects of these trends. The Amazonian population has almost doubled during the last decade – there are nearly 20 million people living in the Brazilian Amazon today, almost two million in Manaus, Amazonas alone. Use of fertilizers, both in aquaculture facilities and in agriculture has likely increased in the Amazon as a way to feed the increasing population. Undoubtedly, deforestation is an important concern, though it is still restricted to the borders of the Amazon. Considering that the Amazon discharges 20% of all freshwater entering the oceans of the world, the impact of these local disturbances on marine hypoxia should be analyzed.

Among the human activities in the Amazon, petroleum production and transport cause concern. Large reserves of petroleum and gas are being exploited near the city of Coari along the Urucu River, a tributary of the Amazon River. The oil, about 20,000 barrels per day, is transported in oil barges to Manaus, nearly 700 kilometers down the river, to be refined (Petrobrás 1998). Although strict safety procedures are observed, there is always a risk of an oil spill both at the wellhead and during transportation. In fact, minor accidents have already been observed in the Amazon. Another risk is a possible rupture of the pipes that transport oil, as occurred at Cururu Lake, near Manaus, an accident that caused great environmental damage. These places are important sites to analyze the effects of crude oil on freshwater ecosystems. The effects of crude oil on freshwater bodies are unknown, despite the massive volume of information about marine and terrestrial environments (Atwood *et al.* 1987, Freedman 1989, Neff 1990, IIAP 1993, Paranhos and Ximenes 2000).

At a given temperature, petroleum compounds are found in gaseous form, known as natural gas, in liquid form, known as crude oil, and in solid or semisolid form, known as asphalt or tar. The water soluble fraction of these compounds is very toxic to aquatic organisms since it contains polyaromatic hydrocarbons. Crude oil also includes short chain hydrocarbons that are volatile and so have a short life in the aquatic environment. However, crude oil includes thousands of long chain hydrocarbons that persist on the top of the water column, as an oil slick, reducing or even eliminating air-water interaction. Such oil slicks shade the water column, reducing photosynthesis and worsening oxygen availability in the affected areas.

After any oil spill, a number of simultaneous processes - collectively known as weathering – natural processes that result in physical and chemical modifications of the original compounds present in the crude oil. Weathering of crude oil includes spreading, evaporation, dispersion, emulsification, biodegradation, dissolution, oxidation and sedimentation. The primary and secondary crude oil compounds affect aquatic organisms both directly, via physical and toxicological effects, and indirectly, via habitat modification, including changes in food availability, changes in competition rates, and changes in predation rates, among others (Malan 1988, Alkindi *et al.* 1996, Brauner *et al.* 1999, Santas *et al.* 1999). In the Amazon, these can be a real threat to fishes.

Since the appearance of the first fish species, dissolved oxygen has been an environmental problem. Dudley (1998), reviewing the levels of atmospheric oxygen over the different geological periods, reported lower levels of oxygen during the Devonian,

Carboniferous, Triassic and Jurassic periods that coincide with the appearance of the main groups of fish that inhabit the Amazon basin today – the lungfish, the bony tongues, and the Ostariophysans. These groups of fishes appeared during low oxygen periods and during their life history had to face episodes of low oxygen in their environment. To do so, these animals developed a myriad of adaptive strategies to exploit all available sources of oxygen or to conform to low oxygen availability. However, they did not pre-adapt to anthropogenic effects in their environment as, for example, the presence of crude oil atop the water column.

This paper reviews the strategies adopted by four fish species of the Amazon, *Colossoma macropomum*, *Arapaima gigas*, *Liposarcus pardalis* and *Astronotus ocellatus*, to deal with hypoxia. We then discuss how crude oil affects these animals and how the strategies they adopted during their evolution to deal with low dissolved oxygen actually increase their vulnerability in crude-oil-polluted environments.

TAMBAQUI, HYPOXIA AND CRUDE OIL

Colossoma macropomum, known locally as tambaqui, is endemic to the Amazon basin. The tambaqui fishery has decreased significantly during recent decades, with catches reduced from 15,000 tons in 1972 to 800 tons in 1996, a consequence of over-exploitation according to Merona and Bittencourt (1988) and Isaac and Ruffino (1996). A hardy nature, rapid growth rate, and ability to tolerate poor water quality make tambaqui a favorite cultured species in many parts of South America. The fish can grow to 1m and can weigh up to 30 kg. *Colossoma* is among the most famous fruit-eating fish. The young fish filter plankton, but the adults eat mainly fruit during the flooded season, using powerful jaws that crush fruits and hard seeds. Because it is a migratory fish, this habit helps to disperse seeds throughout the flooded forest (Araújo-Lima and Goulding 1998).

The hardy nature of tambaqui is related to its hypoxia-tolerance; this species can survive low oxygen levels, near 10% air-saturation. Beyond this point, tambaqui start to breath at the water surface, *i.e.*, under environmental hypoxia the animal comes to the surface and skims the well-oxygenated surface layer of water (reviewed by Val and Almeida-Val 1995). To improve its uptake of oxygen, the animal expands its inferior lips to form a funnel that directs the surface water across the gills. The lips are not involved with gas exchange; they serve strictly to facilitate skimming of the water surface (Braum and Junk 1982, Val and Almeida-Val 1995). As the oxygen concentration returns to normal levels, the lip swelling disappears in about the same time that was required for the expansion, *ca*. two hours. Undoubtedly, this mechanism provides tambaqui and related fish species, such as *Mylossoma* and *Brycon*, an important alternative to ventilation in hypoxic waters.

Under natural hypoxic conditions, *i.e.*, below 15% air saturation, more than 80% of captured specimens of tambaqui presented expanded lips (Val and Almeida-Val, 1995) (Figure 1). Most of the lip-expanded individuals were collected during the night, when oxygen often drops to zero below the top few millimeters of surface water. Under experimental conditions, cannulated tambaqui exposed to hypoxia with access to the water surface for two hours developed no changes in blood oxygenation, compared to normoxia-exposed animals under the

same experimental conditions. However, if access to the water surface was denied, the animals exposed to hypoxia expanded the lips but a massive drop of blood oxygenation occurred (Figure 2). This decrease in blood oxygen was even more drastic if the top of the water column was contaminated with crude oil – blood oxygen in this case decreased to 20% of saturation (Figure 2) and the animal did not survive if it was not transferred to air-saturated water. Interestingly, blood oxygen decreased much faster in the animals exposed to crude oil compared to hypoxia-surface-denied animals, and they did not recover as well as the hypoxia-surface-denied fishes. This suggests that crude oil impairs other mechanisms of oxygen transfer, or even results in higher oxygen consumption.

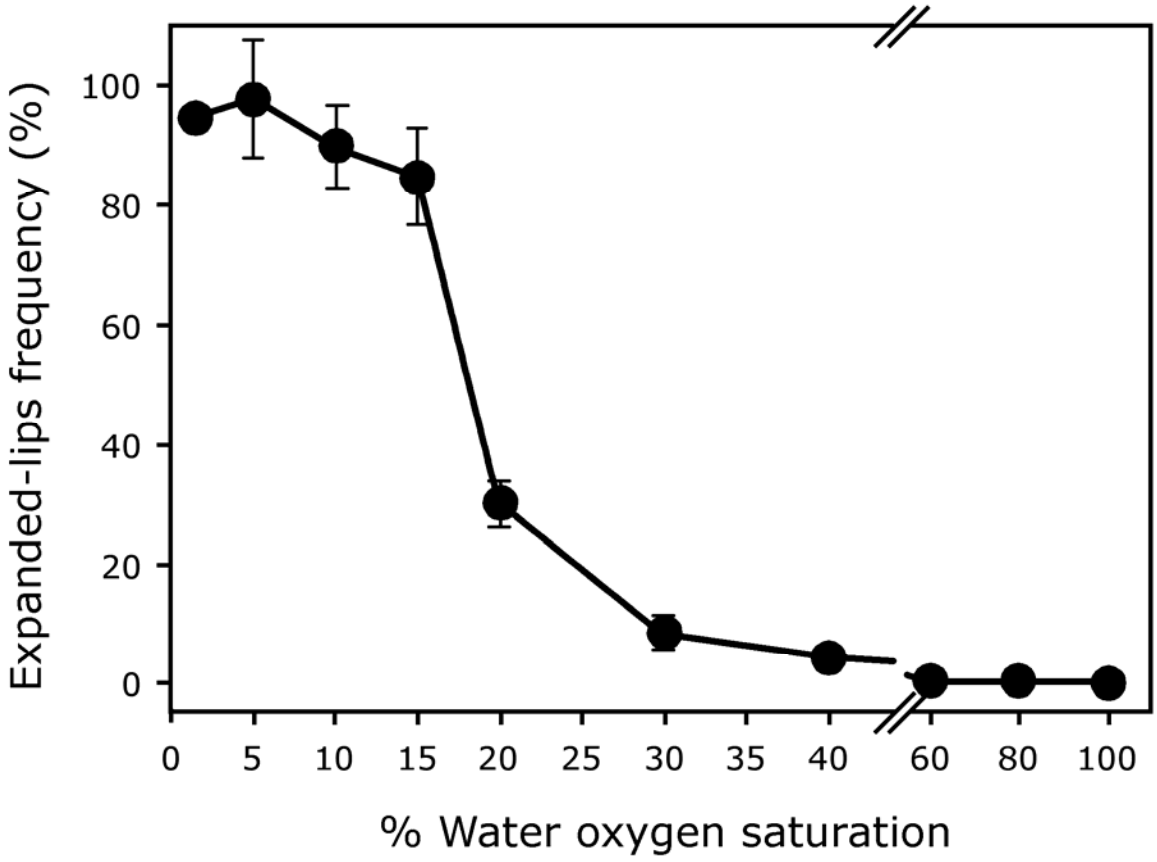

Figure 1. Frequency of specimens of *Colossoma* with expanded lips over different levels of dissolved oxygen. N=20.

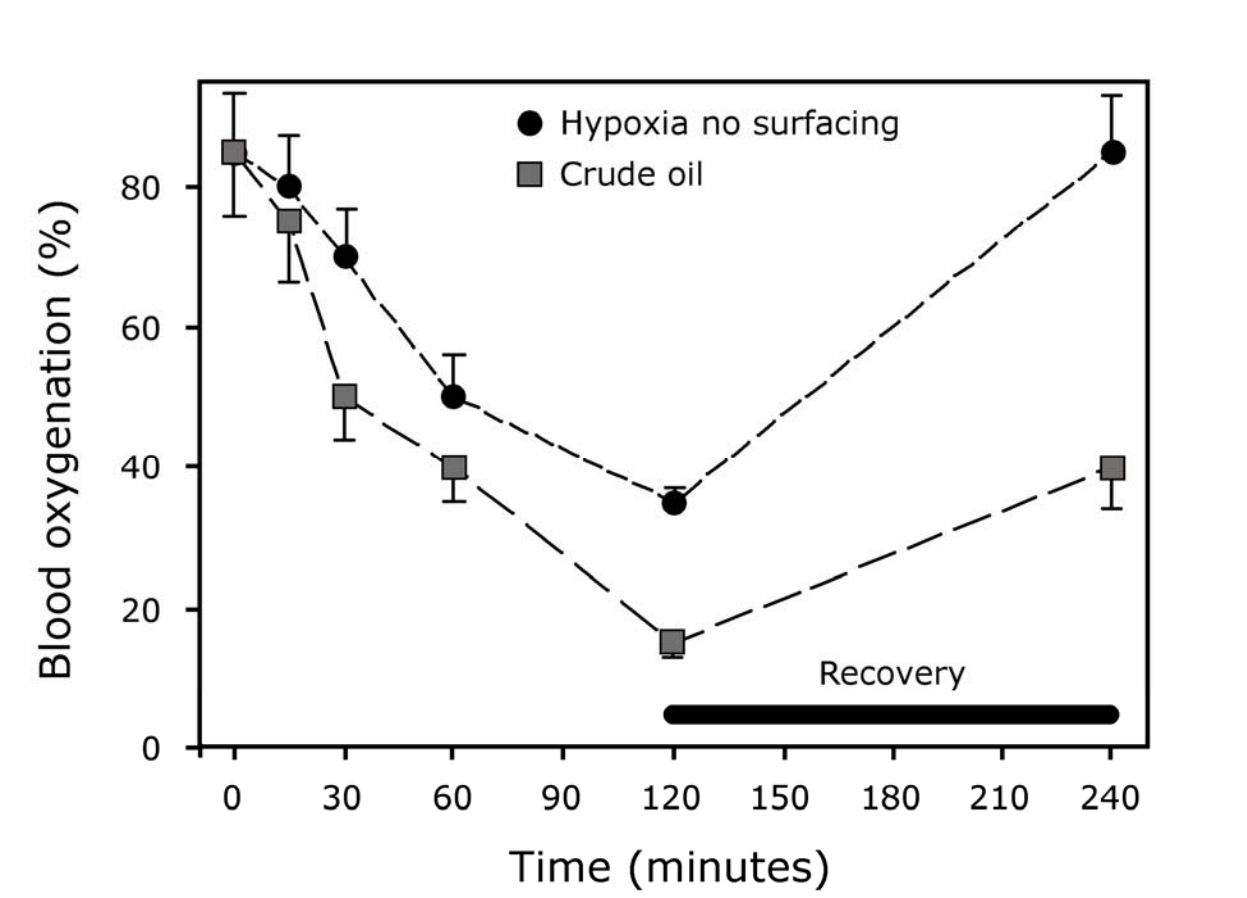

Figure 2. Blood oxygenation of specimens of *Colossoma* exposed to hypoxia and to crude oil over time. Observe that animals exposed to crude oil do not recover as well as animals exposed to hypoxia alone.

In fact, two other changes were evidenced. First, animals exposed to crude oil took up a massive amount of oil compounds into the gall bladder. Gall bladder somatic index (GBSI) increased from values close to 0.05% to values above 1% of the body weight in animals exposed to crude oil for 96 hours. Intriguingly, in animals exposed to crude oil for 340 hours, the GBSI returned to normal levels, suggesting that the gall bladder empties its content into the intestine. That causes no problems to this species, but would represent a challenge to other species that use the stomach and intestine as an accessory air-breathing organ. Second, animals exposed to crude oil experienced a massive increase in met-hemoglobin levels. Met-hemoglobin is unable to reversibly bind oxygen, and so its increased levels result in decreased tissue oxygenation.

ACARI-BODÓ, HYPOXIA AND CRUDE OIL

Liposarcus pardalis, known locally as acari-bodó, is an armored catfish from the family Loricariidae, widely distributed across the Amazon basin. This family includes facultative air-breathing species that use a vascularized stomach and intestine for gas exchange. When facing low dissolved oxygen environments, *Liposarcus* swims to the water surface and gulps air. Mixed with water, this is forced through the digestive system, where oxygen is taken up through the stomach/intestine. This adaptation includes a behavioral change, since the animals exhibit circular movements at regular intervals from the bottom to the water surface (Gradwell 1971). Air-gulping contributes as much as 70% of blood oxygen in animals exposed to hypoxia, as described by Val (1995), suggesting that this species is highly dependent on this adaptation when exposed to poorly oxygenated environments.

During an oil spill, facultative air-breathers such as *Liposarcus* may be subjected to acute toxicity from the oil slick, especially if they are exposed to hypoxia. In this case, the animal gulps and swallows large amounts of crude oil, together with water and air. Ingested crude oil binds to the intestinal epithelium, increasing the diffusion distance by accumulating over the epithelium and causing local edema. Consumption of crude oil contaminated pellets resulted in ultrastructural abnormalities in the intestine of *Oncorhynchus tshawytscha* (Hawkes *et al*. 1980) and some degree of ion regulatory impairment in *Hoplosternum littorale* (Brauner *et al.* 1999). In herring gull chicks, ingestion of crude oil impaired Na^+,K^+ ATPase activity of the nasal salt gland leading to an osmoregulatory impairment (Miller *et al*. 1977).

Following forced crude oil ingestion, *Liposarcus* developed ultrastructural abnormalities in the intestine that resulted in increased diffusion distance (Costa and Farias 1996), impairing oxygen uptake. In fact, following forced crude oil ingestion, *Liposarcus* exhibited a significant increase in hematocrit, hemoglobin levels and circulating red blood cells (Table 1). These changes plus decreased levels of red blood cell ATP and GTP, negative allosteric modulators of Hb-O_2 affinity, are all directed towards an increase in oxygen transfer. This condition imposes some stress to the animals, as evidenced by levels of plasma glucose that are about twice the levels estimated for control animals (Table 1). Thus, it is likely that animals facing internal hypoxia caused by the impairment of oxygen uptake at the air-breathing site make forays to the water surface to gulp air more frequently, which results in even more severe crude oil uptake to the digestive tract, further impairing oxygen transfer from the environment to tissues.

Table 1. Selected blood variables of *Liposarcus* specimens exposed to crude oil. Values marked with an asterisk significantly ($P<0.05$) differ from control.

	Control	Crude oil
Hematocrit (%)	24.6±3.3	33.3±2.1*
Hemoglobin (g/dL)	5.36±0.46	7.46±0.44*
RBC (x 10^6) (cells/mm^3)	1.16±0.10	1.94±0.18*
Glucose (mg/dL)	40.2±3.1	73.5±4.6*
ATP (mM)	2.6±0.2	1.2±0.1*
GTP (mM)	1.5±0.1	0.5±0.04*

The crude oil taken in elicits other adjustments to reduce its toxicity. The liver is an important organ involved in the detoxification of xenobiotics and many toxic compounds accumulate in this organ to harmful levels, causing pathological alterations. Ultra-structural liver alterations have been reported for several fish species exposed to heavy metals in their environment (Koyama *et al.* 1979, Khangarot 1992, Yang and Chen 2003). Liver injury is dependent upon the nature of the toxicant and the length of exposure. Specimens of *Liposarcus* exposed to crude oil produced significantly increased activities of alkaline phosphatase (ALP) and aspartate transaminase (AST) (Table 2), suggesting hepatocellular injury, albeit these enzymes are present in several tissues. So, in addition to the oxygen transfer impairment, crude oil may also cause liver impairment in this fish species.

Table 2. Liver enzyme levels (IU/ml) in *Liposarcus* specimens exposed to crude oil. Values marked with an asterisk significantly ($P<0.05$) differ from control. Crude oil 1 – 120 ml of oil in 30 liters of water (~0.16 mm of oil atop the water column), Crude oil 2 – 480 ml of oil in 30 liters of water (~0.64 mm of oil atop the water column). N=8 for each treatment. Source: Val & Pontes (unpublished data).

	Alkaline Phosphatase (ALP)	Aspartate Transaminase (AST)
Control	0.55±0.07	228±45
Crude oil 1	1.04±0.14*	348±54*
Crude oil 2	1.38±0.14*	506±96*

PIRARUCU AND CRUDE OIL

Arapaima gigas, locally known as pirarucu, is one of the largest freshwater fish in the world. This fish grows up to three meters in length and weighs up to 300 kg. As juveniles, up to 7-10 cm in length, *A. gigas* are obligate water-breathers; as adults they are obligate air-breathers and may drown within 10 minutes without access to air. This transition from water to air-breathing is followed by changes in gill morphology and ultrastructure. In 10g juveniles, the secondary lamellae are well-developed, typical of water-breathing fish, while in larger animals (> 600 g) the secondary lamellae are completely absent (Brauner *et al*. in press). The transition is also followed by a replacement of red blood cell ATP and GTP by IPP (inositol pentaphosphate), a much stronger modulator of hemoblobin-oxygen affinity (Val *et al.* 1992). In adults of *A. gigas*, about 80% of oxygen is taken-up at the well-vascularized swim-bladder and 20% at the gills, while carbon dioxide is preferentially excreted at the gills (~78%) and the rest at the swim-bladder (~15%) and kidney (~7%) (Brauner and Val 1996). So, adults of *Arapaima* are dependent on air uptake through the modified swim-bladder to transfer oxygen to tissues.

In the presence of crude oil atop the water column, individuals of *Arapaima* reduce their forays to the water surface to take-up air by 50%, *i.e.*, air-breathing increases from 5.2 minute intervals under normal conditions to 7.8 minutes for crude-oil-exposed animals (Table 3). Interestingly, as soon as the animals detect the presence of a crude oil slick atop the water column, they start searching for the best place to get through the oil slick. At this point some oil binds to the buccal and gill epithelia and the animals desperately try to clean these surfaces, "spitting out" part of the crude oil taken-in. Although no changes in glucose and lactate are observed (Table 3), this situation requires some hematological adjustments. Hematocrit, circulating red blood cells and hemoglobin concentration are all increased in crude oil exposed animals, suggesting that some internal hypoxia occurs. So, the only way this fish species can reduce the effects of crude oil is by initially increasing air-breath intervals that, in fact, is a poor solution since the animal starts to experience internal hypoxia. Crude oil pollution definitely represents an extreme challenge for this air-breathing fish species.

Table 3. Selected blood parameters and breathing cycle in *Arapaima* exposed to crude oil for three hours. Values marked with an asterisk significantly ($P<0.05$) differ from control. Source: Val (unpublished data).

	Control (n=7)	Crude oil (n=5)
Hematocrit (%)	31.0±1.9	39.5±0.4*
Hemoglobin (g/dL)	5.51±1.71	8.62±0.73*
RBC (x 10^6) (cells/mm^3)	1.92±0.17	2.47±0.13*
Glucose (mg/dL)	55.8±6.9	53.0±7.6
Lactate (mM)	1.79±0.41	1.77±0.10
Breathing cycle (min)	5.2±0.5	7.8±1*

OSCAR, HYPOXIA AND CRUDE OIL

The cichlids (Order Perciformes) are among the most advanced teleosts inhabiting the Amazon basin. They are highly specialized fish, present a high degree of adaptive radiation (reviewed by Val and Almeida-Val 1995), and are considered to have a higher evolutionary rate than their African counterparts (Farias *et al.* 1999). Most Amazonian cichlids that have been studied are hypoxia tolerant. The differences among them depend on their ability to increase anaerobic metabolism and suppress metabolic rates. The cichlid *Astronotus ocellatus* (Oscar) can be considered one of the most hypoxia tolerant fishes in nature (Muusze *et al.* 1998). This ability to tolerate low levels of dissolved oxygen, or no oxygen at all, in adult *Astronotus* results from a combination of anaerobic power and metabolic depression (Almeida-Val *et al.* 1999). Young Oscar individuals practice aquatic surface respiration. However, individuals of this

species reduce the number of incursions to the water surface and increase their anaerobic glycolytic power as they grow by increasing their mass-specific lactate dehydrogenase (LDH) levels resulting in an increase in hypoxia survivorship (Almeida-Val *et al.* 1999, Almeida-Val *et al*. 2000) (Figure 3).

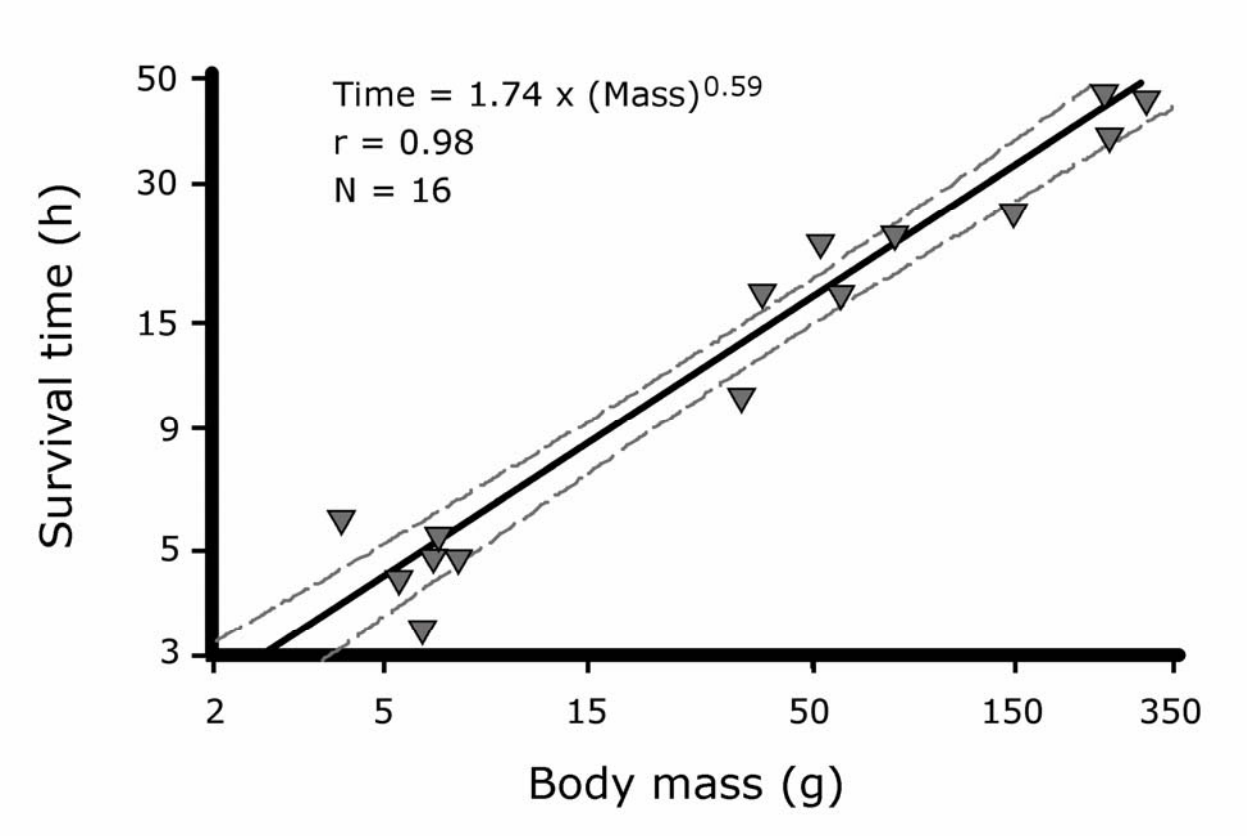

Figure 3. Relationship between body mass and survival time in Oscars exposed to hypoxic conditions ($[O_2]$=30 mmHg). Data from Almeida-Val *et al.* (1999) and Almeida-Val *et al*. (2000).

LDH isoforms are encoded by a family of genes in all vertebrates. Tissue expression of these isoforms in fish changes according to environmental oxygen levels. The relative proportion of LDH isoforms in the brain of Oscar changes when the animal is exposed to low oxygen for long periods. These changes are related to the size of the animals (Almeida-Val *et al.* 1995, Almeida-Val *et al.* 1999). The ability of some fish species to regulate the expression of enzymes and isozymes is important for maintaining metabolic balance when the animal faces environmental challenge. As already mentioned, hypoxia is a continuous challenge for fish of the Amazon. Under hypoxia, those fish species unable to breathe air improve oxygen uptake from water and adjust their anaerobic capacity accordingly. The key point for this adjustment is LDH gene regulation. In fact, the LDH-A gene (muscle type) is regulated differently in young and adult animals. LDH-A expression is suppressed when an adult Oscar is exposed to anoxia.

On the other hand, juveniles activate LDH-A expression in heart and muscle. This suggests that the young animals depend mostly on their anaerobic capacity, while adults rely on metabolic depression to survive anoxia (Oliveira *et al.* submitted). Most likely, the exposure to continuous hypoxia during evolution resulted in a reduction of metabolic rates in Oscar at the whole-organism level compared to other fish species like tilapia and tambaqui (Almeida-Val *et al.* 1999).

With regard to petroleum exposure, compared to air-breathing species the Oscar has an advantage since its hypoxia tolerance allows the animals to suppress their metabolism and become less vulnerable to crude oil. This may be the reason the Oscar presents one of the highest LC_{50} values for crude oil (Table 4).

Table 4. Crude oil LC_{50} for selected fish species of the Amazon under normoxia. Sources: [1]Almeida-Val & Oliveira (unpublished data); [2]Almeida-Val & Cunha (unpublished data); [3]Paula-Silva & Vergueiro (unpublished data).

	Crude oil LC_{50} (ml/L)
Astronotus[1]	36.33
Colossoma[2]	>333.33
Hoplosternum littorale[3]	16.96

CONCLUDING REMARKS

Organisms are expected to respond to novel events as if to familiar events. Hypoxia is a familiar event in the Amazon and so fish respond to it with adaptations shaped over their existence, but in the case of crude oil spills these adaptations play against the animals since this is a new, extreme challenge.

ACKNOWLEDGMENT

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REFERENCES

Alkindi, A.Y A., J.A. Brown, C.P. Waring, and J.E. Collins. 1996. Endocrine, osmoregulatory, respiratory and haematological parameters in flounder exposed to the water soluble fraction of crude oil. Journal of Fish Biology 49:1291-1305.

Almeida-Val, V.M.F., I.P. Farias, M.N.P. Silva, W.P. Duncan, and A.L. Val. 1995. Biochemical adjustments to hypoxia by Amazon cichlids. Brazilian Journal of Medical and Biological Research 28:1257-1263.

Almeida-Val, V.M.F., M.N. Paula-Silva, W.P. Duncan, N.P. Lopes, A.L. Val, and S.C. Land. 1999. Increase of anaerobic potential during growth of an Amazonian cichlid, *Astronotus ocellatus*. Survivorship and LDH Regulation after Hypoxia Exposure. *In:* Biology of Tropical Fishes. A.L. Val and V.M.F. Almeida-Val (eds.). INPA, Manaus, AM. pp. 437-448.

Almeida-Val, V.M.F., A.L. Val, W.P. Duncan, F.C.A. Souza, M.N. Paula-Silva, and S. Land. 2000. Scaling effects on hypoxia tolerance in the Amazon fish *Astronotus ocellatus* (Perciformes, Cichlidae): contribution of tissue enzyme levels. Comparative Biochemistry and Physiology 125B:21-226.

Araújo-Lima, C., and M. Goulding. 1998. So Fruitful a Fish: Ecology, Conservation and Aquaculture of the Amazon's Tambaqui. Columbia University Press.

Atwood, D.K., F.J. Burton, J.E. Corredor, G.R. Harvey, A.J. Mata-Jiminez, A. Vasquez-Botello, and B.A. Wade. 1987. Results of the CARIPOL petroleum pollution monitoring project in the wider Caribbean. Marine Pollution Bulletin 18:540-548.

Braum, E., and W.J. Junk. 1982. Morphological adaptation of two Amazonian characoids (Pisces) for surviving in oxygen deficient waters. International Revue ges Hydrobiology 67:869-886.

Brauner, C.J., C.L. Ballantyne, M.M. Vijayan, and A.L. Val. 1999. Crude oil affects air-breathing frequency, blood phosphate levels and ion regulation in an air-breathing teleost fish, *Hoplosternum littorale*. Comparative Biochemistry and Physiology 123C:127-134.

Brauner, C. J., V. Matey, J. M. Wilson, N. J. Bernier, and A. L. Val. In press. Transition in organ function during the evolution of air-breathing; Insights from *Arapaima gigas*, an obligate air-breathing teleost from the Amazon. Journal of Experimental Biology.

Brauner, C.J., and A.L. Val. 1996. The interaction between O_2 and CO_2 exchange in the obligate air breather, *Arapaima gigas*, and the facultative air breather *Liposarcus pardalis*. *In:* Physiology and Biochemistry of the Fishes of the Amazon. A.L. Val, V.M.F. Almeida-Val, and D. J. Randall (eds.). INPA, Manaus. pp. 101-110.

Costa, O.T.F., and A.R.L. Farias. 1996. Histopathology of gills and accessory air-breathing organ of *Lipossarcus pardalis* exposed to crude oil. *In:* The Physiology of Tropical Fishes. International Congress on the Biology of Fishes. A.L. Val, D.J. Randall, and D. MacKinlay (eds.). American Fisheries Society, San Francisco. pp. 85-88.

Diaz, J., and R. Rosenberg. 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioral responses of benthic macrofauna. Oceanography and Marine Biology Annual Review 33:245-303.

Dudley, R. 1998. Atmospheric oxygen, giant Paleozoic insects and the evolution of aerial locomotor performance. The Journal of Experimental Biology 201:1043-1050.

Farias, I.P., G. Ortí, I. Sampaio, H. Schneider, and A. Meyer. 1999. Mitochondrial DNA phylogeny of the family Cichlidae: Monophyly and fast molecular evolution of the neotropical assemblage. Journal of Molecular Evolution 48.

Freedman, B. 1989. Environmental Ecology. The Impacts of Pollution and Other Stresses on Ecosystem Structure and Function. Academic Press, San Diego.

Gradwell, N. 1971. A photographic analysis of the air breathing behavior of the catfish, *Plecostomus punctatus*. Canadian Journal of Zoology 49:1089-1094.

Hawkes, J.W., E.H. Gruger, and O.P. Olson. 1980. Effects of petroleum hydrocarbons and chlorinated biphenyls on the morphology of the intestine of chinook salmon (*Oncorhynchus tshawytscha*). Environmental Research 23:149-161.

IIAP. 1993. Estudio de los impactos de la contaminación sobre los ecosistemas acuáticos y el hombre de la Anazonía Peruana - Alternativas de solución. IIAP, Proyecto de Investigación para la Protección del Medio Ambiente. Iquitos, Perú. 41 pp.

Isaac, V., and M. Ruffino. 1996. Population dynamics of Tambaqui *Colossoma macropomum* Cuvier 1818, in the Lower Amazon, Brazil. Fisheries Management and Ecology 3:315-333.

Junk, W.J., P.B. Bayley, and R.E. Sparks. 1989. The flood pulse concept in river-floodplain systems. *In:* Proceedings of the International Large River Symposium. D.P. Dodge (ed.). Canadian Special Publication of Fisheries and Aquatic Science, Canada 106:110-127

Junk, W.J., M.G. Soares, and F.M. Carvalho. 1983. Distribution of fish species in a lake of the Amazon River floodplain near Manaus (lago Camaleao), with special reference to extreme oxygen conditions. Amazoniana 7:397-431.

Khangarot, B.S. 1992. Copper-induced hepatic ultrastructural alterations in the snake-headed fish, *Channa punctatus*. Ecotoxicology and Environmental Safety 23:282-293.

Koyama, F., M. Fujita, and Y. Itayawa. 1979. Effects of oral administration of cadmium on fish. IV. Effects on ultrastructure of hepatic and renal cells of carp and progy. Bulletin of Japanese Society of Scientific Fisheries 45:429-436.

Malan, D.E. 1988. The effects of Qatar light crude oil on the saltmarsh crab *Sesarma catenata* and its implications in the field: toxicity to adults and larvae. South African Journal of Marine Sciences 7:37-44.

Merona, B., and M. Bittencourt. 1988. A Pesca na Amazônia através dos Desembarques no Mercado de Manaus: Resultados Preliminares. Memoria Sociedad de Ciencias Naturales La Salle XLVIII:433-453.

Miller, D.S., D.B. Peakall, and W.B. Kinter. 1977. Ingestion of crude oil: sublethal effects in herring gull chicks. Science 199:315-317.

Muusze, B., J.L. Marcon, G. Van den Thillart, and V.M.F. Almeida-Val. 1998. Hypoxia tolerance of Amazon fish. Respiratory and energy metabolism of the cichlid *Astronotus ocellatus*. Comparative Biochemistry and Physiology 120A:151-156.

Neff, J.M. 1990. Composition and fate of petroleum and spill-treating agents in the marine environment. *In:* Sea Mammals and Oil: Confronting Risks. J.R. Geraci and D.J. St Aubin (eds.). Academic Press, London. pp. 1-32.

Oliveira, A.R., A.L. Val, N.P. Lopes, A.R. Chippari-Gomes, M.N. Paula-Silva, C.F.M. Menck, and V.M.F. Almeida-Val. Anoxia- and hypoxia-induced expression of LDH-A in Oscar (*Astronotus crassipinis*). The Journal of Experimental Biology. (Submitted)

Paranhos, R., and M. S. Ximenes. 2000. Verão Negro no litoral carioca. Ciência Hoje 27:48-49.

Petrobrás. 1998. Concluída a primeira fase do AM-028. Jornal Exploração e Produção Petrobrás AM 4.

Santas, R., D.P. Hader, and P. Santas. 1999. Is crude oil bioremediation affected by changes in ambient ultraviolet radiation? Marine Pollution Bulletin 38:1022-1025.

Val, A.L. 1995. Oxygen transfer in fish: morphological and molecular adjustments. Brazilian Journal of Medical and Biological Research 28:1119-1127.

Val, A.L. 1996. Surviving low oxygen levels: Lessons from fishes of the Amazon. *In:* Physiology and Biochemistry of the Fishes of the Amazon. A.L. Val, V.M.F. Almeida-Val, and D.J. Randall (eds.). INPA, Manaus pp. 59-73.

Val, A.L., E.G. Affonso, R.H.S. Souza, V.M.F. Almeida-Val, and M.A.F. Moura. 1992. Inositol pentaphosphate in erythrocytes of an Amazonian fish, the pirarucu (*Arapaima gigas*). Canadian Journal of Zoology 70:852-855.

Val, A.L., and V.M.F. Almeida-Val. 1995. Fishes of the Amazon and their Environments: Physiological and Biochemical Features. Springer Verlag, Heidelberg. 232pp.

Wu, R. 1999. Eutrophication, trace organics and water-borne pathogens: pressing problems and challenge. Marine Pollution Bulletin 39:11-22.

Wu, R. 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Pollution Bulletin 45:35-45.

Yang, J.L., and H.C. Chen. 2003. Serum metabolic enzyme activities and hepatocyte ultrastructure of common carp after gallium exposure. Zoological Studies 42:455-461.

REGULATION OF BLOOD HEMOGLOBIN CONCENTRATION IN HYPOXIC FISH

Mikko Nikinmaa and Virpi Tervonen[1]

ABSTRACT

A common response to hypoxic conditions in fish is an increase in blood hemoglobin concentration. Three major factors appear responsible for this increase that has both rapid (minutes-hours) and longer-term (hours-days) components. The rapid response involves liberation of preformed erythrocytes, especially from the spleen, and a reduction of plasma volume. Ongoing experiments suggest that the reduction of plasma volume is caused by an increase in plasma cardiac peptide concentration and consecutive diuresis. The longer-term increase in hemoglobin concentration appears to be caused by the production of new erythrocytes in the kidney. While the mechanism of erythropoiesis in all vertebrates appears similar involving hypoxia-inducible factor and erythropoietin, no published data are currently available about upregulation of erythropoietin gene expression in hypoxic fish.

INTRODUCTION

A common response of fish and other vertebrates to hypoxic conditions is an increase in blood hemoglobin concentration (Grant and Root 1952, Nikinmaa 1990). Increased blood hemoglobin concentration helps the loading of oxygen in the respiratory epithelia despite its decreased availability. Adjustment of hemoglobin concentration is achieved primarily by adjusting the number of circulating erythrocytes per unit volume of blood, although it is possible for fish erythrocytes to produce hemoglobin while in circulation (Speckner *et al.* 1989, Lund *et al*. 2000). The increase in the number of circulating erythrocytes and hemoglobin concentration can be divided into two temporal components (Figure 1). Rapid adjustments of hemoglobin concentration can be caused by liberation of erythrocytes from storage organs, mainly the spleen, and by adjustments of plasma volume. Long term production of red blood cells is the result of hypoxia-induced activation of erythropoiesis. The purpose of this paper is to evaluate the current knowledge about the role of these different mechanisms in adjusting erythrocyte number per unit volume and comment on the plausible regulatory mechanisms involved.

FACTORS AFFECTING THE AMOUNT OF OXYGEN AVAILABLE TO TISSUES

Other factors in addition to the red cell number (and their hemoglobin concentration) affect the availability of oxygen in tissues. In hypoxic conditions, the oxygen affinity of hemoglobin is clearly increased (Weber 1982, Weber and Jensen 1988, Jensen *et al*. 1993, Nikinmaa 2001); this finding pertains both to interspecific differences between species inhabiting oxygen-rich and oxygen-poor environments, and intraspecific responses to hypoxia. The increase in oxygen affinity will increase the amount of oxygen loaded in the gills. The

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venous reserve of oxygen bound to hemoglobin is also associated with the oxygen equilibrium curve. In normoxic conditions hemoglobin remains approximately 50% saturated with oxygen. However, this venous reserve is markedly reduced in hypoxia, *e.g.*, 24 h hypoxia (oxygen tension ca. 6 kPa at 18°C) in rainbow trout decreased the venous oxygen saturation of hemoglobin from 51.5% to 22.5%. Consequently, oxygen utilization was barely affected despite the decrease in arterial oxygen saturation (Figure 2). Capillary density within oxygen-requiring tissue and blood flow via the capillaries in hypoxia also markedly affect tissue oxygen availability. Notably, formation of blood vessels (angiogenesis) is one of the most characteristic responses occurring in hypoxic conditions (Giordano and Johnson 2001, Wagner 2001, Maxwell and Ratcliffe 2002). Furthermore, local oxygen level appears to be one of the factors controlling the opening and closing of precapillary sphincters and thus directing the flow of erythrocytes to different capillary beds. In fish, hypoxic rearrangements of blood flow have so far been unequivocally described for gill vasculature (Sundin and Nilsson 1997). At least partially as a result of vascular rearrangements, the proportion of circulating erythrocytes varies markedly between different sites. Such variability, and its adjustments during hypoxic conditions, influences both the oxygen delivery to the tissues and the viscosity of blood in the tissue.

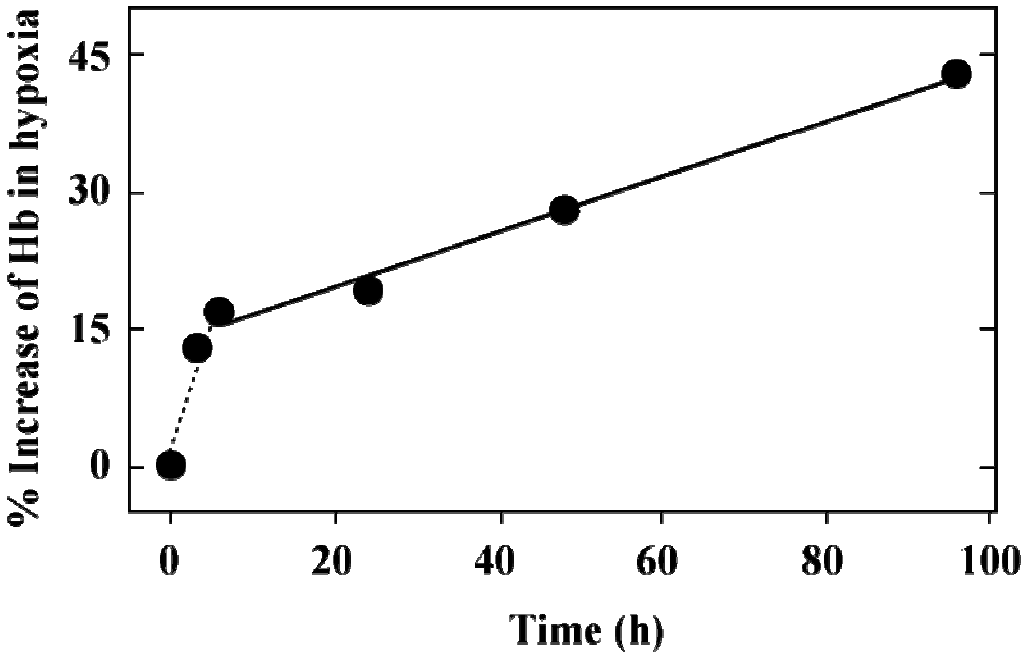

Figure 1. The % increase of hemoglobin concentration of hypoxic (6 kPa oxygen at 11°C), chronically cannulated rainbow trout as compared to normoxic (>16 kPa oxygen) rainbow trout sampled with similar frequency. The increase has two components, rapid (dotted line, occurring within 6 h hypoxia) and more sustained (solid line, continuing for at least 4 days). Original data from Soivio *et al.* (1980).

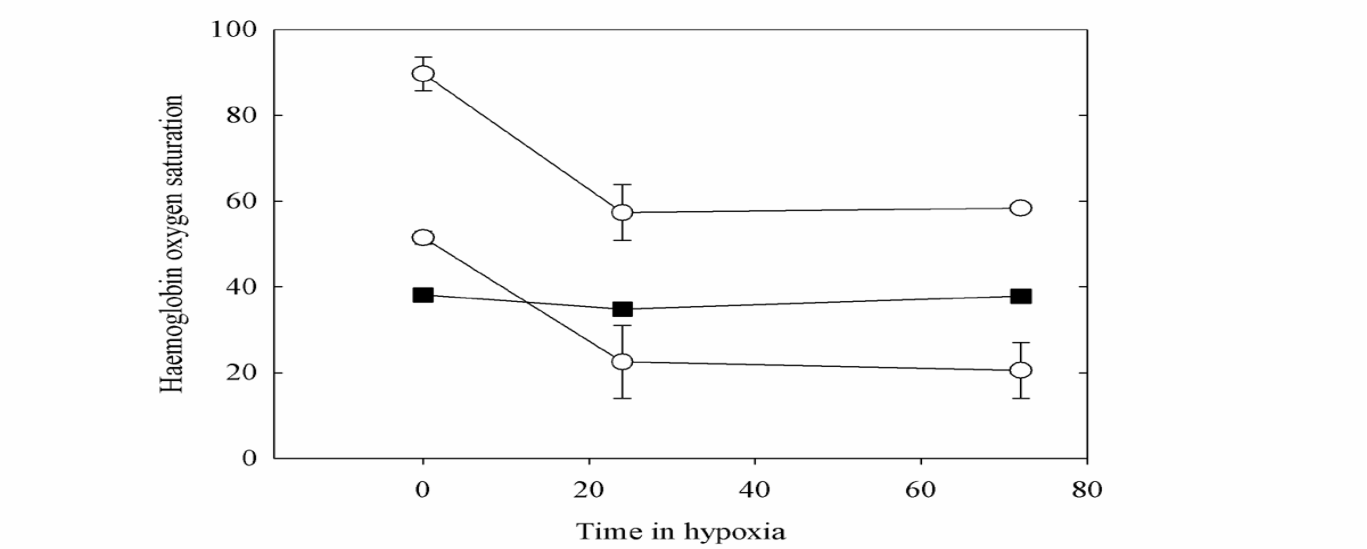

Figure 2. The hemoglobin oxygen saturation of rainbow trout in dorsal (upper line) and ventral aorta (lower line) in normoxia (0, >16 kPa oxygen at 18°C) and at 24 and 72 h hypoxia (6 kPa oxygen), N = 5. The oxygen saturation of both dorsal and ventral aortic blood was significantly decreased between normoxia and 24 h hypoxia (Wilcoxon matched-pairs, signed-ranks test). The decrease in ventral aortic oxygen saturation from ca. 50% to ca. 20% indicates the pronounced reduction of venous oxygen reserve. Largely owing to the decrease in venous oxygen reserve, the percentage utilization of oxygen (black squares) remained unaltered between normoxia and hypoxia. Original data from Nikinmaa and Soivio (1982).

RAPID RESPONSES OF ERYTHROCYTE NUMBER TO HYPOXIC CONDITIONS

Spleen Contraction

In many species of fish, the spleen is the primary storage site of erythrocytes (Gallaugher and Farrell 1998). Liberation of erythrocytes from the spleen is under adrenergic nervous or humoral control (Nilsson 1983). Thus, erythrocyte liberation can occur very rapidly, and is known to take place as a response to both exercise and hypoxia (Yamamoto *et al.* 1980, Yamamoto *et al*. 1983, Yamamoto 1987, Gallaugher and Farrell 1998). Although splenic contraction can play a role in hypoxic hemoconcentration, its importance between species varies markedly. Furthermore, season, and possibly temperature, have significant effects on spleen size (Gallaugher and Farrell 1998), although the influence of these factors on splenic contraction and consecutive changes in erythrocyte number have not been investigated.

Reduction of Plasma Volume

The pioneering study by Swift and Lloyd (1974) showed that in acute hypoxia the urine flow rate of rainbow trout increased simultaneously with an increase in blood hematocrit value. The increased excretion of water causes a reduction in plasma volume, as is observed in rainbow trout using plasma volume measurements with dye dilution technique (Figure 3). A decrease in plasma volume will increase the number of erythrocytes per unit volume, and since cardiac output remains unaltered in hypoxia (Holeton and Randall 1967), will cause an increased flux of erythrocytes (and oxygen) through the circulation system. However, while increased urine flow rate and associated decrease in plasma volume are commonly observed in hypoxic vertebrates, the mechanism for these changes has remained unclear. One possible mechanism for regulating urine flow in hypoxia is an increased release of cardiac peptides. Atrial natriuretic peptide (ANP) release from the heart increases in hypoxia/anoxia (Skvorak *et al.* 1996), and hypoxia increases ANP gene expression (Chun *et al.* 2003). Since the recently cloned cardiac peptide of salmonids (sCP) is a volume-regulating hormone causing diuresis (Tervonen *et al.* 2002), an increase in its release during acute hypoxia could result in the observed response. Our ongoing experiments with rainbow trout have shown that cardiac peptide release does indeed increase in hypoxic conditions (Figure 4) simultaneously with diuresis. The mechanism by which the increase in the peptide release occurs in hypoxia is not clear at the moment, but could be either a result of direct effects of hypoxia, or increased stretch of the heart in hypoxia. Hypoxic exposure is commonly associated with bradycardia (Hughes 1973). Since the cardiac output does not change significantly, the stroke volume is markedly increased in hypoxic conditions (Holeton and Randall 1967). An increase in stroke volume necessarily causes the heart muscle to stretch, which is a powerful stimulus for cardiac peptide release (Tervonen *et al.* 1998, Kokkonen *et al.* 2000).

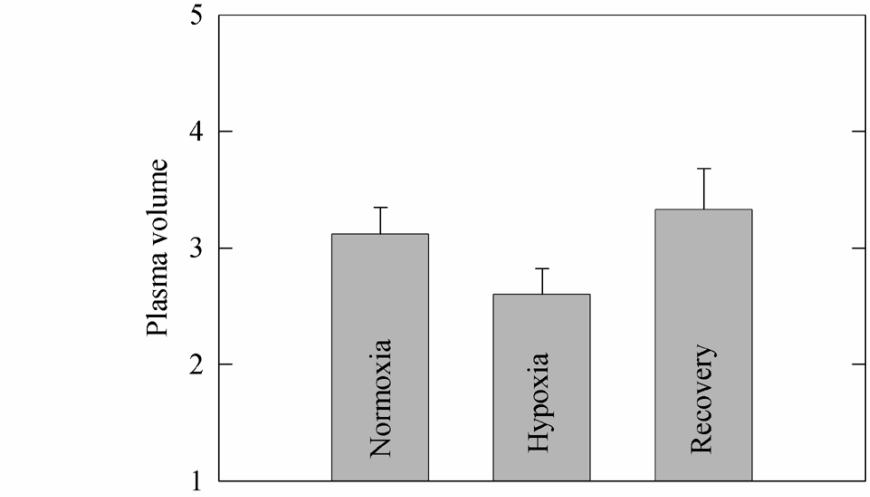

Figure 3. The plasma volume (% of total volume of fish) of rainbow trout (N = 6) in normoxia (>16 kPa oxygen at 18°C) and after 24 h hypoxia (6 kPa oxygen). For three rainbow trout, the plasma volume was further determined after a 24 h recovery period in normoxic water. The plasma volume was measured with the Evans blue dye dilution technique as described earlier (Smith 1966, Nikinmaa *et al.* 1981). The plasma volume of hypoxic rainbow trout was significantly ($P < 0.05$) lower than that of the normoxic fish (t-test for paired samples). Unpublished data of M. Nikinmaa, S. Egginton, E. Railo and A. Soivio.

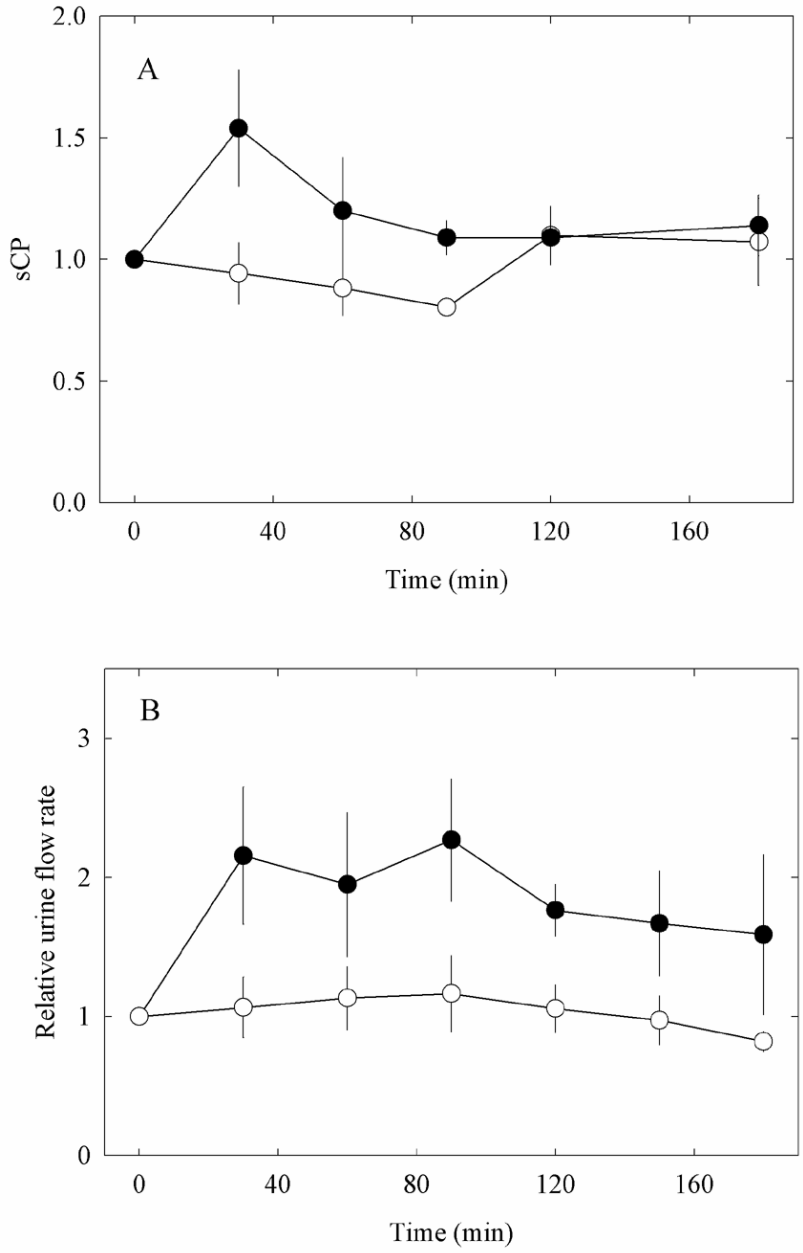

Figure 4. (A) The relative immunoreactive cardiac peptide concentration (sCP) in ventral aortic blood in normoxia (oxygen concentration of water ca. 9 mg/l at 12ºC; empty circles; N = 6, mean ± SEM given) and in hypoxia (decrease of oxygen concentration to 3 mg/l in 90 min; filled circles; N = 6). According to the t-test, the immunoreactive cardiac peptide concentration was significantly elevated at 30 and 90 min hypoxia as compared to controls ($P<0.05$). (B) Relative urine flow rate in normoxic and hypoxic rainbow trout. The number of fish sampled in normoxia = 5 and in hypoxia 6. Legend as in (A). Owing to hypoxia the relative urine flow rate elevated as compared to normoxia ($P < 0.05$; ANOVA). Unpublished data of V. Tervonen, O. Vuolteenaho and M. Nikinmaa.

LONG-TERM ADJUSTMENTS OF ERYTHROCYTE NUMBER

An increase in erythropoiesis has been observed in all vertebrates as a response to a decrease in the ratio of cellular oxygen availability/oxygen demand (Grant and Root 1952). Thus, both anemia (reduced maximal oxygen capacity) and hypoxia (reduced oxygen content without a change in maximal oxygen capacity) hasten erythrocyte formation in fish (McLeod *et al.* 1978, Härdig *et al.* 1978). The newly formed erythrocytes, mostly produced in the anterior kidney, are round and have a smaller volume than mature erythrocytes (Figure 5). The molecular mechanism of hypoxia-induced erythropoiesis in mammals has recently been clarified in detail (Semenza 2000, Semenza 2001, Jelkmann and Hellwig-Burgel 2001, Wenger 2002). Hypoxic conditions stabilize the transcription factor, the hypoxia-inducible-factor 1α (HIF-1α) that is transported to the nucleus, forms dimers with ARNT (aryl hydrocarbon receptor nuclear translocator), and binds to the hypoxia response element in the promoter region of the erythropoietin gene. Consequent to the DNA-binding by the dimer, the erythropoietin gene is activated and produces the hormone erythropoietin, the major activatory hormone of erythropoiesis. In mammals, erythropoietin is mostly produced in the peritubular cells of the kidney (Lacombe *et al.* 1988). Although human erythropoietin can stimulate erythropoiesis in fish (Weinberg *et al*. 1976), clear upregulation of the erythropoietin gene in hypoxic conditions has, as yet, not been reported in the literature (Gracey *et al*. 2001). One possible reason for this is the fact that data on the anterior kidney, the major erythropoietic site in fish, are presently lacking. On the other hand, gene expression profiling of hypoxic *Gillichthys* liver indicated that several genes associated with insulin or insulin-like growth factors are upregulated (Gracey *et al.* 2001). This observation suggests the possibility that insulin-like growth factors could be involved in the activation of erythropoiesis in fish as in mammals (Nikinmaa 1990).

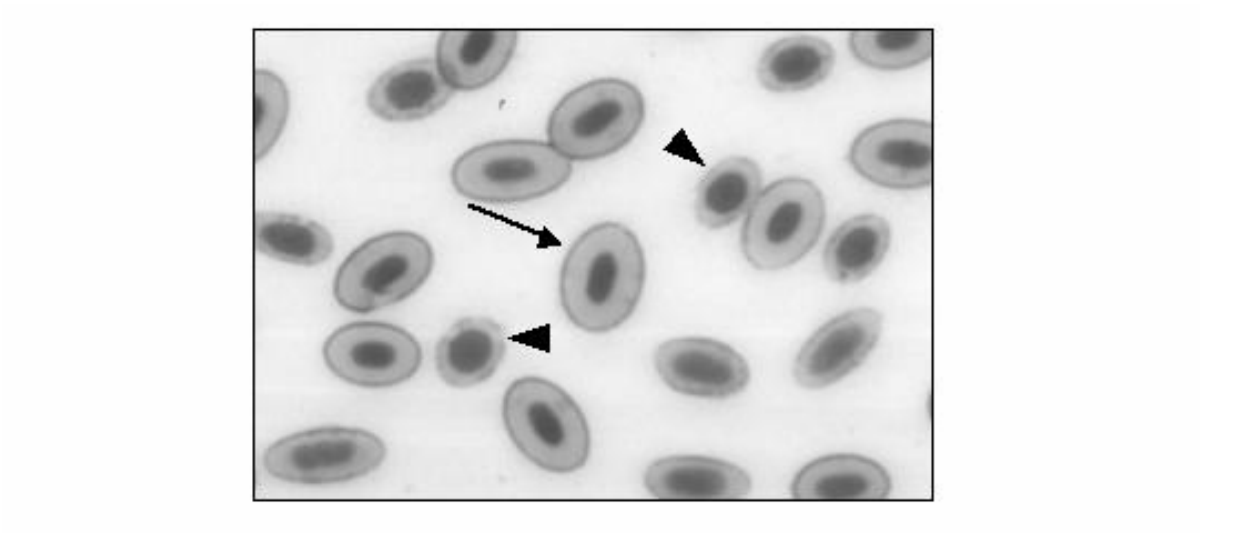

Figure 5. Immature (indicated by triangles) and mature (indicated by arrow) erythrocytes of rainbow trout after stimulation of erythropoiesis by anemia (50% reduction of hematocrit value by bleeding). Experimental details in Lecklin *et al*. (2000).

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REFERENCES

Chun, Y.S., J.Y. Hyun, Y.G. Kwak, I.S. Kim, C.H. Kim, E. Choi, M.S. Kim, and J.W. Park. 2003. Hypoxic activation of the atrial natriuretic peptide gene promoter through direct and indirect actions of hypoxia- inducible factor-10. Journal of Biochemistry 370:149-157.

Gallaugher, P. and A.P. Farrell. 1998. Hematocrit and blood oxygen-carrying capacity. *In*: Fish Respiration, S.F. Perry and B.L. Tufts (eds.). Academic Press. San Diego. Volume 17:185-227.

Giordano, F.J. and R.S. Johnson. 2001. Angiogenesis: the role of the microenvironment in flipping the switch. Current Opinion and General Development 11:35-40.

Gracey, A.Y., J.V. Troll, and G.N. Somero. 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proceedings of the National Academy of Sciences 98:1993-1998.

Grant, W.S. and W.S. Root. 1952. Fundamental stimulus for erythropoiesis. Physiology Review 32:449-498.

Härdig, J., L.A. Olsson, and L.B. Höglund. 1978. Autoradiography on erythrokinesis and multihemoglobins in juvenile *Salmo salar* L. at various respiratory gas regimes. Acta Physiologica Scandinavica 103:240-251.

Holeton, G.F. and D.J. Randall. 1967. The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. Journal of Experimental Biology 46:317-327.

Hughes, G.M. 1973. Respiratory responses to hypoxia in fish. American Zoology 13:475-489.

Jelkmann, W. and T. Hellwig-Burgel. 2001. Biology of erythropoietin. Advances in Experimental Medicine and Biology 502:169-187.

Jensen, F.B., M. Nikinmaa, and R.E. Weber. 1993. Environmental perturbations of oxygen transport in teleost fishes: causes, consequences and compensations. *In:* Fish Ecophysiology, F.B. Jensen and J.C. Rankin (eds.). Chapman & Hall. London. Pp. 161-179.

Kokkonen, K., H. Vierimaa, S. Bergstrom, V. Tervonen, O. Arjamaa, H. Ruskoaho, M. Jarvilehto, and O. Vuolteenaho. 2000. Novel salmon cardiac peptide hormone is released from the ventricle by regulated secretory pathway. American Journal of Physiology and Endocrinal Metabolism 278:E285-E292.

Lacombe, C., J-L. Da Silva, P. Bruneval, J-G. Fournier, F. Wendling, N. Casadevall, J-P. Camilleri, J. Bariety, B. Varet, and P. Tambourin. 1988. Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. Journal of Clinical Investigation 81:620-623.

Lecklin, T., A. Tuominen, and M. Nikinmaa. 2000. The adrenergic volume changes of immature and mature rainbow trout (*Oncorhynchus mykiss*) erythrocytes. Journal of Experimental Biology 203:3025-3031.

Lund, S.G., M.C.L. Phillips, C.D. Moyes, and B.L. Tufts. 2000. The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. Journal of Experimental Biology 203:2219-2228.

Maxwell, P.H. and P.J. Ratcliffe. 2002. Oxygen sensors and angiogenesis. Seminars in Cell Development Biology 13:29-37.

McLeod, T.F., M.M. Sigel, and A. A. Yunis. 1978. Regulation of erythropoiesis in the Florida gar, *Lepisosteus platyrhinchus*. Comparative Biochemistry and Physiology 60A:145-150.

Nikinmaa, M. 1990. Vertebrate red blood cells. Springer. Berlin-Heidelberg-New York.

Nikinmaa, M. and A. Soivio. 1982. Blood oxygen transport of hypoxic *Salmo gairdneri*. Journal of Experimental Zoology 219:173-178.

Nikinmaa, M., A. Soivio, and E. Railo. 1981. Blood volume of *Salmo gairdneri*: influence of ambient temperature. Comparative Biochemistry and Physiology 69A:767-769.

Nikinmaa, M. 2001. Hemoglobin function in vertebrates: evolutionary changes in cellular regulation in hypoxia. Respiration Physiology 128:317-329.

Nilsson, S. 1983. Autonomic nerve function in the vertebrates. Springer. Heidelberg

Semenza, G.L. 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. Journal of Applied Physiology 88:1474-1480.

Semenza, G.L. 2001. HIF-1, O_2, and the 3 PHDs: How animal cells signal hypoxia to the nucleus. Cell 107:1-3.

Skvorak, J.P., E.T. Sutton, P.S. Rao, and J.R. Dietz. 1996. Mechanism of anoxia-induced atrial natriuretic peptide release in the isolated rat atria. American Journal of Physiology 271:R237-R243.

Smith, L.S. 1966. Blood volumes of three salmonids. Journal of Fisheries Research Board of Canada 23:1439-1446.

Soivio, A., M. Nikinmaa, and K. Westman. 1980. The blood oxygen binding properties of hypoxic *Salmo gairdneri*. Journal of Comparative Physiology and Biology 136:83-87.

Speckner, W., J.F. Schindler, and C. Albers. 1989. Age-dependent changes in volume and hemoglobin content of erythrocytes in the carp (*Cyprinus carpio* L.). Journal of Experimental Biology 141:133-149.

Sundin, L. and G.E. Nilsson. 1997. Neurochemical mechanisms behind gill microcirculatory responses to hypoxia in trout: In vivo microscopy study. American Journal of Physiology 272:R576-R585.

Swift, D.J. and R. Lloyd. 1974. Changes in urine flow rate and hematocrit value of rainbow trout *Salmo gairdneri* (Richardson) exposed to hypoxia. Journal of Fisheries Biology 6:379-387.

Tervonen, V., O. Arjamaa, K. Kokkonen, H. Ruskoaho, and O. Vuolteenaho. 1998. A novel cardiac hormone related to A-, B- and C-type natriuretic peptides. Endocrinology 139:4021-4025.

Tervonen, V., H. Ruskoaho, T. Lecklin, M. Ilves, and O. Vuolteenaho. 2002. Salmon cardiac natriuretic peptide is a volume-regulating hormone. American Journal of Physiology and Endocrinology Metabolism 283:E353-E361.

Wagner, P.D. 2001. Skeletal muscle angiogenesis - A possible role for hypoxia. Advances in Experimental Medicine and Biology 502:21-38.

Weber, R.E. 1982. Intraspecific adaptation of hemoglobin function in fish to oxygen availability. *In:* Exogenous and endogenous influences on metabolic and neural control, A.D.F. Addink and N. Spronk (eds.). Pergamon Press. Oxford. Pp. 87-102.

Weber, R.E. and F.B. Jensen. 1988. Functional adaptations in hemoglobins from ectothermic vertebrates. Annual Review of Physiology 50:161-179.

Weinberg, S.R., J. LoBue, C. D. Siegel, and A.S. Gordon. 1976. Hematopoiesis of the kissing gourami (*Helostoma temmincki*). Effects of starvation, bleeding, and plasma-stimulating factors on its erythropoiesis. Canadian Journal of Zoology 54:1115-1127.

Wenger, R.H. 2002. Cellular adaptation to hypoxia: O-2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O-2- regulated gene expression. FASEB Journal 16:1151-1162.

Yamamoto, K-I. 1987. Contraction of spleen in exercised cyprinid. Comparative Biochemistry and Physiology 87A:1083-1087.

Yamamoto, K-I, Y. Itazawa, and H. Kobayashi. 1980. Supply of erythrocytes into the circulating blood from the spleen of exercised fish. Comparative Biochemistry and Physiology 65A:5-13.

Yamamoto, K-I., Y. Itazawa, and H. Kobayashi. 1983. Erythrocyte supply from the spleen and hemoconcentration in hypoxic yellowtail. Marine Biology 73:221-226.

THE ROLE OF HYPOXIA, STARVATION, β-NAPHTHOFLAVONE AND THE ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR IN THE INHIBITION OF REPRODUCTION IN FISH

David Randall and Huiping Yang[1]

ABSTRACT

Hypoxia has been shown to affect egg and sperm production, as well as gamete quality, percent hatching, and larval survival, in the carp. Hypoxia reduces food intake and this has been shown to also inhibit egg production in the zebrafish. Thus the inhibition of reproduction during hypoxia could be a direct effect of hypoxia or an indirect effect caused by reduced food intake; most likely both play a role. Hypoxia, and perhaps starvation, result in increased levels of Hypoxia Inducing Factor 1α (HIF-1 α), and this combines with the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) to form HIF-1. In turn, HIF-1 binds to DNA and modulates expression of a number of genes involved in cellular responses to hypoxia, such as erythropoiesis and angiogenesis. β-naphthoflavone (βNF), via the aryl hydrocarbon receptor (Ahr), binds to ARNT and inhibits egg production and food intake in zebrafish. Formation of this βNF/Ahr/ARNT complex causes an increase in ethoxyresorufin-O-deethylase (EROD) activity in the liver. Steroid levels remain low during both hypoxia and exposure to βNF. It is possible that both βNF and HIF-1 compete for ARNT, reducing ARNT activity, and this reduction in ARNT leads to an inhibition of reproduction. ARNT also dimerizes with the transcription factor SIM1, as well as HIF-1 and Ahr. The ARNT/SIM1 dimer is involved in the regulation of the hypothalamic-pituitary axis. βNF has been reported to act on pituitary gonadotrophs, disturbing sexual maturation, however, we found no change in zebrafish serum gonadotropin levels in response to hypoxia or βNF exposure.

OXYGEN IN THE AQUATIC ENVIRONMENT

Most of the earth's gaseous oxygen is in the atmosphere; only a small amount is dissolved in water. Oxygen levels in the surface layers of water equilibrate with oxygen levels in the atmosphere. Oxygen is used in respiration of organisms and by oxidation of organic and inorganic matter in the aquatic environment. Photosynthesis results in oxygen production, but only if light is available. At depth, in the absence of light, oxygen must be transported from the surface by mixing and diffusion. The latter is a very slow process and of limited importance. In the absence of mixing, water at depth becomes hypoxic. In fact, hypoxia is a common feature of the aquatic environment. Because of the absence of photosynthesis, nocturnal hypoxia is common in tropical lakes and lagoons. This is especially so if the lagoon is poorly flushed. During the day, with high levels of photosynthetic activity, the oxygen level in water may rise above that in the air and the water becomes hyperoxic. Thus, during the night there can be a net

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flux of oxygen from air into the water, but during the day oxygen can move in the opposite direction (Figure 1). Water bodies often stratify because of density differences due to temperature and/or salinity. Hypoxia is common at depth in these unmixed waters. There is a minimum-oxygen layer or zone in oceans below the photic zone (Randall and Farrell 1997). This layer can have oxygen levels of around 10 to 20% of air saturation levels and it extends for vast distances through the oceans. Hypoxia occurs in lakes beneath ice because of reduced light penetration and mixing.

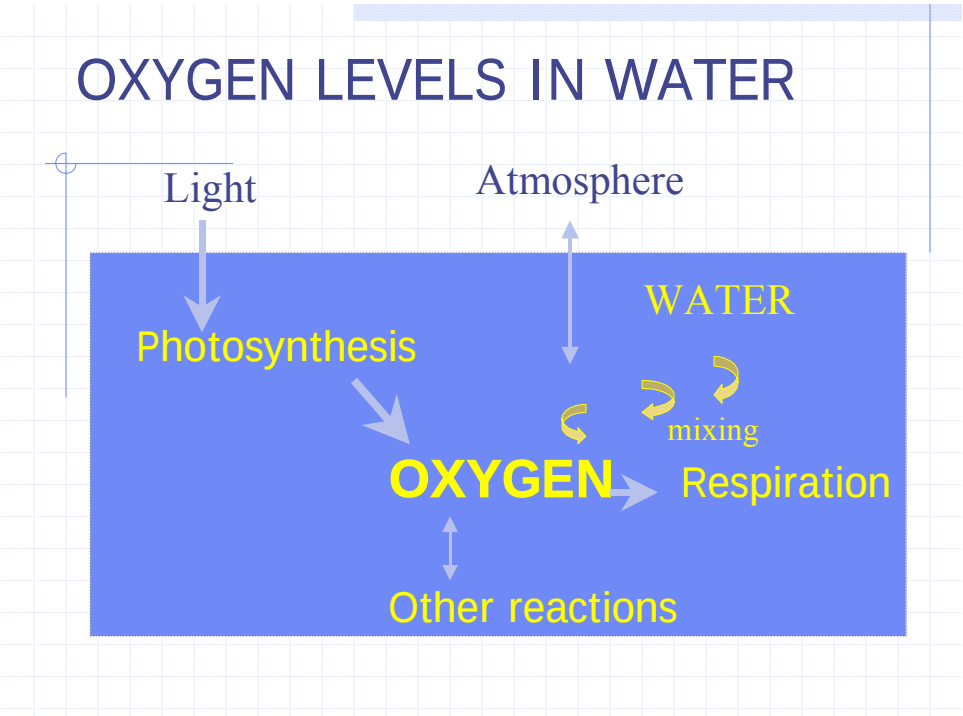

Figure 1. Oxygen levels in water are determined by production due to photosynthesis, used by organisms in respiration, and the oxidation of substances in the water column. There is also some exchange between water and air.

EFFECTS OF HUMAN ACTIVITIES ON AQUATIC OXYGEN LEVELS

Nearly one hundred years ago Arrhenius (1908) predicted that increased carbon dioxide levels, due to industrial activity, would result in an increase in atmospheric temperature via the "greenhouse effect." Oxygen solubility and, therefore, concentration decreases with increasing water temperature. Oxygen tends to enter waters that are cooling and then return to the atmosphere as the waters warm. Much of the oxygen entering the oceans does so in polar regions and is then distributed by ocean currents. Recent observations have shown that the oxygen levels of the Southern Oceans (the so- called "lungs" of the world's oceans) are dropping as a result of climate change.

Large increases in the human population over the last century have resulted in increased industrial, fertilizer, and sewage effluent discharge into rivers and oceans. This discharge has caused eutrophication that, in turn, has led to increased aquatic hypoxia in rivers and coastal regions of oceans around the world where there are large human populations. Examples include the Gulf of Mexico, the Black Sea, and the Danube (National Research Council 1999).

EFFECTS OF HYPOXIA IN THE AQUATIC ECOSYSTEM

Animals have not had time to adapt to these rapid changes in aquatic oxygen levels caused by humans that have gained in strength over the last decade. Hypoxia in coastal marine waters has been associated with a major change in fish species composition, and a reduction in the number of demersal fishes. Aquatic hypoxia has caused changes in species composition, as some organisms leave and other more sensitive (or less mobile) species die out. One possible explanation of this phenomenon is the impairment of gonadal development and failure in spawning, fertilization, hatching and survival. What is left are those species that are more tolerant of hypoxia. The overall effect of aquatic hypoxia is a reduction in species diversity and a marked reduction in biomass (Diaz and Rosenberg 1995).

PHYSIOLOGICAL RESPONSES TO HYPOXIA IN FISH

Animals that live where hypoxia is a long-time periodic event have adapted to it. For example, many tropical fish become quiescent during periods of nocturnal hypoxia in coral lagoons, reducing energy requirements during periods of reduced oxygen availability. Another example of fish adaptation to oxygen availability is the temperate freshwater fish *Amia calva* that uses air breathing to supplement oxygen supplies during summer, when elevated temperatures raise fish oxygen requirements but reduce oxygen content in the water. The female fish from lakes in Ontario, Canada, grow eggs during the winter months when they are under ice and do not breathe air. Raising the temperature of these winter fish in a holding facility killed only the females (Daxboeck and Randall, personal observation) because there was not enough room in the peritoneal cavity for eggs and an air-filled bladder. That is, egg production and air breathing cannot occur at the same time in *Amia*. Egg production occurs when air breathing is not required to maintain oxygen requirements.

At the individual level, animals exposed to hypoxic conditions attempt to maintain oxygen delivery to the tissues in the face of reduced levels in the environment. Fish increase gill ventilation and gill diffusing capacity to enhance the transport of oxygen across the gills into the blood. Heart rate is reduced but stroke volume is increased and the changing pattern of blood flow through the gills increases gill diffusing capacity for oxygen (Randall 1982). Decreased red blood cell phosphate levels result in an increase in hemoglobin oxygen affinity and this also facilitates oxygen uptake at the gills (Val and de Almeida-Val 1995). Blood erythrocyte levels are increased initially due to release from the spleen and subsequently due to erythropoiesis in response to the hormone erythropoietin (EPO), produced by the kidney. Hypoxia has been shown to result in increased EPO levels in the kidney and spleen of rainbow trout (Kakuta and

Randall unpublished observations). Anaerobic metabolism increases during hypoxia (Randall 1982, van den Thillart and van Waarde 1985). There is an up-regulation of anaerobic enzymes, increased glucose transport and utilization of liver glycogen. The magnitude of the glycogen stores is an important determinant of hypoxic survival. There is a down-regulation of energy expenditure coupled to the up-regulation of anaerobic metabolic pathways.

Hypoxic areas are often patchy in distribution, and many fish respond to aquatic hypoxia by initially increasing activity in an attempt to leave the area. Fish decrease swimming activity during prolonged hypoxia and tend to move to colder waters (Schurmann and Steffensen 1994). This decrease in temperature reduces energy metabolism and is associated with an increase in the oxygen content of water. Fish exhibit reduced food intake during hypoxia (Zhuo *et al.* 2001) and this also decreases energy expenditure. Experiments with zebrafish (*Danio rerio*) showed that they stopped feeding about six hours after the onset of hypoxia (Yang and Randall, unpublished data). Protein synthesis was reduced by 40% during anoxia in carp (Smith *et al.* 1996, 1999), with the liver showing a much larger reduction (85%) than the muscle (40%). Protein synthesis in the brain was maintained at the normal low rates seen under normoxic conditions.

HYPOXIA INHIBITS REPRODUCTION

Hypoxia, starvation, and β-naphthoflavone (β-NF) exposure inhibit reproduction in fish. The inhibition is dose dependent but the exact nature of the relationship is not clear. The more hypoxic and the greater the level of β-NF in the water the more complete is the inhibition of reproduction.

Wu *et al.* (2003) reported on the effects of hypoxia on reproduction of the common carp (*Cyprinus carpio*). Gonad development was reduced when fish were exposed to hypoxia for eight weeks. There was a significant reduction in the number of spermatocytes and spermatids, lowered incidence of mitosis, decreased lobular diameter of testes and reduced sperm motility in male carp. In female carp, oocytes from hypoxic fish remained in the early stages of the developmental process, whereas normoxic females had oocytes that were near completion of the developmental process. Successful spawning females were 71.4% in the normoxic group, significantly higher than the hypoxic group (8.3%). There also was a marked decrease in the percentages of fertilization success (99.4% in normoxia and 55.5% in hypoxia); hatching (98.8% in normoxia and 17.2% in hypoxia); and survival of larvae (93.7% in normoxia and 46.4% in hypoxia) (Figure 2). Zebrafish normally produce eggs every day, but hypoxia (0.5 – 0.8 mgO_2/L), starvation, and β-NF (1.0 mg/L) reduced egg production (Yang and Randall, unpublished data) (Figure 3). There were increased numbers of undeveloped eggs, but no effect was observed on hatching rates when the eggs were allowed to develop under normoxic conditions. There were significant decreases in serum testosterone and estradiol levels in carp exposed to hypoxia (Wu *et al.* 2003). The size of the gonads was related to steroid levels in the blood. What is clear is that hypoxia results in reduced egg production, reduced sperm motility and reduced larval survival and this could account for the disappearance of a number of fish species seen in areas subjected to hypoxia.

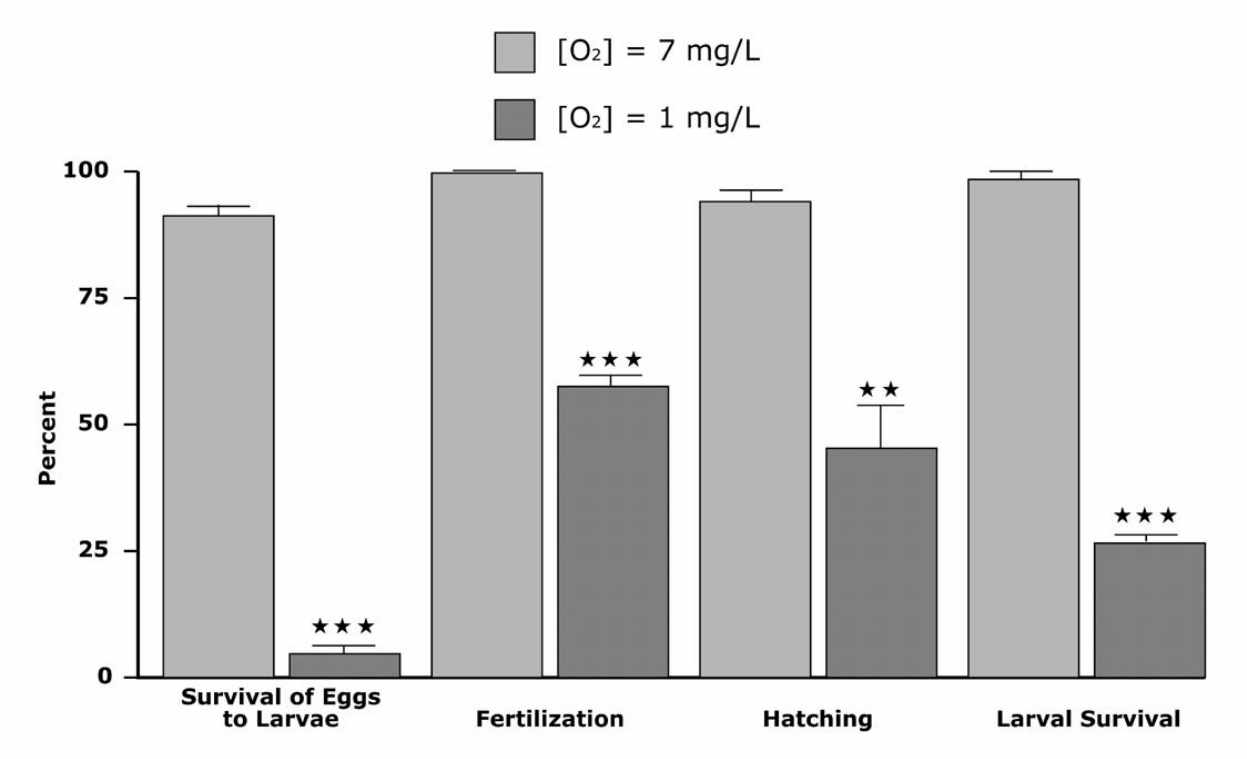

Figure 2. Effects of hypoxia on common carp: (a) percent survival of eggs to larvae; (b) percent of fertilization, hatching rate and larval survivorship of *C.carpio* upon exposure to 7.0 or 1 mg O_2/L for 12 weeks. Values significantly different from the control are indicated by asterisks. (n = 6, mean ± SE). (**: p<0.01; ***p<0.001). (adapted from Wu *et al.* 2003).

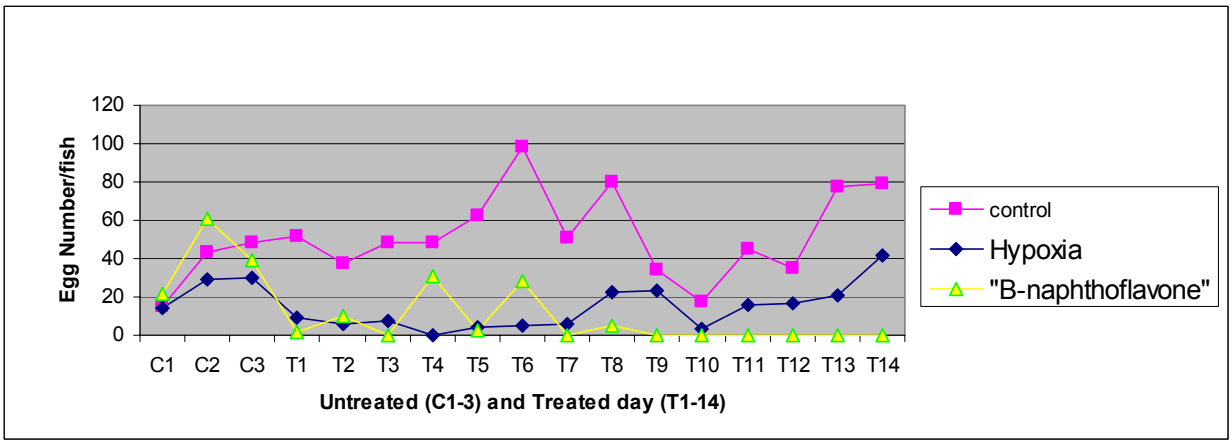

Figure 3. Both hypoxia (0.5-0.8 mgO_2/L) and β-NF (1 mg/L) inhibit egg production in zebrafish. (Yang and Randall, unpublished data)

HYPOXIA-INDUCING FACTOR 1α (HIF-1α) IN FISH

Hypoxia-induced changes in gene expression in *Gillichthys mirabilis*, a marine intertidal goby, have been reported by Gracey *et al.* (2001). The changes are similar, but not identical, to the response observed in mammals. In muscle there is a down-regulation of genes associated with cell growth and protein synthesis, whereas in the liver there is a change in expression pattern away from growth to processes essential for survival. Little change is observed in the brain, as expected. Hypoxia-Inducing Factors (HIFs) are produced by most cells. HIF-1α is continually produced but degraded during normoxia, but this degradation is inhibited by hypoxia. HIF-1α combines with the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT=HIF-1β) to form HIF-1. HIF-1α has been cloned and described for rainbow trout (Soitamo *et al*. 2001; *Oncorhynchus mykiss*), for grasscarp (*Ctenopharyngodon idella;* Kong & Law, unpublished data) and for common carp (*Cyprinus carpio*; Poon and Hung, unpublished data). HIF-1 down-regulates transcription in general, but up-regulates production of erythropoietin, vascular endothelial growth factor, glucose transporters, apoptosis, and anaerobic enzymes. In other words, HIF-1 is involved in a number of mechanisms and is an important mediator of cellular and systemic oxygen homeostasis (Jonathan and Ratcliffe 1998, Seagroves *et al*. 2001, Semenza 2001a & b, Minet *et al*. 2000, Riva *et al*. 1998).

THE ARNT MOLECULE AND REPRODUCTION IN FISH

Hypoxia, starvation and β-NF inhibit reproduction in zebrafish. HIF-1 may also be involved indirectly in the inhibition of reproduction during hypoxia and starvation. There are many similarities in the physiological responses to hypoxia and starvation, and both may involve HIF-1 activation. β-NF binds to the aryl hydrocarbon receptor (AhR) that, in turn, binds to ARNT. Exposure to β-NF increases EROD activity in the zebrafish liver, presumably via formation of the β-NF/AhR/ARNT complex. Hypoxia inhibits this EROD activation in some mammalian cell lines, presumably because of competition between HIF-1α and β-NF/AhR for the ARNT molecule (Gradin *et al.* 1996, Nie *et al.* 2001). Hypoxia and β-NF exposure may reduce the availability of ARNT and this reduction in availability may be involved in the inhibition of reproduction. ARNT also heterodimerizes with another basic Helix-Loop-Helix (bHLH)-PAS transcription factor, SIM1 (Michaud *et al.* 2000, Hosoya *et al.* 2001). SIM1 is a critical regulator of neuronal differentiation in the hypothalamus. The paraventricular (PVN), anterior periventricular (aPV) and supraoptic (SON) nuclei fail to develop in the absence of SIM1 gene function in mice. These nuclei are major components of the hypothalamic-pituitary axis. ARNT2, the *in vivo* dimerization partner of SIM1 during development of the PVN/SON in mammals, is also found in fish brains. Andersson *et al.* (1993) suggested that β-NF acts on pituitary gonadotropins, and that the possibility existed that ARNT2/SIM1 may be involved in this process. The observation, however, that zebrafish serum gonadotropin levels were unaffected by hypoxia and β-NF exposure (Yang and Randall, unpublished observations) indicated that this system may not be the one inhibiting reproduction during hypoxia. In addition, there were no observed differences in ARNT2 in dioxin-sensitive and dioxin-resistant populations of *Fundulus heteroclitus* (Powell *et al.* 2000), indicating that dioxin resistance is not related to ARNT activity. Thus, the mechanism by which hypoxia inhibits reproduction remains obscure, at least to us.

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REFERENCES

Andersson, T., L. Forlin, S. Olsen, A. Fostier and B. Bretin. 1993. Pituitary as a target organ for toxic effect of P4501A1 – inducing chemicals. Molecular and Cellular Endocrinology 91: 99-105.

Arrhenius, S. 1908. Das Werden der Welten. Hirzel, Leipzig, 108 pp.

Diaz, R. J. and R. Rosenberg. 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. Oceanography and Marine Biology Annual Review. 33: 245-303.

Gradin, K., J. McGuire, R .H. Wenger, I. Kvietikova, M .L. Whitelaw, R. Toftgard, L. Tora, M. Gassmann and L. Poellinger. 1996. Functional interference between hypoxia and dioxin signal transduction pathways: Competition for recruitment of the Arnt transcription factor. Molecular and Cellular Biology 16: 5221-5231.

Gracey, A. Y., Troll, J. V., Somero, G. N. 2001. Hypoxia-Induced Gene Expression Profiling in the Euryoxic Fish, *Gillichthys mirabilis*. Proceedings of the National Academy of Sciences 98: 1993 - 98.

Hosoya, T., Y. Oda, S. Takahashi, M. Morita, S. Kawauchi, M. Ema, M. Yamamoto and Y. Fujii-Kuriyama. 2001. Defective development of secretory neurons in the hypothalamus of Arnt2-knockout mice. Genes to Cells 6: 361-374.

Jonathan, M. G. and P. J. Ratcliffe. 1998. Hypoxia and the regulation of gene expression. Molecular Medicine Today 4:122-129.

Michaud, J.L., C. DeRossi, N.R. May, B.C. Holdener and C.M. Fan. 2000. ARNT2 acts as the dimerization partner of SIM1 for the development of the hypothalamus. Mechanisms of Development 90: 253-261.

Minet, E., G. Michel, J. Remacle, and C. Michiels. 2000. Role of HIF-1 as a transcription factor involved in embryonic development, cancer progression and apoptosis (review). International Journal of Molecular Medicine 5: 253-259.

National Research Council. 1999. Clean Coastal Waters: Understanding and Reducing the Effects of Nutrient Pollution. National Academy Press, New York

Nie, M., A.L. Blankenship and J. P. Giesy. 2001. Interactions between aryl hydrocarbon receptor (AhR) and hypoxia signaling pathways. Environmental Toxicology and Pharmacology 10: 17-27.

Powell, W. H., R. Bright, S.M. Bello and M.E. Hahn. 2000. Development and tissue-specific expression of AHR1, AHR2, and ARNT2 in dioxin-sensitive and –resistant populations of the marine fish *Fundulus heteroclitus*. Toxicological Sciences 57: 229-239.

Randall, D.J. 1982. The control of respiration and circulation in fish during exercise and hypoxia. Journal of Experimental Biology. 100: 275-288.

Randall, D.J. and A.P. Farrell. 1997. Deep-Sea Fishes. Fish Physiology Vol. 16. Academic Press, New York. 388 pp.

Riva, C., C. Chauvin, C. Pison, and X. Leverve. 1998. Cellular physiology and molecular events in hypoxia-induced apoptosis. Anticancer Research 18: 4729-4736.

Schurmann, H. and J. F. Steffensen. 1994. Spontaneous swimming activity of Atlantic Cod *Gadus morhua* exposed to graded hypoxia at three temperatures. Journal of Experimental Biology 197: 129-142.

Seagroves, T. N., H. E. Ryan, H. Lu, B. G. Wouters, M. Knapp, P. Thibault, K. Laderoute, and R. S. Johnson. 2001. Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. Molecular and Cellular Biology 21: 3436-3444.

Semenza, G. L. 2001a. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends in Molecular Medicine 7: 345-350.

Semenza, G. L. 2001b. HIF-1, O2, and the 3 PHDs: How animal cells signal hypoxia to the nucleus. Cell 107:1-3.

Smith, R. W., D. F. Houlihan, G. E. Nilsson, and J. G. Brechin. 1996. Tissue-specific changes in protein synthesis rates in vivo during anoxia in crucian carp. American Journal of Physiology 40: R897-R904.

Smith, R. W., D. F. Houlihan, G. E. Nilsson, and J. Alexandre. 1999. Tissue-specific changes in RNA synthesis in vivo during anoxia in crucian carp. American Journal of Physiology 277: R690-R697.

Soitamo, A. J., C. M. Råbergh, M. Gassmann, L. Sistonen, and M. Nikinmaa. 2001. Characterization of a hypoxia-inducible factor (HIF-1α) from rainbow trout. Journal of Biological Chemistry 276: 19699-19705.

Val, A. L. and V. M. F. de Almeida-Val. 1995. Fishes of the Amazon and their environment: physiological and biochemical aspects. Zoophysiology. vol. 32. Springer-Verlag, Heidelberg. 224 pp.

Van den Thillart, G. and A. van Waarde. 1985. Teleosts in hypoxia: Aspects of anaerobic metabolism. Molecular Physiology 8: 393-409.

Wu, R. S. S., B. S. Zhou, D. J. Randall, N.Y. S. Woo and P. K.S. Lam. 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environmental Science and Technology 37: 1137

Zhou, B. S., R.S.S. Wu, D.J. Randall and P.K.S. Lam. 2001. Bioenergetics and RNA/DNA ratios in the common carp (*Cyprinus carpio*) under hypoxia. Journal of Comparative Physiology B 171:49-57.

COMPOSITION AND RATE OF RELEASE OF CHEMICAL SUBSTANCES INTO WATER BY DEAD FISH

Alexander Kasumyan[1] and Arvo Tuvikene[2]

INTRODUCTION

Massive fish death has occurred often in natural water bodies, caused by various environmental factors. The most common is hypoxia that occurs often during winter in many eutrophic water bodies, mainly in the temperate and high latitudes (Nikolski 1963, Ravera *et al.* 1986, Renaud 1986, Merceron 1988). Local strong and sharp shift of water temperature can also lead to high mortality of fish (Michaelis 1985). Marine fish perish frequently in red tides caused by toxic dinoflagellates (Mortensen 1985, Riley *et al.* 1989, Adnan 1989). Adults of some fish like osmerids (*Osmerus eperlanus*) and anadromous salmonids (*Oncorhynchus, Salmo*) die naturally shortly after spawning (Wootton 1998). Pollution causes fish kills, especially in closed water bodies (Haslouer 1983, Sanzin 1989). It seems obvious that substances released into the water after mass fish death can strongly affect the local water quality, and could even lead to temporary aggravation of eutrophication.

The composition of chemical substances released by dead fish into water as well as the rate of release, are unknown. It was possible to find in the literature only one paper concerning this topic (Wold and Selset 1978). That paper was devoted to the study of the composition of body mucus collected in successive time intervals (0-2, 3-5 and 6-8 hours) after the death of Arctic char *Salvelinus alpinus*. The particular emphasis was the mucin glycoprotein composition in mucus samples. It was found that the proportions of both chloroform-methanol-soluble and water-soluble material decreased from the 0-2 hour to the 6-8 hour mucus samples. The mucus fraction that was insoluble in chloroform-methanol and in water (70%) consisted largely of protein. The water-soluble glycoprotein samples contained the common mucin sugars fucose, galactose, N-acetylgalactosamine and sialic acid. The investigators proposed that the mucin glycoprotein in the 6-8 hour period was secreted – at least partly – as incomplete glycoprotein molecules.

There are data showing that dead fish are a source of chemical substances that regulate fish behavior. An alarm pheromone of cyprinids passes out spontaneously from the undamaged skin of dead fish, but does not release into the water from live fish in the absence of skin wounds or scratches (von Frisch 1941). It has been noted that sharks avoid remaining in water where dead sharks have been kept for several days (Groop 1975). The water extract of decaying shark's meat contains substances that have a repellent effect for sharks (Gilbert and Gilbert 1973). The smell of dead whitefish *Coregonus lavaretus* evoked an avoidance response in Arctic char, *Salvelinus alpinus* (Höglund *et al.* 1975). On the other hand, substances released into the water from dead groupers *Epinephelus merra* were more attractive for sharks than the smell of resting or agitated fish (Tester 1963a, 1963b). Both dead fish and pieces of fish meat usually are more attractive food items than living fish,

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especially for predators that are nocturnal or bottom and deep-water feeders (Brawn 1969). Many scavengers like decapods easily find dead fish using olfaction (Zimmer-Faust and Case 1982, Zimmer-Faust 1989).

The study reported in the present article was designed to learn what kinds of substances are released into the water during the first 24 hours after the death of fish. We also attempted to investigate the rate at which different substances pass out of dead fish. Observation of alarm pheromone appearance in the water surrounding dead cyprinid fish was chosen as the main approach for this study.

MATERIALS AND METHODS

Subjects

European minnow, *Phoxinus phoxinus* (5-7 cm total length), common carp *Cyprinus carpio* (10-12 cm) and crucian carp *Carassius carassius* (11-12 cm) were used for these experiments. Fish were caught by net in natural waters (minnow and crucian carp) or were obtained from a fish farm near Moscow (common carp). Before the experiments, fish were kept in aerated holding tanks. Water in the aquaria was partially replaced each week with freshly- aerated tap water. Living bloodworms (*Chironomidae* larvae) were used as fish food.

Sample Collection

Minnows and common carp were killed by asphyxia. Living fish (a single minnow or a group of 5-8 common carp) were placed into a glass flask (100 ml for minnow and 3 liters for common carp) filled with anoxic water and exposed for around one hour. Crucian carp were killed by injection of 0.2 ml KCl (1 M) into the heart followed by asphyxia in open air. Dead fish were rinsed with distilled water and placed into a glass beaker filled with distilled water (50 ml, minnow; 200-500 ml, common carp; 200 – 400 ml, crucian carp). The water temperature varied between 17 and 23°C.

In the first series of experiments, one dead minnow was exposed in the water for 15 or 30 minutes, 1, 2, 4 and 12 hours. In the second series of experiments, one dead minnow was exposed for one hour and then transferred to a new beaker with fresh distilled water each hour for eight hours after death. In the third series of experiments, 5-8 dead common carp were held in fresh, distilled water for one hour during the first, second, third, sixth, twelfth and twenty fourth hours after death. In the fourth series of experiments, with crucian carp, one dead fish was exposed in artificial pond water (NaCl $2.9 * 10^{-2}$, KCl $3.7 * 10^{-3}$, $CaCl_2$ $5.8 * 10^{-2}$, Na HCO_3 $1.6 * 10^{-2}$ g/L). The water exchange procedure was the same as in the second series of experiments. Water samples for electro–olfactogram (EOG) measurements were taken after 30 minutes, 1, 2, 3, 4, 5, 6, 9, 12, and 24 hours. The experiment was repeated five times with different fish. Water from the dead fish was collected for fractionation 0.5 and 24 hours after fish death.

All water samples were filtered through coarse Watman filter paper. Samples intended for both gel-chromatography and electrophoresis were lyophilized and kept in the freezer. Freshly collected samples were used in both behavioral trials and EOG-recordings.

Behavioral Experiments

For each trial, 7-9 minnows were placed into a flow-through fluviarium (115x20x20 cm) 30 minutes before the beginning of the experiments. The fluviarium was supplied by fresh river water from a local reservoir. The water velocity in the fluviarium was 5-7 l/min. Fish usually showed positive reotaxis and stayed together near the mesh-screen just below the upstream end of the fluviarium. The test-solution of 50 ml volume was introduced into the fluviarium in one dose over 2-3 seconds, five cm upstream of the mesh-screen. The intensity of fish behavioral response was estimated by the 6-point scale shown in Table 1.

Table 1. Six-point scale used to quantify the intensity of the fish fright reaction.

Numeric rating	Fish Behavior
0	No reaction
1	Orientation of fish with the water current is disturbed; some fish slowly retreat downstream or turn 180º and depart into a part of the school that is lower in the current. As a result the school elongates but soon resumes its initial shape and position.
2	The number of fish reacting by active departure increases but does not exceed one-half the total fish in the school.
3	The majority of fish actively depart by sudden movements; the school moves downstream.
4	Fish orient to the stimulus, crowding together into a compact school and quickly swimming downstream. Fish movements are sudden and jerky; fish try to hide on the bottom for 10-20 seconds.
5	Fish orient to the stimulus, crowding together into a compact school, rushing downstream and swimming about in a disorderly fashion; some fish jump out of the water or try to swim upstream, but then turn back. Periods of violent motor activity alternate with prolonged (1-3 minutes) position-maintenance.

Electro–olfactogram Recording

We investigated the olfactory sensitivity to water containing dead fish by recordings of EOG of adult crucian carp. The fish were anaesthetized with Saffan (Alfaxalone, 24 $mg \cdot kg^{-1}$, Schering-Plough Animal Health, England) and immobilized with tubocurarin (Jexin 10 $mg \cdot kg^{-1}$, Duncan, Flockhart and Co Ltd., England) before being placed in a fish-holder. The gills were perfused with tap water. The skin covering the left olfactory organ was removed and the olfactory rosette exposed to extracts of alarm substance by a stimulator device placed above the olfactory rosette. Artificial pond water was flushed over the rosette at a rate of 3.5 ml/min. The recording electrode was placed between two lamellae in the caudal end of the olfactory rosette and the reference electrode under the skin of the fish head. The EOG responses were amplified and recorded by means of a PowerLab system (ADInstruments, Castle Hill, NSW, Australia). Olfactory stimulation was standardized to 10-second periods with 60 s between each stimulus during recording. Each fish was first tested with artificial pond water and 10^{-5}molar L-serine to ensure recording quality. Stimulation with blank water and L-serine was conducted repeatedly during experiments. From each response of dead fish water the response of the nearest tested blank water was subtracted.

Fractionation and Chemical Analysis

The gel chromatography with sephadex G-25 had these specifications: column volume 37 ml and height-to diameter ratio 48:1; elution by phosphate buffer (0.35 M, pH 6.8); each sample applied on the column contained 5-10 mg dry materials dissolved in 1-2 ml distilled water; dextran blue (molecular weight (MW) 2,000,000), diphosphopiridinucleotide sodium salt (MW 709), nicotinamidadenindinucleotide ($NADNH_2$, MW 665), folic acid (MW 441) and tyrosine (181) were used for preparing the calibration curve; a UV-detector (280 nm) was used for recording the UV-absorbing fraction. Electrophoresis in polyacrylamide gel: the concentration of polyacrylamide was 3.5% and 7.5%, and the pH was 6.7 and 8.6, respectively for preliminary concentration and separation; tris-glycine buffer, pH 8.3; temperature 24°C; 200 volt and 1.4 mA per tube for the concentration process and 2.8 mA for the separation process; Kumasi brilliant blue 0.0015% solution in 10% trichloro-acetic acid was used for determination of the peptide fraction. The protein content in samples was estimated by Lowry's method (Lowry *et al.* 1951). Fractions of skin extract and water from dead fish taken for EOG recordings were obtained by means of gel chromatography (column 80 ml filled with sephadex G-25 (fine)). Elution speed was 1ml/min. The mobile phase was artificial pond water. To study the EOG activity of skin extract, the fish skin was homogenized with artificial pond water in a mortar and then centrifuged 15 min at 5000 rpm. The supernatant was used for fractionation.

RESULTS

Behavioral Experiments

The water surrounding the dead fish became repellent within 0.5 hr after fish death. It was found that water samples collected during the first hour after fish death evoked a typical fright reaction in minnow (Figure 1). Several seconds after introducing 50 ml of this water into the fluviarium, fish calmly swimming near the upstream mesh-screen decreased their motor activity and oriented toward the source of the odor, and the rate of gill movements increased. Schools of fish became denser than before stimulation. Fish turned into the current and rushed abruptly downstream; they sometimes stopped midway and oriented to the current for a short time, then continued rapid retreat, showing random jerky swimming. In the final stage, fish hid in a compact group near the bottom at the downstream end of the fluviarium. The time for which fish remained hidden varied from 1-2 to 3-5 minutes and even longer. After this time, the duration of which depended on the strength of the response, fish gradually calmed down and some returned to their preferred position near the upper mesh-screen. The repellent activity of water samples depended strongly on the duration of their original exposure to dead fish. It increased sharply up to a two-hour exposure, when it reached a stable level (Figure 1).

The aim of the second series of experiments was to study more precisely the rate of alarm pheromone release by dead fish. It was confirmed that repellent activity appeared in the water surrounding the dead fish during the first hour after fish death. The alarm pheromone release process reached its maximum between one and two hours after fish death, then decreased slowly during the next several hours (Figure 2).

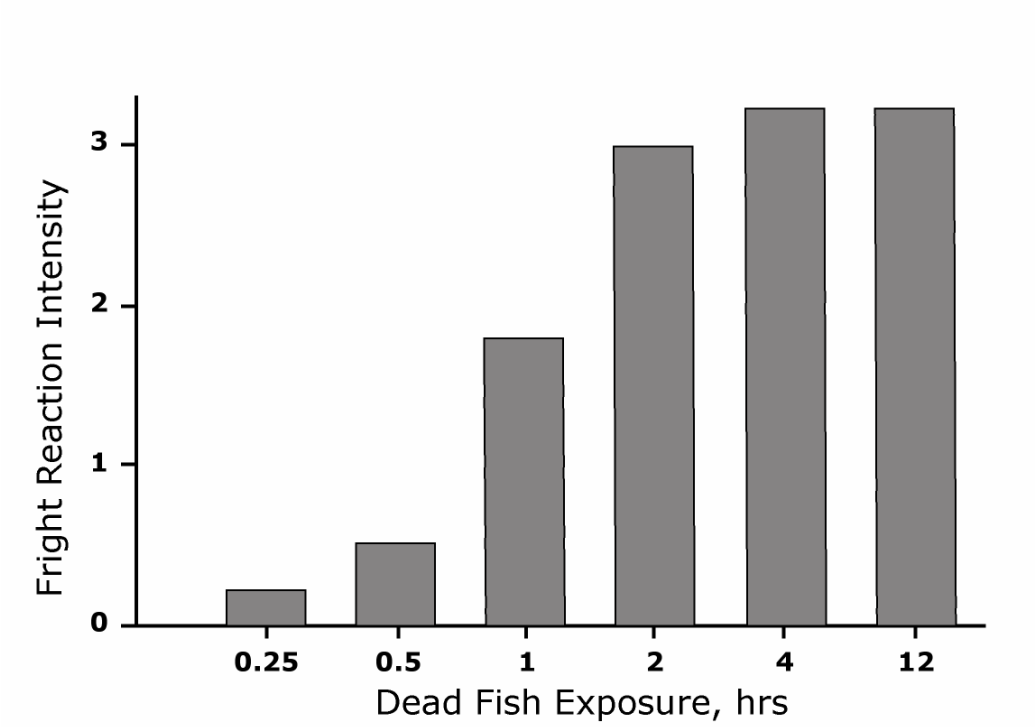

Figure 1. The fright reaction of European minnow, *Phoxinus phoxinus*, to water samples of dead fish depending on the length of exposure of the dead fish in the water. Intensity scale 0-5 (5 maximum).

EOG Recordings

The EOG response reached its maximum during the first 0.5 hour after fish death, decreased for the next six hours, then started to increase again (Figure 3). The EOG response after 24 hours was comparable to that after 0.5 hour, although the 0.5 hour water sample contained more EOG-active fractions (as measured by gel chromatography) than the 24 hour water sample (Figure 4). The most potent substances from skin extract responsible for high EOG-response were of relatively low MW (around 500 Da) (Figure 5). After fish death, the electrophysiologically most potent substances released from skin into water had lower MW than substances from the skin extract. Soon after fish death, the substances released had higher MW and were more potent in provoking an EOG response than the substances released after 24 hours.

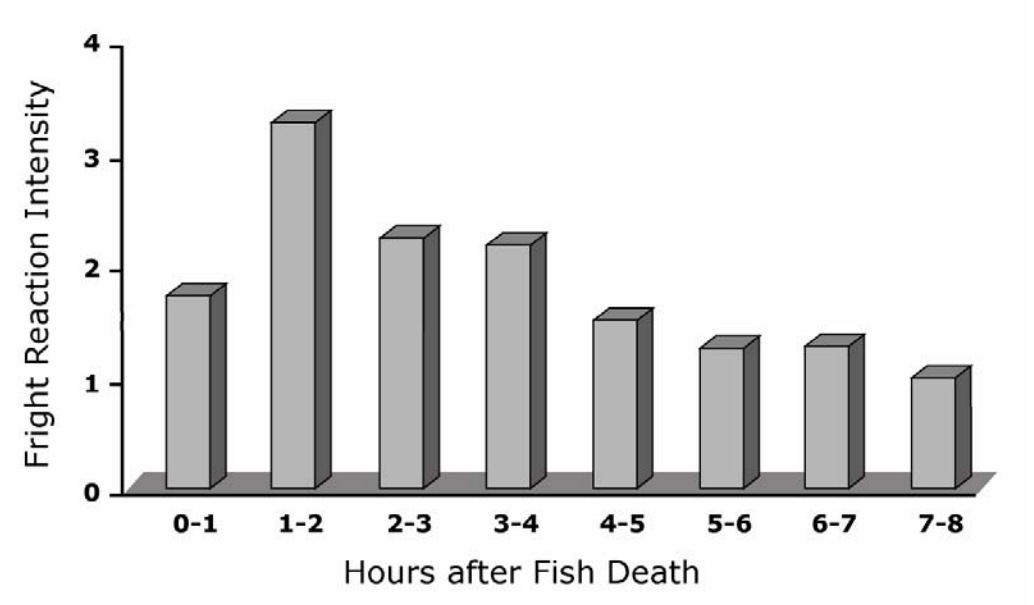

Figure 2. The fright reaction of European minnow, *Phoxinus phoxinus*, to water samples exposed for one hour to dead fish, depending on the elapsed time since fish death. Intensity scale 0-5.

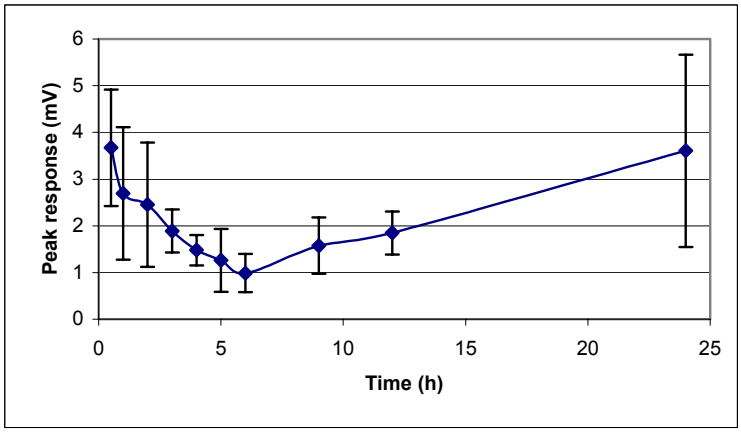

Figure 3. Electro-olfactogram responses of Crucian carp to the water of dead fish depending on the time elapsed since fish death.

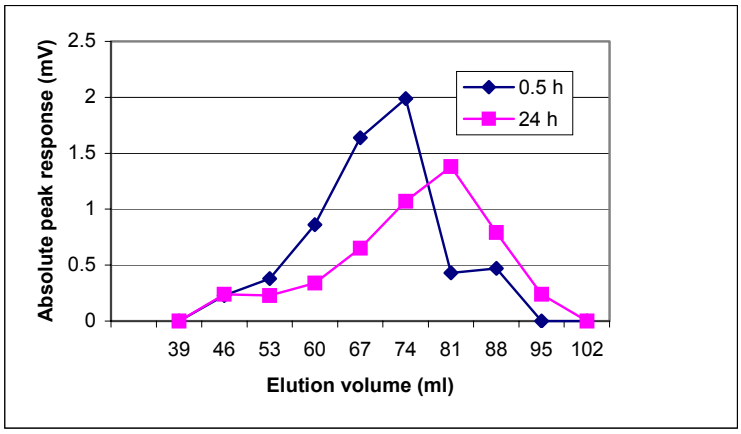

Figure 4. EOG responses in Crucian carp to the gel-chromatography fractions of dead fish water. Elution volume 42 ml = molecular weight 5000, 68 ml = MW 100; see Table 2.

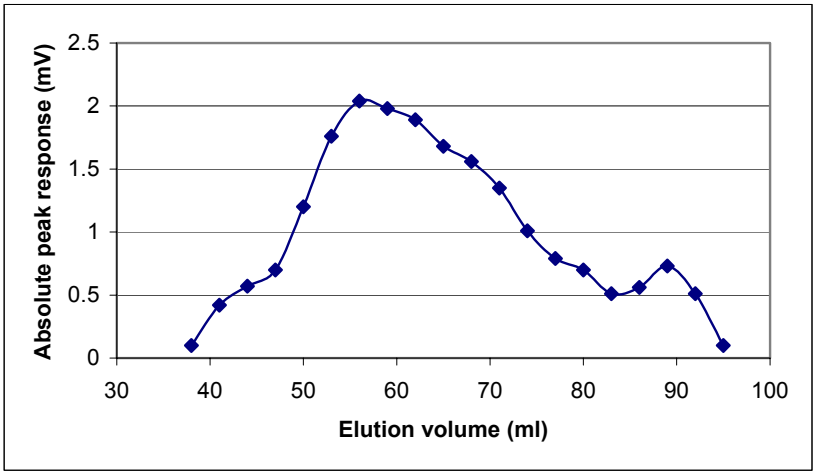

Figure 5. EOG responses in Crucian carp to the gel-chromatography fractions of fish skin. Elution volume 42 ml = molecular weight 5000, 68 ml = MW 100.

Chemical Substances Released by Dead Fish

Gel chromatography showed that water samples that contacted dead common carp contained a composite mixture of substances with different molecular weights (Figure 6). These substances differed in their release rate from dead fish. For example, the fraction with the highest-molecular-weight substances (more than 5,000) decreased in water samples during the first six hours then increased to the 24th hour. Fractions with the largest exit volume, 49-52 ml (MW less than about 200-300 Da), had a similar dynamic. Fractions with exit volume between 31-42 ml (MW 600-1200 Da) were most common in all samples (Table 2).

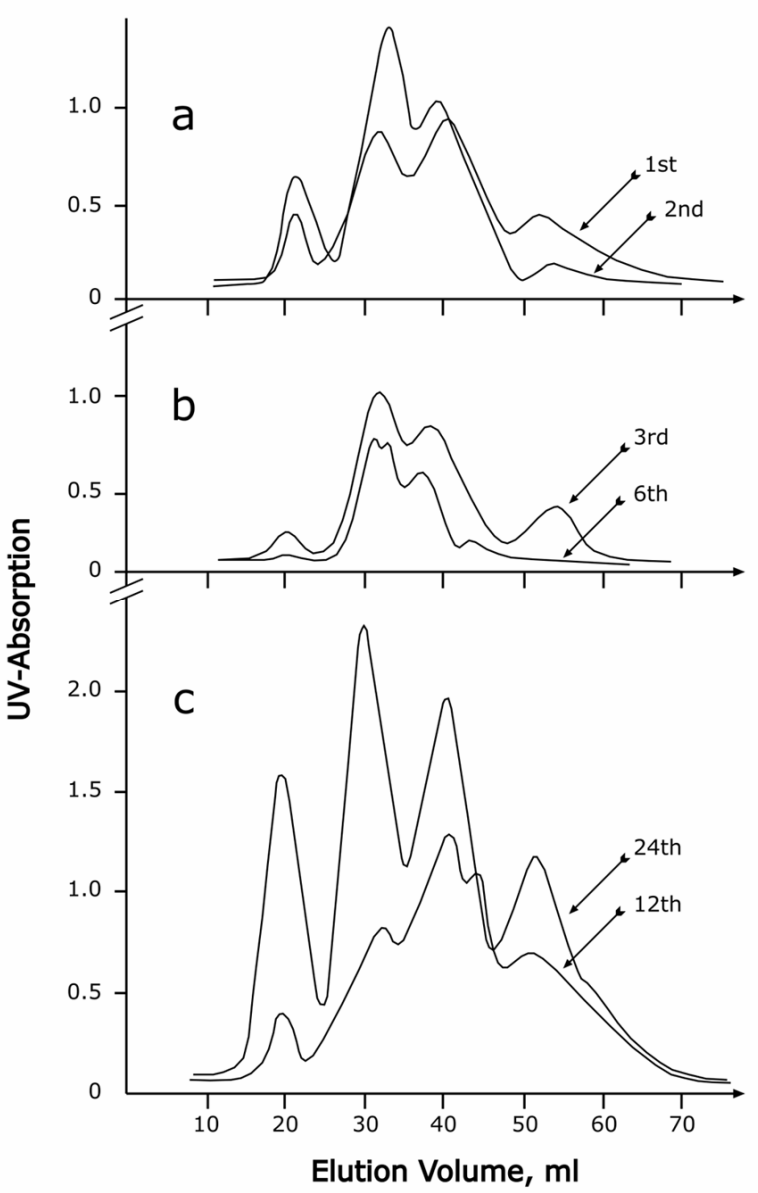

Figure 6. UV absorption @280 nm of chromatographically-separated fractions of water samples collected during one-hour time intervals after the death of common carp, *Cyprinus carpio*: a) first and second hours; b) third and sixth hours; c)12th and 24th hours.

Table 2. Chromatographic parameters for fractions obtained by sephadex G-25 separation of water samples obtained after exposure to dead common carp, *Cyprinus carpio* (n.e. – not estimated).

Time after fish death	Fraction peak, ml	Molecular weight	Peak area, %	Partition coefficient	UV-absorption, conventional units
1st hour	19	> 5,000	9.4	0	0.8
	31	~ 1200	29.6	0.8	1.7
	38	n.e.	46.2	1.26	1.8
	49	n.e.	14.8	2.0	0.8
2nd hour	19	> 5,000	7.0	0	1.25
	31	~ 1200	43.8	0.8	2.65
	37	n.e.	45.6	1.1	2.0
	50	n.e	3.6	2.1	0.3
3rd hour	19	> 5,000	1.6	0	0.4
	31	~ 1200	36.4	0.8	1.85
	37	n.e.	52.2	1.2	1.55
	52	n.e	9.8	2.2	0.6
6th hour	31	~ 1200	17.2	0.8	1.3
	32	~ 1100	37.9	0.86	1.3
	37	n.e	44.9	1.2	1.0
12th hour	19	> 5,000	2.7	0	0.8
	31	~ 1200	22.8	0.8	1.75
	40	n.e.	33.3	1.4	2.75
	42	n.e	20.9	1.7	2.3
	50	n.e.	19.4	2.1	1.5
24th hour	19	> 5,000	16.8	0	3.3
	31	~ 1200	32.1	0.8	5.0
	40	n.e.	31.7	1.4	4.25
	52	n.e	19.5	2.2	2.5

Table 3. Comparison of parameters for fractions obtained by sephadex G-15 separation of skin water extract of freshly killed European minnow *Phoxinus phoxinus* and skin water extract of dead minnow exposed in water for 14 and 25 hours (modified after Lebedeva et al. 1975).

Fraction peak, ml	Molecular weight	Peak area, %			Partition coefficient
		0 hour	14th hour	25th hour	
12	> 1500	30	53	77	0
19	~ 950-1200	51	28	23	0.4
35	~ 350-500	19	19	0	1.4

The number of peptide fractions was relatively low in water samples collected during the first six hours after common carp death and increased 3-4 times in the period between the 6th and 24th hours (Figure 7). The quantity of proteins in the water increased dramatically to the end of the first 24-hour period of dead fish exposure (Figure 8).

DISCUSSION

These results indicate that dead fish were a strong source of various chemical substances that appeared in the surrounding water shortly after fish death. The release rate differed strongly between substances and depended on their molecular weight. Relatively heavy molecules with molecular weight more than 5,000 were found to increase to the end of the first 24-hour period after fish death. In the same period, both the number of peptide fractions and the total amount of proteins rose strongly.

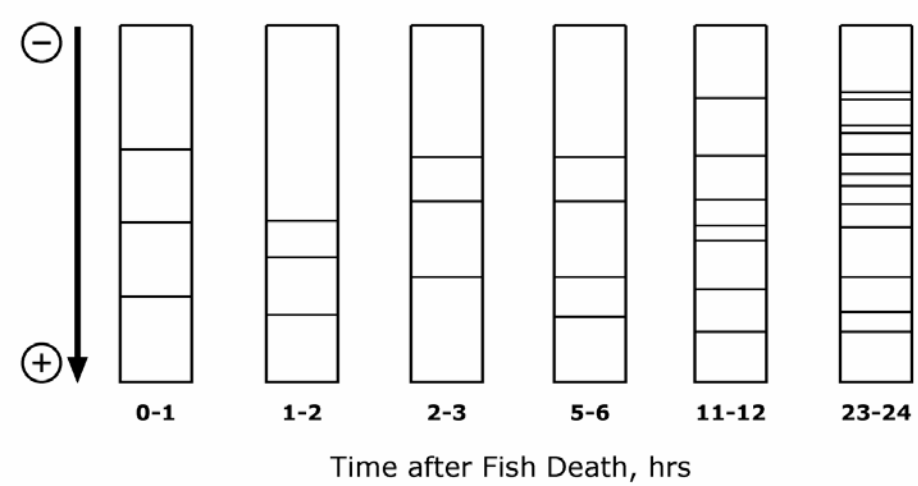

Figure 7. Electro-phoregram for dead common carp, *Cyprinus carpio*, water samples collected during the 1st, 2nd, 3rd, 6th, 12th and 24th hours after fish death.

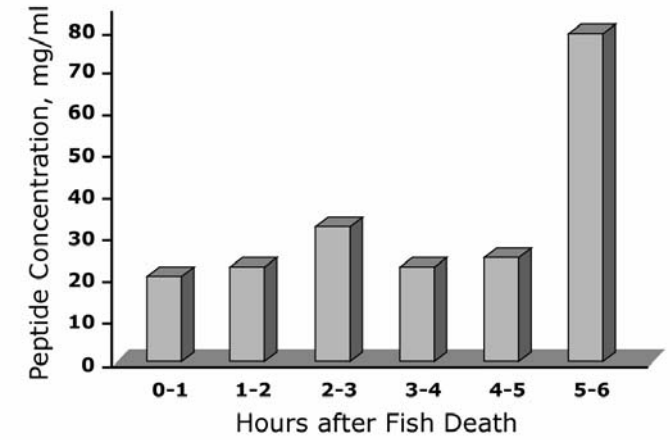

Figure 8. Peptide concentrations in dead common carp, *Cyprinus carpio*, water samples collected during the first, second, third, fourth, fifth and sixth hours after fish death. Data from 12 common carp with 80 g mean body weight, water volume 700 ml, water temperature 23°C.

Substances with relatively low molecular weight, like alarm pheromone, were released at a high rate earlier than peptides or proteins during the first several hours after fish death. The chemical nature of ostariophysian alarm pheromone is not yet known. There are two hypotheses: that alarm pheromone is either a pterine-like substance (hypoxanthine-3(N)-oxide) (Pfeiffer 1982) or a more complicated substance with molecular weight around 1100 (Kasumyan and Lebedeva 1979, Kasumyan and Ponomarev 1987).

Observation on the alarm pheromone appearance in the water surrounding the dead minnow showed that the samples collected during the first hour after fish death possessed repellent activity and evoked obvious fright reactions in fish. The alarm pheromone release process reached its maximum two hours after fish death, and then slowly decreased over several more hours. This finding corresponds well with data obtained earlier showing that skin of dead minnow rapidly loses repellent activity: skin water extract prepared from freshly killed minnow evoked the fright reaction much more strongly than extract prepared 14 and 25 hours later (Lebedeva *et al*. 1975). Sephadex G-15 chromatography revealed that substances with relatively low molecular weight decreased in the skin of dead minnow much faster than substances with higher molecular weight. These data indicated that the skin of dead fish is the source for substances that appeared in the water soon after fish death. Fish body mucus is another source, as was found by Wold and Selset (1978).

Based on results presented in Tables 2 and 3 and Figures 2 and 3, it is possible to suggest that there are two phases of substance release in the first 24 hours after fish death. In the first phase, relatively low-molecular-weight substances are released from body mucus and fish skin during the first 1-3 hours. The second phase begins about 12 hours after fish death, and is accompanied by the release of more diverse substances than in the first phase; the released substances may, in part, be products of large protein molecule destruction.

Experiments with electrophysiological methods also showed that substances with relatively low MW are released quickly from the skin of dead fish. After fish death the electrophysiologically most potent substances released from skin into the surrounding water had lower MW than substances from the skin extract. The substances released soon after fish death had higher MW than the substances released 24 hours after fish death.

Dead fish may cause fast augmentation of dissolved organic material into the surrounding water. In turn, this can cause a dissolved oxygen deficit that aggravates other unfavorable conditions, and could lead to massive fish mortality. Knowledge about dead fish impacts on the water condition and processes in the water ecosystem is still poor, and would benefit from more investigation.

REFERENCES

Adnan, Q. 1989. Red tides due to *Noctiluca scintillans* (Macartney) Ehrenb and mass mortality of fish in Jakarta Bay. Proceedings of the 1st International Symposium on Red Tides. Takamatsu, 1987.

Brawn, V.M. 1969. Feeding behaviour of cod (*Gadus morhua*). Journal of the Fisheries Research Board of Canada 26(3):583-596.

Gilbert, P.W., and C. Gilbert. 1973. Sharks and sharks deterrents. Underwater Journal 5:69-79.

Groop, B. 1975. Wohin sind all die Haie verschwunden? Delphin 22(9):9-12.

Haslouer, S. 1983. Natural pollution caused fish kills in Kansas during 1979-1980. Transactions of the Kansas Academy of Sciences 86:136-143.

Höglund, L.B., A. Bohman, and N.A. Nilsson. 1975. Possible odour responses of juvenile Arctic char (*Salvelinus alpinus* (L.)) to three other species of subarctic fish. Reports of the Institute of Freshwater Research, Drottingholm 54:21-35.

Kasumyan, A.O., and N.E. Lebedeva. 1979. New data on the nature of the alarm pheromone in cyprinids. Journal of Ichthyology 19(5):109-114.

Kasumyan, A.O., and V.Yu. Ponomarev. 1987. Biochemical features of alarm pheromone in fish of the order Cypriniformes. Journal of Evolutionary Biochemistry and Physiology 23:20-24.

Lebedeva, N.E., G.A. Malyukina, and A.O. Kasumyan. 1975. The natural repellent in the skin of cyprinids. Journal of Ichthyology 15(3):472-480.

Lowry, O.H., N.I. Rosebrougth, H.L Farr, and R.I. Randall. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193.

Merceron, M. 1988. Baie de Vilaine; juillet 1982, mortalité massive de poisons: l'analyse des causes et des mécanismes du phénomêné les propositions d'action. Equinoxe 21:4-9.

Michaelis, H. 1985. Strandungen der Brachsenmakrele *Brama brama* an der deutchen Nordseeküste nach einer Zählung vom Herbst 1979. Senkenbergiana maritima 17(1/3):25-31.

Mortensen, A.M. 1985. Massive fish mortalities in the Faeroe Islands caused by a *Conyaulax excavate* red tide. Proceedings of the 3rd International Conference "Toxic Dinoflagellates," St. Andrews, 1985. pp. 165-170.

Nikolski, G.V. 1963. The Ecology of Fishes. Academic Press, London. pp. 352.

Pfeiffer, W. 1982. Chemical signals in communication. *In:* Chemoreception in fishes, T.J. Hara (ed), Elsevier Science Publishers, Amsterdam, pp. 307-326.

Ravera, O., M.R. Dal, and S. Zarini. 1986. Un effetto dell'eutrofizzazione; la moria di pesci nel Lago di Comabbio. Acqua aria. 8:791-795.

Renaud, M. 1986. Hypoxia in Louisiana coastal waters during 1983: Fisheries. Fisheries Bulletin 84(1):19-26.

Riley, C.M., S.A. Holt, G.J. Holt, E.J. Buskey, and C.R. Arnold. 1989. Mortality of larval red drum (*Sciaenops ocellatus*) associated with a *Ptychodiscus brevis* red tide. Contributions in Marine Science 31:137-146.

Sanzin, D. 1989. Fischterben, 1987. Fischer und Teichwirt. 40(11) 320-324.

Tester, A.L. 1963a. Olfaction, gestation and the common chemical sense in sharks. *In:* Sharks and Survival. P.W. Gilbert (ed.), Boston, pp. 173-181.

Tester, A.L. 1963b. The role of olfaction in shark predation. Pacific Science 17(2):145-170.

von Frisch, K. 1941. Über einen Schreckstoff der Fischhaut und seine biologische Bedeutung. Zeitschrift vergleichenes Physiologie. 29(1):46-145.

Wold, J.K., and R. Selset. 1978. Glycoproteins in the skin mucus of the char (*Salmo alpinus* L.). II. Production of mucus after death of fish. Comparative Biochemistry and Physiology B 61:271-273.

Wootton, R.J. 1998. Ecology of teleost fishes. Kluwer Academic Publishers, Dordrecht.

Zimmer-Faust, R.K. 1989. The relationship between chemoreception and foraging behavior in crustaceans. Limnology and Oceanography 34:1367-1374.

Zimmer-Faust, R.K., and J.F. Case. 1982. Odors influencing foraging behavior of the California spiny lobster, *Panulirus interruptus*, and other decapod crustaceans. Marine Behavioral Physiology 9:35-58.

DEPRESSION OF LIPOLYSIS IN FISHES: A POSSIBLE HYPOXIA PROTECTION MECHANISM

Guido van den Thillart and Gerjanne Vianen[1]

ABSTRACT

Catecholamines are usually released under stress conditions, including hypoxia. These hormones have a strong lipolytic action in mammals. Under hypoxia, β-oxidation hardly proceeds, resulting in accumulation of fatty acids together with intermediates of β-oxidation. This process causes severe cellular damage in mammalian tissues, but not in fishes. In fishes, we even find a decrease of free fatty acids, despite the fact that both epinephrine and norepinephrine increase strongly under hypoxia. From both in-vivo and in-vitro studies with different fish species, we have found evidence that norepinephrine, in particular, inhibits lipolysis in adipose tissue via β_1-adrenoceptors. In addition, there is some evidence that β_2-adrenoceptors in the liver are involved in the stimulation of lipolysis. The suppression of lipolysis in fishes under hypoxia can be considered as a survival mechanism. For water-breathers, hypoxia is a natural phenomenon, while it is pathological for the air-breathing mammals.

INTRODUCTION

Hypoxia is a condition that occurs often in water bodies. This is mainly due to the limited solubility of oxygen in water and the much lower diffusion rate in water compared to the gas phase. While for air-breathers hypoxia is a pathological condition, it is often a regular phenomenon for water-breathers. Even when hypoxia occurs only a few times during a life cycle, it can act as a selection mechanism in evolution, and thus specific adaptations can be expected in water-breathers.

Hypoxia restricts maximal aerobic energy generation that declines rapidly at low oxygen levels (1-2 ppm). The critical oxygen level is the point at which the resting oxygen consumption becomes dependent on the ambient oxygen concentration (PO_2). This point is the ultimate lethal level (Fry 1947) since the oxygen extraction at this point is just enough for organism maintenance. Energy consumption must remain in balance with energy production, and below the critical PO_2 energy generation must come via anaerobic metabolism that causes lactic acid accumulation, or the animal can depress its metabolism below the standard metabolic rate (SMR). The first metabolic response is temporary and becomes lethal if prolonged; the second response inactivates the animal but extends survival time. It is obvious that hypoxia elicits many physiological responses, and also that an animal under hypoxia has several options to choose from. Hormones like catecholamines and cortisol increase in response to stress in mammals as well as in fishes. We expect that there are differences in the release and effect of these hormones between mammals and fishes with respect to hypoxia.

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Under normoxic conditions, lipids and proteins are the major fuels for the energy metabolism of teleost fishes, whereas carbohydrate catabolism appears to be of minor importance (Cowey and Walton 1989, Van den Thillart and Van Raaij 1995). However, under hypoxia and anoxia carbohydrate becomes the major substrate for energy metabolism. During hypoxia, glycogen stores are mobilized, resulting in hyperglycemia and elevated blood lactate levels (Van den Thillart and Van Raaij 1995).

Although lipid metabolism has been studied extensively in relation to nutrition, relatively little is known about the regulation of lipid metabolism in fish, certainly with respect to hypoxia. Free fatty acids (FFAs) are considered the most dynamic form of lipid transport from storage to the oxidation site in the different tissues. Triglycerides in the blood are considered as the principal form of transport to the storage site in the adipose tissues. Mobilization occurs in the adipocytes via activation of hormone sensitive lipase. The fatty acids diffuse into the plasma and bind to plasma proteins (albumin), and from there they diffuse into the different cell types for oxidation. The last steps of transport involve the esterification with CoA and transport into the mitochondria as carnitine ester. In the mitochondria, the CoA ester is regenerated from the carnitine ester, then broken down by β-oxidation. In mammals, lipolysis is stimulated by catecholamines. This process starts with activation of the β-adrenoceptors on adipose tissue. The transduction mechanism involves sequentially the increase of cAMP, activation of protein kinase A, and activation of hormone-sensitive lipase. Catecholamines are usually released under stress conditions, including hypoxia. Under the latter condition, lipid mobilization is, however, not very useful since β-oxidation is impaired due to oxygen shortage. The combination of phospholipid hydrolysis, inhibition of β-oxidation, and the lipolytic action of catecholamines causes an increase of the plasma and tissue fatty acid levels. These fatty acids and end products of β-oxidation make biomembranes leaky and ultimately cause major cell and tissue damage (Katz 1981, Moore 1985, Hoekstra and Golovina 2002).

TELEOSTS AND MAMMALS HAVE CONTRASTING HYPOXIA RESPONSES

During hypoxia, exposure glucose levels have been shown to increase, but free fatty acid levels decrease in the blood of carp (van Raaij *et al*. 1996a), trout (van Raaij *et al*. 1996a/1996b, Vianen 1999), and tilapia (Vianen *et al*. 2002). Severe hypoxia caused the epinephrine levels in carp plasma to rise from almost zero (0.04) to 2 ng/ml and norepinephrine from zero (0.25) to 50 ng/ml. The effect on the catecholamine levels in trout was different: epinephrine increased from 0.06 to 12 ng/ml, and norepinephrine increased from 0.5 to 9 ng/ml. At the same time, there was a strong decline of plasma FFA levels in carp and a small decline in the plasma of trout. These observations are quite contrary to the response in mammals. Since under hypoxia a simultaneous increase of catecholamines was observed together with a decline in fatty acids, it was assumed that these hormones are involved in the inhibition of lipolysis. This was tested by infusion experiments (van Raaij *et al.* 1995). Carp were cannulated in the dorsal aorta and after two days of recovery a saline solution containing norepinephrine or epinephrine was infused for 90 minutes, such that the plasma levels were increased to about 50 ng/ml. The effect of both infusions was a transient increase of eight mM glucose that peaked shortly after the infusion and reached control values at about eight hours after the infusion. The effect on plasma FFA levels differed between epinephrine and norepinephrine. While the first caused an increase of FFA

levels, norepinephrine within 15 minutes caused a significant decline that lasted for about eight hours after infusion.

These short-term hypoxia exposure trials suggested a controlling effect of catecholamines. The hypoxia effects on metabolites and catecholamines, however, were transient. Therefore, prolonged hypoxia of 48h was applied to cannulated carp and trout (Vianen 1999). Carp were exposed to 20% air saturation and trout to 40% saturation at 20° and 15°C, respectively. Figure 1 shows the changes in hormone levels in carp. Epinephrine increased from 0.05 to 0.6 ng/ml, then declined to control levels within three hours. In contrast, norepinephrine increased from 0.12 to 3.9 ng/ml, and stayed at that level for the whole period of 48 hours. A similar pattern was observed in trout: the epinephrine increased from 0.04 to 0.4 ng/ml and the norepinephrine increased from 0.17 to 4 ng/ml. Both hormones increased continuously for about six hours, and thereafter remained at the elevated level. For about 50% of the trout the condition was lethal. Those animals that died showed in an early phase increased levels of catecholamines and lactate. In both carp and trout, the plasma fatty acid levels dropped quickly after the onset of hypoxia and remained at about 50% of the control value throughout the whole hypoxic period. These results show that norepinephrine is likely the controlling factor inhibiting lipolysis during hypoxia.

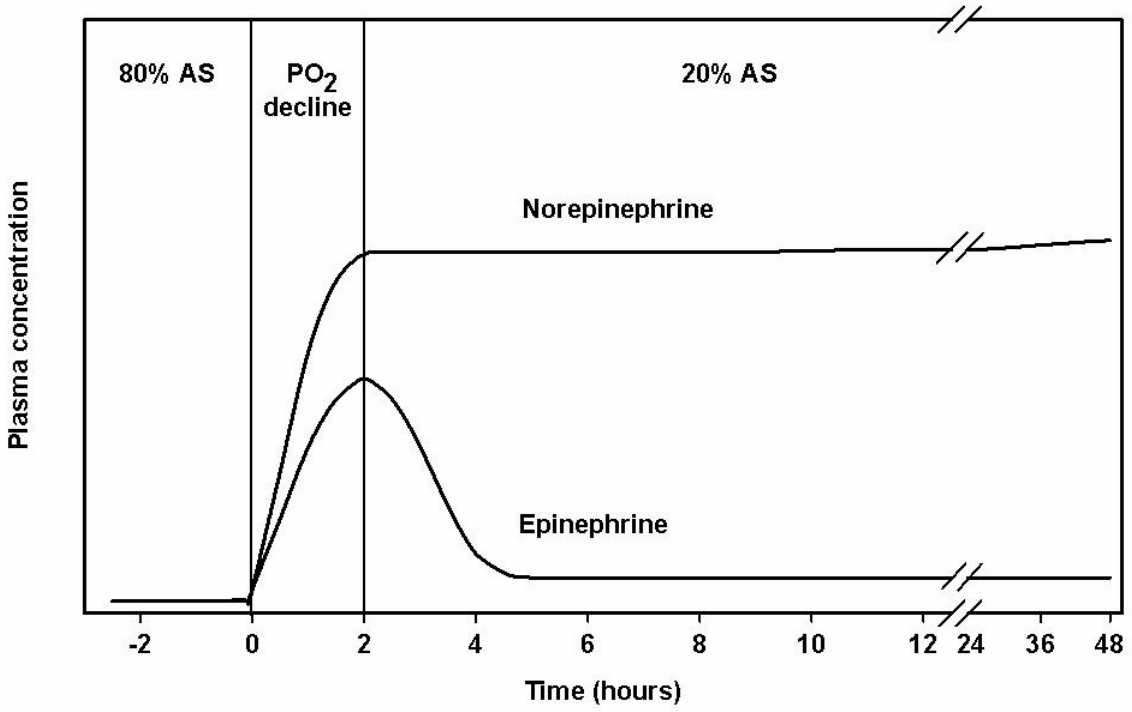

Figure 1. The effect of chronic hypoxia on the catecholamine levels in carp. Carp kept at 20°C were cannulated in the dorsal aorta. After two days' recovery at > 80% air saturation (AS), the oxygen level was decreased in a linear fashion over two hours from 80% to 20%. Blood samples were taken at regular intervals over a period of 48h for measurement of glucose and free fatty acids (see text).

MECHANISMS OF ADRENERGIC CONTROL OF LIPOLYSIS

The opposite effects of epinephrine and norepinephrine suggest an action via both β- and α_2-adrenoceptors, since norepinephrine has a higher affinity for α_2-adrenoceptors while epinephrine has a higher affinity for β-adrenoceptors (Lafontan *et al.* 1997). The general transduction mechanism of both adrenoceptors is depicted in Figure 2. The binding to $ß_1$-and/or $ß_2$-adrenoceptors causes, sequentially, activation of Gs proteins, activation of adenylate cyclase, increase of cAMP, and activation of protein kinase A. Protein kinase A in turn activates hormone sensitive lipase (HSL) that induces lipolysis. The function of this multi-step transduction process is an amplification of the signal by a factor of about 10^{10}. Inhibition by catecholamines is possible via α_2-adrenoceptors that bind to Gi-proteins. These Gi-proteins prevent the production of cAMP by inhibiting adenyl cyclase. This mechanism could explain the stimulation of lipolysis by epinephrine as well as the inhibition of lipolysis by norepinephrine. The opposite effects of the two hormones may result from selective binding to different adrenoceptors on different cell types. Adipose tissue and liver are the most important with respect to mobilization of lipids (Sheridan 1988, 1994). Therefore, proof must come from experiments with specific agonists and antagonists in combination with in-vitro studies. In several studies, the mechanism was analyzed using specific α_2- and $ß_{1/2}$- agonists and antagonists: norepinephrine (α- and ß- agonist), isoproterenol (ß- agonist), clonidine (α_2-agonist), yohimbine (α_2- antagonist), ICI-118.551 ($ß_2$- antagonist), and atenolol ($ß_1$- antagonist).

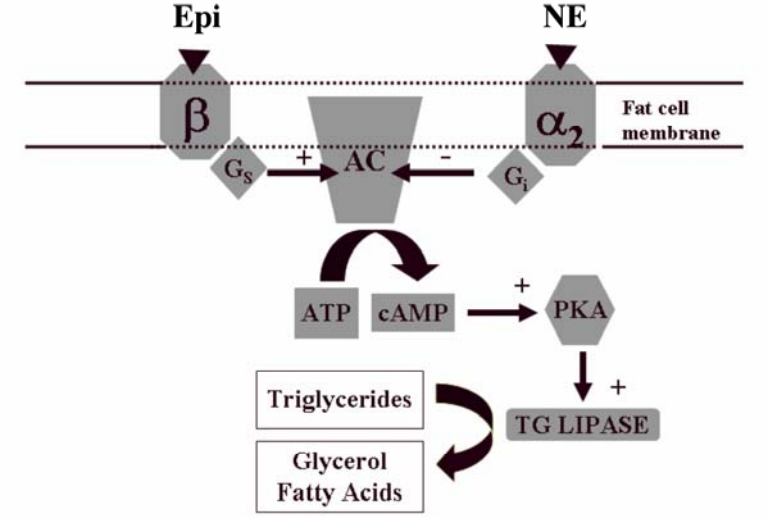

Figure 2. Control of lipolysis by epinephrine (Epi) and norepinephrine (NE). Epinephrine has a higher affinity for β-adrenoceptors, while norepinephrine has a higher affinity for α_2-adrenoceptors. Gs proteins stimulate adenylcyclase (AC) via β-activation, while α_2-activation stimulates Gi proteins to inhibit adenylcyclase. The increase of cAMP activates protein kinase A (PKA) that, in turn, activates (via phosphorylation) triglyceride lipase. TG lipase or hormone sensitive lipase is translocated to the lipid droplets where it gets access to the stored triglycerides.

IN-VIVO EXPERIMENTS

Infusion experiments with carp were carried out using different saline solutions (van den Thillart *et al*. 2001). The first series tested the involvement of α_2-adrenoceptors. The animals were infused for 1.5h with norepinephrine, norepinephrine + yohimbine (α_2- antagonist), and clonidine (α_2- agonist). Upon infusion with norepinephrine, plasma glucose levels began to rise and FFA levels fell, reaching +60% for glucose and -50% for FFA at about 2h after starting (Figure 3). When the animals were previously injected with yohimbine the glucose response did not differ, but the decline of FFA was retarded. Thus, during infusion with norepinephrine FFA only decreased by about 10%. The same inhibition as without norepinephrine was reached after five hours, suggesting that the effect of yohimbine wears off in a few hours. Infusion with the α_2-agonist clonidine resulted in a transient 10% decline of plasma FFA, independent of the applied concentration. This suggests that α_2-adrenoceptors can be involved only indirectly, possibly via an effect on tissue perfusion.

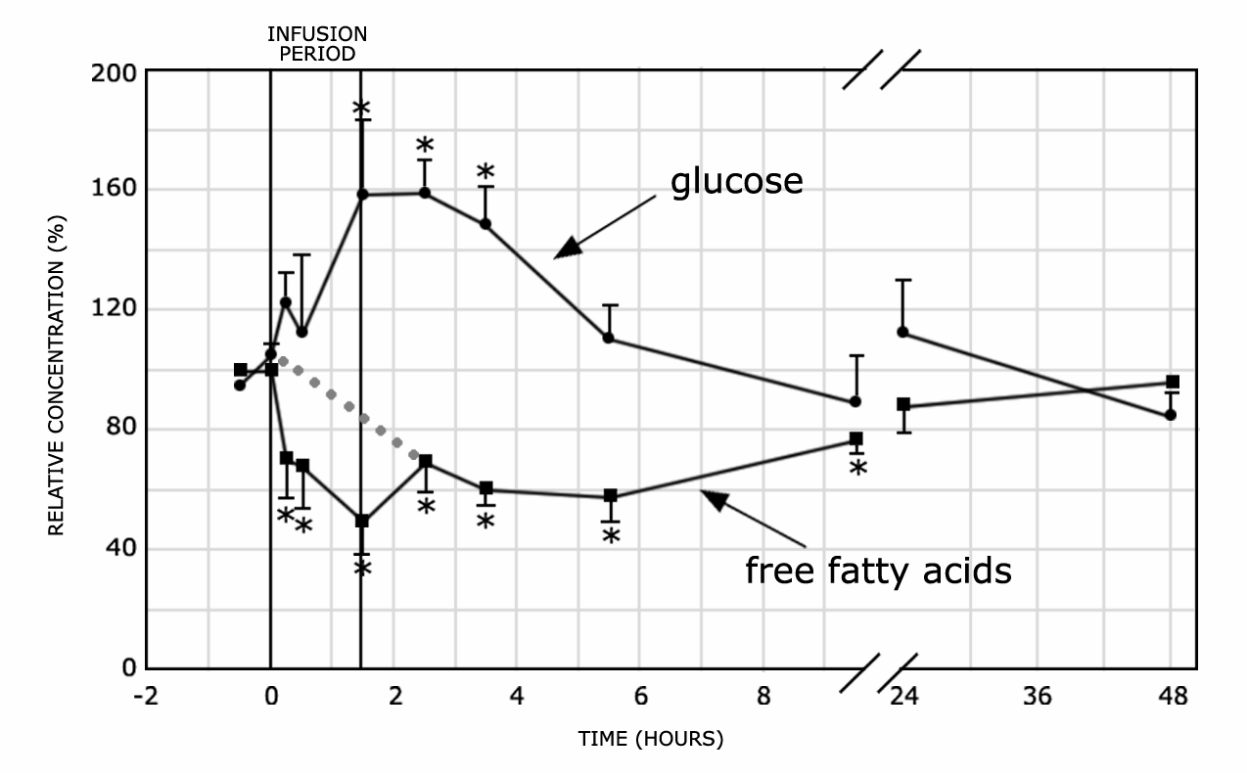

Figure 3. Effect of infusion with norepinephrine on plasma glucose and fatty acid levels in cannulated carp. Norepinephrine was infused during 90 minutes. The dashed line shows the FFA decline after previous injection with the α_2-blocker yohimbine. Yohimbine had no effect on glucose levels. Significant differences, $P<0.05$, infusion vs control group, are denoted by *.

Stimulation of β-adrenoceptors is mediated by the β-selective agonist isoproterenol. Infusion of isoproterenol according to the same protocol as with norepinephrine showed a similar stimulation of glucose release but inhibition of FFA release to the same magnitude and speed as with norepinephrine. This unexpected result suggests that the direct action of norepinephrine on inhibition of lipolysis is via β-adrenoceptors and not via α_2-adrenoceptors.

To determine the type of β-adrenoceptor involved, we applied ICI-118.551 (a β_2-antagonist) or atenolol (a β_1-antagonist) in combination with isoproterenol. The combination shows the respective actions of β_1- or β_2-stimulation. The strong increase of plasma glucose stimulated by isoproterenol appears to be attenuated by both antagonists in the same way: the peak value is about 10% lower and the control levels are reached two hours earlier, *i.e.* six instead of eight hours after the onset of infusion. This suggests that β-stimulation of glycogenolysis (in the liver) is mediated by both β_1- and β_2-adrenoceptors. A different picture can be seen with plasma FFA levels: the decline of plasma FFA by isoproterenol was deeper with the addition of ICI-118.551, while the addition of atenolol caused an increase of plasma FFA levels. This result suggests that there are two opposite actions on FFA release. The stronger decline by addition of ICI-118.551 shows an inhibitory action of β_1-adrenoceptors that are likely located on cell membranes of adipose tissues. The opposite effect of atenolol, showing an increase of plasma FFA, suggests that β_2-adrenoceptors (likely located on liver cells) can stimulate lipolysis.

IN-VITRO EXPERIMENTS

To verify the hypothesis that β-stimulation leads to inhibition of lipolysis in adipose tissue, we carried out experiments with isolated adipocytes from tilapia mesenteric fat tissue (Vianen et al. 2002). These experiments confirmed that norepinephrine reduces the FFA release of adipocytes at concentrations of $\geq 10^{-7}$M. No effect of phentolamine ($\alpha_{1,2}$-antagonist) was observed, indicating that no α-adrenoceptor was involved. On the other hand, a clear effect of timolol ($\beta_{1,2}$-antagonist) showed that the inhibition was caused by a β-adrenoceptor. We also found evidence for inhibitory β_3-adrenoceptors; however, since they are activated only at concentrations of isoproterenol $\geq 10^{-5}$ M, it is unlikely that they have physiological relevance. With other fish species (trout, catfish, and sea bream), we verified that activation of β-adrenoceptors on adipocyte membranes results in reduced release of fatty acids (van Heeswijk, Van den Thillart, Vianen, unpublished results).

CONCLUDING REMARKS

Knowledge of lipid metabolism in fishes, particularly its hormonal control, is limited. Recent findings show that fish lipid mobilization, in particular, operates opposite to that in mammals. While in mammals both epinephrine and norepinephrine stimulate lipolysis, we found clear evidence that norepinephrine inhibits fish lipolysis mediated by β_1-adrenoceptors. We assume that the general occurrence of the inhibiting β-adrenoceptors in adipose tissue of

fishes is a hypoxia survival mechanism. Fish can easily be confronted with environmental hypoxia. In combination with increased levels of stress hormones and a strongly reduced capacity for β-oxidation, the accumulation of lipophilic compounds can become life threatening.

ACKNOWLEDGEMENT

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REFERENCES

Cowey, C.W., and M.J. Walton. 1989. Intermediary Metabolism. *In*: Fish Nutrition. J.E. Halver (ed.) New York, Academic Press.

Fry, F.E.J. 1947. Effects of the environment on animal activity. University of Toronto Studies in Biology Series 55:1-62.

Hagve, T.A., H. Sprecher, and C.M. Hohl. 1990. The effect of hypoxia on lipid metabolism in isolated adult rat cardiac myocytes. Journal of Molecular and Cellular Cardiology 22:1467-1475.

Hoekstra, F.A., and E.A. Golovina. 2002. The role of amphiphiles. Comparative Biochemistry and Physiology A 131:527-533.

Katz, A.M., and F.C. Messineo. 1981. Lipid membrane interactions and the pathogenesis of ischemic damage to the myocardium. Circulation Research 48:1-16.

Lafontan, M., P. Barbe, J. Galitzky, G. Tavernier, D. Langin, C. Carpéné, A. Bousquet-Melou and M. Berlan. 1997. Adrenergic regulation of adipocyte metabolism. Human Reproduction 12:6-20.

Moore, K.H. 1985. Fatty acid oxidation in the ischemic heart. Molecular Physiology 8:549-564.

Sheridan, M.A. 1988. Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. Comparative Biochemistry and Physiology B 90:679-690.

Sheridan M.A. 1994. Regulation of lipid metabolism in poikilothermic vertebrates. Comparative Biochemistry and Physiology B 107:495-508.

Van den Thillart, G., and M. Van Raaij. 1995. Endogenous Fuels: Noninvasive Versus Invasive Approaches. *In*: Biochemistry and Molecular Biology of Fishes. Volume 4:33-63. Hochachka and Mommsen (eds), Elsevier Science BV. Amsterdam.

Van den Thillart G., G. Vianen, M. Campos Ponce, H. Leleiveld, M. Nieveen, M. Van Raaij, A. Steffens, and J. Zaagsma. 2001. Differential role of adrenoceptors in control of plasma glucose and fatty acids in carp, *Cyprinus carpio* (L.). American Journal of Physiology 281:R615-624.

van Heeswijk R., G. van den Thillart, and G. Vianen. β-Adrenergic stimulation of lipolysis in fish adipocytes. (unpublished)

Van Raaij, M., G. Van den Thillart, D. Pit, P. Balm, and A. Steffens. 1996a. Substrate mobilization and hormonal changes in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) during deep hypoxia and subsequent recovery. Journal of Comparative Physiology (B) 166:443-452.

Van Raaij, M.T.M., D.S.S. Pit, P.H.M. Balm, A.B. Steffens, and G. Van den Thillart. 1996b. Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. Hormones and Behavior 30:85-92.

Van Raaij, M.T.M., G. Van den Thillart, M. Hallemeesch, P.H.M. Balm, and A.B. Steffens. 1995. Effect of arterially infused catecholamines and insulin on plasma glucose and free fatty acids in carp. American Journal of Physiology 2768:R1163-1175.

Vianen, G.J. 1999. Hormonal control of energy metabolism in teleost fish during hypoxia. Ph.D. Thesis. Leiden University.

Vianen, G.J., P.P. Obels, G. Van den Thillart, and J. Zaagsma. 2002. β-Adrenoceptors mediate inhibition of lipolysis and adipocytes of tilapia (*Oreochromis mossambicus*). American Journal of Physiology 282:E318-325.

WATER QUALITY CONDITIONS OF NEVA BAY AND THE EASTERN GULF OF FINLAND

B.G. Skakalsky[1]

ABSTRACT

This study is based on data obtained during long-term observations of the Russian state monitoring network and the results of various expeditions by Russian research institutes and joint Russian-Finnish research on the ecological situation of the eastern Gulf of Finland. The Gulf of Finland has been estimated to be one of the most pollution-loaded areas within the Baltic Sea. In addition, the eastern part of the Gulf of Finland is the arena of interaction of fresh waters of the Neva River and brackish waters of the Gulf of Finland. The intensity of this interaction is affected by the variability of the Neva discharge and the dynamics of water macrocirculation in open areas of the Gulf of Finland. Long-term, complex hydrological-ecological studies have established the basic characteristics of the functioning water system under the influence of man's activities in the basin. Principle among these are wastewater discharge from St. Petersburg and other populated areas, and completion of the dam complex for protection of the city from floods. Due to an excessive inflow of nutrients into the eastern Gulf of Finland, there is anthropogenic eutrophication. The most poisonous blue-green algae blooms occur in June-August along the entire coast behind the site of the flood protection complex, and expand westward into the open part of the Gulf. Accumulations of thread-like and blue-green algae during these months considerably degrade the water quality, often making it impossible to use the beaches for recreation.

INTRODUCTION

About 80 million inhabitants live in the watershed of the Baltic Sea. They are residents of 10 industrially-developed countries who conduct a significant part of their foreign trade by overseas transport, and actively use those marine resources.

As a result of excessive anthropogenically-induced loading, the ecosystem of the Baltic Sea has been polluted and gradually degraded since the mid-1960s. According to hydrobiological examinations, at the end of the 1960s there was a transition of the marine ecosystem from oligotrophic to mesotrophic, testifying to the accelerated eutrophication of the Baltic Sea (HELCOM 1996, Wulff 1991, Savchuk 2002).

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Hydrographic and hydrometeorological features of the Baltic Sea boost the sensitivity of its ecosystem to human actions. A vertical stratification of the water body into two basic strata is typical for the Baltic Sea and of essential ecological importance. These strata are the well-aerated upper desalinated waters, and a lower stratum with an elevated salt content and oxygen deficit.

These factors render an adverse influence on the self-purification ability of the marine environment for pollutants of anthropogenic origin, and create conditions for their accumulation within the ecosystem. Monitoring data from the Baltic Sea show that the pollution structure of the aquatic environment has not remained constant, but has undergone changes related to the implementation of protection measures. For example, after passage of recommendations of the Helsinki Convention on Protection of the Marine Environment of the Baltic Sea Region (1974), a decrease in the level of oil pollution of the sea took place. Now most experts consider the processes of eutrophication and accumulation of heavy metals as the most dangerous to the ecology of many regions of the Baltic Sea (HELCOM 1996).

One of the most ecologically-compromised regions of the Baltic Sea is the Gulf of Finland that receives enormous volumes of wastewater from the territories of the Russian Federation, Finland and Estonia (HELCOM 1998). A general scheme of the Gulf of Finland is shown in Figure 1. The eastern part of the Gulf is the arena of interaction of fresh Neva River waters and brackish waters of the Baltic Sea. This interaction is influenced by a number of factors, most notably the variability of Neva River runoff and the dynamics of macrocirculation of the waters in the open Gulf.

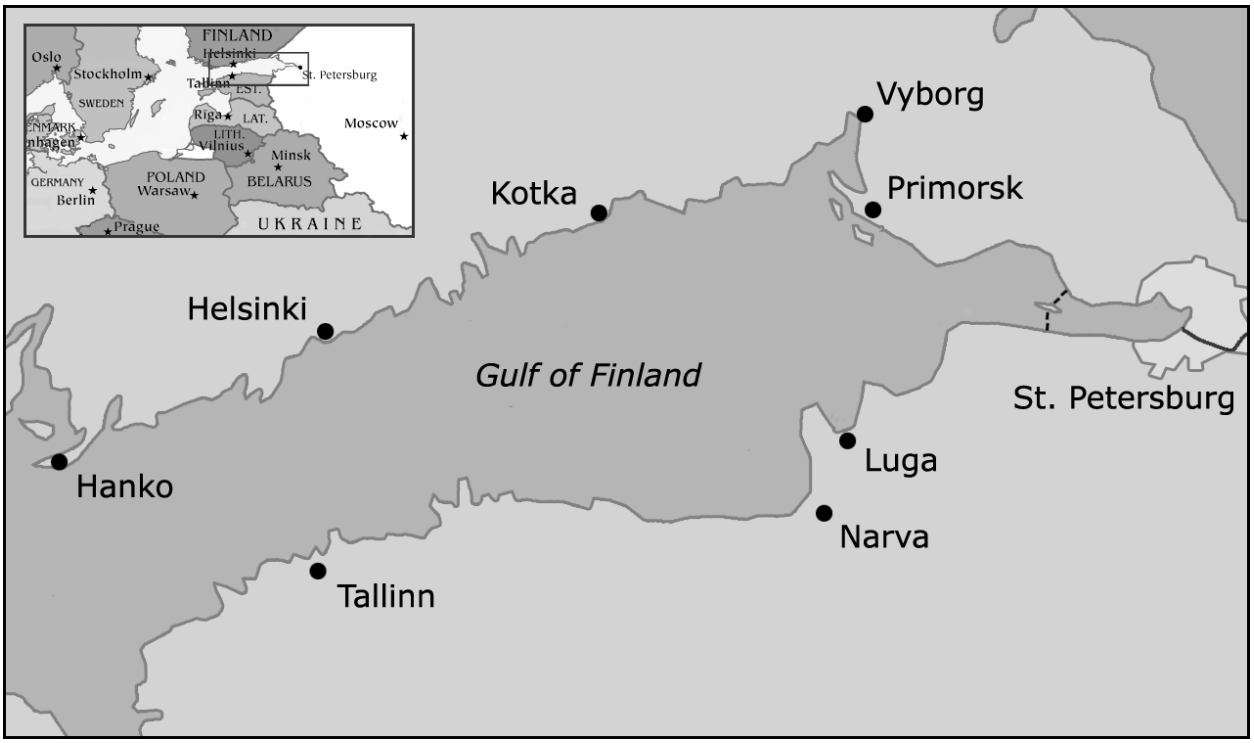

Figure 1. The Gulf of Finland

In the anthropogenic loading of pollution to its waters, a special role is played by the watershed of Lake Ladoga - River Neva - Neva Bay that supplies about 70 % of the inflow to the Gulf (Figure 2). The Neva carries wastewater from the territory of St. Petersburg, the largest city on the Baltic Sea coast with a population of about five million. The total volume of wastewater coming from the St. Petersburg area to the Gulf of Finland is about three million m^3 per day, including about one million that has not been treated.

The lower Neva Bay water system is the most polluted area of the Gulf of Finland, indeed, of the entire Baltic Sea. The inflows of polluted water coming out of Neva Bay render considerable influence on the chemical and biological state of the entire eastern Gulf of Finland. They can be seen in images taken from space and in shipboard measurements, extending westward to the borders of the territorial waters of Russia (Pitkanen *et al*. 1993).

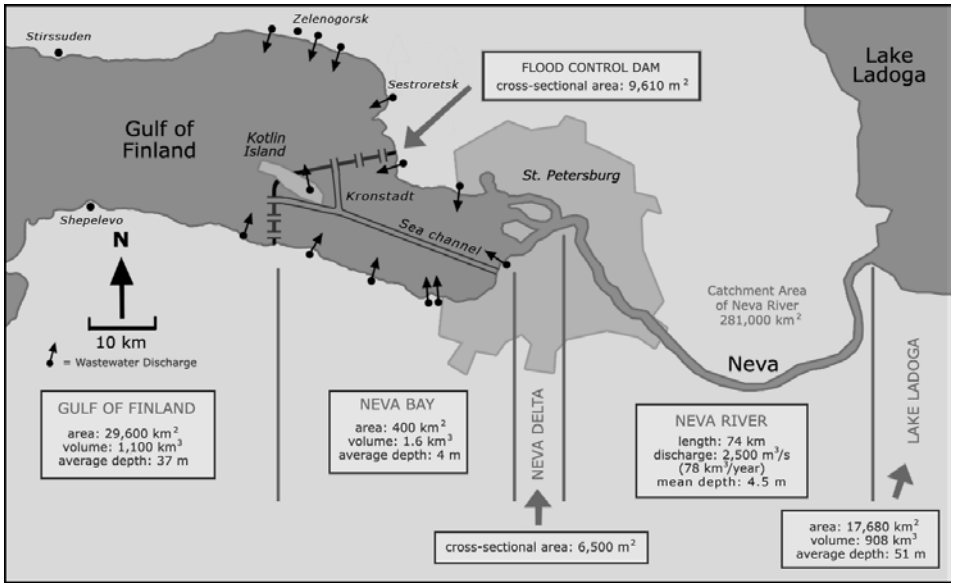

Figure 2. The Neva Estuary and the inner Gulf of Finland

MATERIALS AND METHODS

In the present paper, the study area is divided into two parts according to hydrographic characteristics. To the east is the shallow Neva Bay, 400 km^2 in area, with a depth of 3-5 m. The Bay is bounded on the west by a flood protection barrier (the dam) that is perforated by sea gates in its northern and southern portions. To the west of the dam is the eastern Gulf of Finland. The innermost part of the Gulf, described in this paper, has a surface area of 9000 km^2 and an average depth of 37 meters.

Our study is based on data from 35 monitoring stations in the marine waters. Also cited are data obtained in joint Finnish-Russian research expeditions by the Finnish R/V Muikku during 1990-96 (Pitkanen *et al*. 1993). Other data were collected by expeditions of the State

Hydrological Institute (St. Petersburg) and Russian State Hydrometeorological University (RSHU) during 1982-2001 (Nekrasov *et al.* 2002). The frequency of sampling was 2- 4 times in summer and twice during winter, as a rule. The basic water quality parameters (nutrients, oxygen, pH, salinity, heavy metals etc.) and biological indices were analyzed from discrete samples using standard analytical methods (HELCOM Recommendations 1988, 1994).

RESULTS AND DISCUSSION

Hydrography: The water quality in Neva Bay is determined by a number of factors of both natural and human origin: the quality of Lake Ladoga water, anthropogenic loading to the River Neva above St. Petersburg, wastewaters from the territory of St. Petersburg, natural processes within the Bay, and the constructed flood protection barriers joining the coasts with Kotlin Island. The most important natural factor is the mixing of fresh river waters with slightly-brackish waters: in the hydrochemical regime of Neva Bay the influence of brackish waters of the Gulf of Finland is clear. Inflow of brackish waters occurs to various degrees depending on hydrometeorological conditions. The volume of such inflows as a proportion of the total volume of water in the bay is rather insignificant. These inflows influence the hydrochemical characteristics of the bay on the whole only weakly, essentially having an effect on water salinity only in a band south of the sea channel, where the average salinity rises to 0.2‰.

The determinants of water quality in the eastern Gulf of Finland from Kronstadt west to the Stirssuden – Shepelevo region (the western border of the inner Neva estuary) have the following features. First, unlike in Neva Bay the depth in the central part of this region reaches 12-30 m. Second, the transitional type of water exchange characteristic of Neva Bay is replaced here by water exchange of a marine type where the vertical exchange is controlled by a salinity gradient and there is a current westward from the mouth of the Neva River. The northern part of this region is strongly influenced by anthropogenic sources of pollution from the northern coast because of the limited water exchange. This polluted region includes a strip 1-2 km wide stretching between the cities of Sestroretsk and Zelenogorsk (see Figure 2).

Salinity: The spatial distribution of salinity in Neva Bay is characterized by considerable inhomogeneity. The least saline water is observed in the northern part of the Bay, north of the sea channel and the city of Kronstadt, west to the northern gate of the dam. Through almost this entire region the salinity is that of the Neva waters. Immediately adjacent to the northern gate it rises to 0.1‰.

In the southern part of the bay there is a greater increase in salinity, and in the region of the southern gate it reaches on average 0.15-0.25‰. The waters of Neva Bay for the most part are Ca-HCO_3 type waters, like the Neva River. From east to west the concentrations of chloride and sodium increase gradually, and near the sea gates the ionic composition of the water assumes features of the brackish waters of the Gulf of Finland. The salinity increases sharply to the west of Kotlin Island, reaching 2-3‰ in the surface layer and 4-6 ‰ at the bottom in the vicinity of the cape of Stirssuden.

Nutrients: In the chemistry of Neva Bay waters and the inner Gulf of Finland the most dynamic components are nutrients, in which seasonal changes are well expressed. Nitrogen compounds prevail, with concentrations approximately an order of magnitude higher than those of phosphorus (Table 1).

Table 1. Mean concentrations of nutrients in the surface waters of Neva Bay and the Eastern Gulf of Finland, 1997-98.

Component	Neva Bay	Eastern Gulf of Finland
Total nitrogen, µg/L	**769**	**748**
Total phosphorus, µg /L	**31**	**31.5**
N_{tot} /P_{tot}	**24.8**	**23.7**

In Neva Bay, the content of nitrate- and nitrite-nitrogen is lowest in the center of the Bay and highest along the southern coastline, especially in the southeast, where zones of nitrite pollution appear regularly. There, the concentration of nitrite-N is greater than Russia's Maximum Permissible Concentration (MPC) of 20 µg /L. The same zones of pollution are observed near wastewater outfalls of municipal treatment plants.

During the warm months, the nitrogen concentration in the central Bay varies within rather narrow limits (700-800 µg /L), increasing slightly in the direction of coastal zones with limited water exchange. In Neva Bay, the organic forms of nitrogen prevail, and among the mineral forms the obvious prevalence of oxidized compounds - nitrates - is measured. Along both coasts of the Bay there are bands with the nitrate content elevated 25-75 % over that measured in the center of the Bay.

The major ammonia pollution (NH_4 -N > 390 µg/L) is found in a band to the south of the sea channel. The extremely high values exceed by 2-3 times Russia's Maximum Permissible Concentration.

The seasonal trends of all nutrient concentrations are characterized by peak values in winter, tied to impairment at this time of the biological processes of self-purification. This is shown most sharply for ammonia-nitrogen. Thus, in the vicinity of the southern and northern gates of the Bay the winter concentrations of NH_4-N exceed summer concentrations by 3 to 11 times. In the central Bay, this excess is more moderate - about 2 times. This is the result of weakened oxidation processes in winter, when wastewater nitrogen is predominantly in reduced forms.

Moving westward from the central Neva Bay to the central zone of the eastern Gulf there is a well-expressed reduction of concentration in both mineral and organic nitrogen. This general trend does not include nitrite pollution that remains at the same level in the Gulf as in the western Neva Bay. The northern coastal zone shows considerably higher concentrations of nitrogen compounds than the central region of the inner Gulf. The MPC value of 20 µg NO_2-N/L is consistently exceeded in the northern coastal zone.

A typical feature that distinguishes the deep-water, central Gulf of Finland from the shallow Neva Bay is the well-expressed vertical stratification of phosphorus concentration related to its mineral form (Figure 3). The concentration of mineral phosphorus in the bottom layer is always higher than at the surface. In deeper waters this excess measures about 2-5 times.

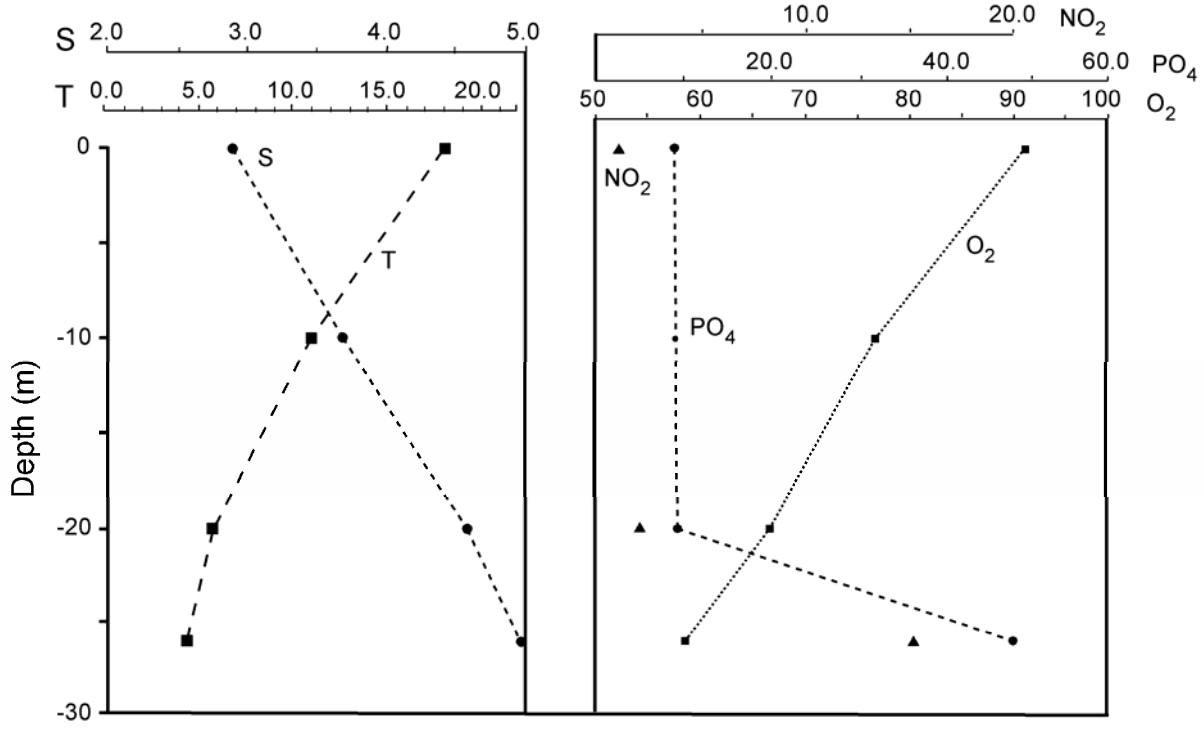

Figure 3. Vertical distribution of temperature (T, ^{o}C), salinity (S, ^{o}/oo), NO_2-N (µg/L), PO_4-P (µg/L) and O_2 (% saturation) at a station in the southern Gulf of Finland near Luga Bay. Sampling occurred during the month of August.

Based on long-term observations in open parts of the Gulf of Finland, it has been found that time trends of phosphorus concentration are well expressed. These are positive in the surface layers (*i.e.* increasing with time) and negative at a depth of 70 m. The increasing phosphorus concentration in the upper, productive layer testifies to the hydrochemical conditions for acceleration of eutrophication in the Gulf of Finland (Savchuk 2002).

Organic Matter: Compared to the Gulf of Finland proper, a distinctive hydrochemical feature of Neva Bay is the elevated content of dissolved organic matter, originating from natural organic matter in Neva River water and the discharges from the cities. The zones of high organic matter (exceeding the MPC of 2.0 mg/L BOD_5) are found adjacent to the northern coastal wastewater treatment plant and in a southern coastal zone where unpurified wastewater from the southern part of St. Petersburg is discharged. As a rule, the BOD_5 in these zones does not exceed values of 1-2 times the MPC.

Moving from the mouth of the Neva River to the west, in spite of additional discharges of labile organic material in wastewater, the concentration of these substances in Bay water decreases. This is apparently caused by increases in the microbiological and sedimentation removal processes. In the central zone of the eastern Gulf of Finland, as compared to Neva Bay, the water is only slightly polluted or contaminated by labile organic matter ($BOD_5 \approx$ 2-2.5 mg/L).

Oxygen Regime: One of the most important indicators of the ecological state of an aquatic ecosystem is the dissolved oxygen content of the water. According to effective sanitary and fishery requirements, the oxygen concentration should not be less than 4 mg/L in winter and 6 mg/L in summer (USSR State Committee on Environmental Protection 1991). For the Gulf of Finland, with its salinity and temperature ranges, this means the abundance of oxygen should not be reduced below 70% of saturation.

The essential characteristics of the oxygen regime of the Neva Bay and eastern Gulf of Finland have been measured. Data from long-term observations show that the content of the dissolved oxygen in the waters of Neva Bay is 8-12 mg/L, and the degree of saturation of water by oxygen is about 80-95 %. No major difference between summer and winter values was observed. Such a favorable oxygen regime is caused by the high content of oxygen in Neva River water and good conditions for aeration in the shallow Neva Bay. Most of Neva Bay lacks any appreciable vertical stratification of oxygen content. Spatially, it is possible to distinguish among the areas of Neva Bay based on the oxygen content. The highest degree of enrichment of water by oxygen is peculiar to the most dynamic part of the Bay, north of the sea channel, where the oxygen content is typically 90-100 % of saturation.

It is necessary to note that in recent years, owing to the amplification of eutrophication in narrow coastal zones, during the aquatic plant growing season values of >100 % O_2 saturation have been measured within the Bay.

Features of the oxygen regime have been observed outside the dam. In near-bottom waters, oxygen concentrations are somewhat decreased in moving from the open part of the Gulf (60-70% saturation) towards the Neva estuary (50-60%), in spite of decreasing water depths. The lowest values (30-50%) have been measured in the semi-enclosed basins along the Finnish coast near the city of Kotka. Statistical analysis of the dissolved oxygen concentration measurement in the surface and bottom layers of water of the eastern part of the Gulf of Finland has shown that oxygen saturation is generally lower than 70 % (usually about 40-50 %) in the bottom layer at all stations of the central region.

Ecotoxic Pollutants: The most widespread pollutants of mainly human origin that are present in water of the Neva Bay and the eastern Gulf of Finland are petroleum hydrocarbons, phenols, detergents, and heavy metals. The concentration of each pollutant differs both in space and in time. This is caused first by the irregular mode of their influx from anthropogenic sources, and second from the effect of self-purification processes. Therefore the degree of pollution of Neva Bay and the inner Gulf of Finland by synthetic substances varies considerably (Table 2). Since monitoring began, the highest pollutant concentrations have always been found

in shallow coastal zones. This is caused by limited water exchange, high local loading and the influence of wastewater from centralized sewer discharges.

Table 2. Mean concentrations of selected pollutants in surface waters, 1997-98 (µg /L). Source: City of St. Petersburg (1999).

Constituent	Neva Bay	Eastern Gulf of Finland	Russian MPC
Oil	73	58	50
Phenol	0.71	0.75	1
Fe	140	208	100
Mn	10.6	18.5	10
Pb	10	35	100
Cu	6.5	9	5

Among the heavy metals, those that most frequently exceed the MPC values for waters that support fisheries are copper, iron and manganese. The maximum level of pollution by copper is observed nearly annually in a zone to the south of the sea channel, where single samples can display more than 10 MPC, or 50µg /L. The level of pollution of water masses near the bottom is higher than in surface waters, especially in the eastern part of the Gulf of Finland.

Concerning the pollution of Neva Bay by pesticides, it is necessary to note that a decrease of their concentrations has lately been observed. In recent years, the following concentration ranges have been measured (in µg /L): DDT - 0.03-0.07; hexachlorocyclohexane - 0.001-0.003.

The pollution by phenols, oil, and detergents in the eastern Gulf of Finland is characterized by the following basic features. The north coastal zone, used for recreation, is more polluted than the central region of the eastern Gulf. It is necessary to view the zone around the dam separately. However, the effect of the dam remains insignificant and is limited to the zone immediately adjacent to the sea gates of the Bay (Shiklomanov and Skakalsky 1991, 1996). This conclusion is supported by comparison of the variability in total phosphorus concentrations at stations located to the east of the dam (stations K1, K2, 11, 14, and 17) and to west of it (stations K1', K2', 26 and 26a) (Figure 4).

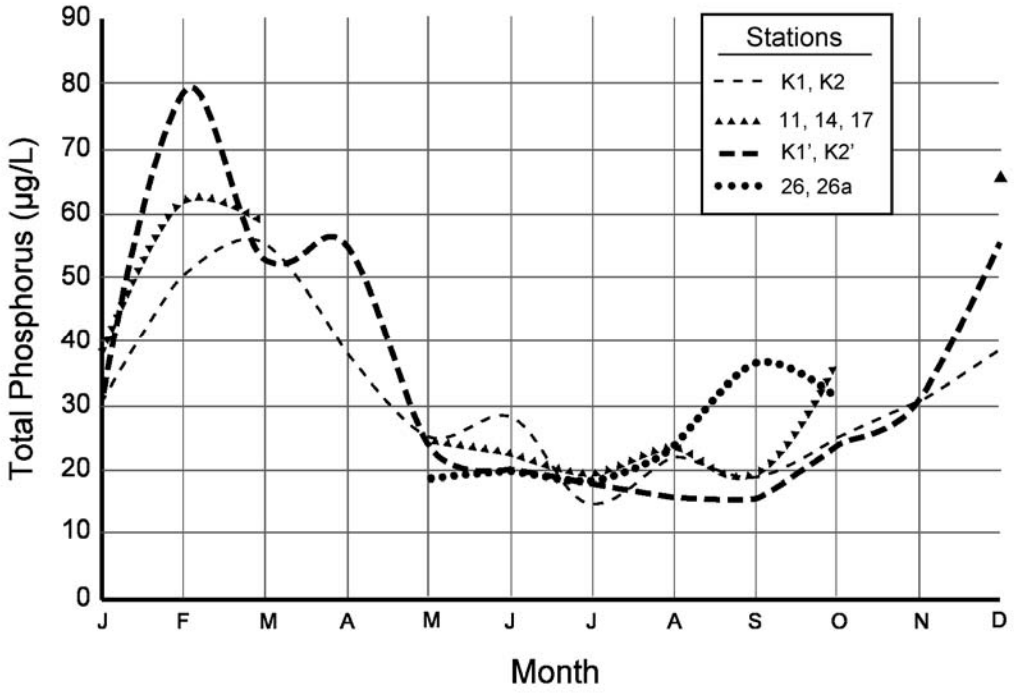

Figure 4. Total phosphorus concentrations in surface water at stations east and west of the southern sea gate (µg/L).

Time Trends: In the eastern part of the Gulf of Finland, the northern coastal strip in all periods is characterized by the highest levels of pollution. There, because of the influx of great masses of nutrients there is accelerated eutrophication. Developments of poisonous blue-green algae occur consistently in June-August all along the coast from the dam to the west. This phenomenon considerably worsens the quality of the water, and frequently makes it impossible to use the beaches for recreational purposes. In the summer, phytoplankton blooms are observed in almost all investigated areas of the Gulf of Finland.

Trend-analysis of long-term changes by Spearman's criteria have shown that the concentrations of total nitrogen, NO_3 -N, BOD, oil, phenols, and manganese all have marked downward concentration trends for most of the investigated waters. Additionally, investigators have found for Neva Bay and the shallow waters of the eastern Gulf (east of the dam) downtrends of concentration of detergents, oil, and BOD, and upward trends of the concentrations of Cu and Cd. The area west of the dam (especially in the open part of the Gulf of Finland) is characterized by increasing concentrations of mineral phosphorus in bottom waters. No long-term trend of oxygen concentrations in deep waters was evident because the regime of dissolved oxygen is significantly affected by periodic salt water intrusions from the open Baltic Sea. However, since the 1990s areas with hypoxic conditions (2-4 mg O_2/L) in deep waters have been observed to develop more often (Pitkanen *et al.* 1997).

The processes of self-regulation and pollution loading that occur in the eastern Gulf of Finland determine how effectively the Ca-HCO_3 system is able to maintain the observed values of water pH. It is noted that in all studied waters alkalinity predominated before about 1990, but since then acidity has been observed. The previously-mentioned downtrend in the content of oxygen in the water, occurring while the percent oxygen saturation rose slightly or stayed invariable, is probably attributable to a rise in water temperature. All of these factors influence the redox conditions of the Gulf.

For example, in the vicinity of Vyborg on the northern Gulf of Finland (see Figure 1) time trends for the main components of the Ca-HCO_3 system can be estimated by the calculated values of the Spearman criterion (for surface waters):

Parameter	Spearman criterion	Estimation of tendency
Temperature	0.38	increase
pH	-0.03	insignificant change
$[HCO_3]$	0.63	increase
$[O_2]$	-0.80	decrease
O_2 saturation	0.05	insignificant change

CONCLUSIONS

It is possible to conclude that the ecosystem of the eastern Gulf of Finland is becoming more unstable. One of the reasons is the ever-present potential for blooms of algae in various regions of the Gulf driven by incremental increases in the concentrations of phosphorus and nitrogen in the euphotic layer. There has been a general decrease in the concentration of oxygen in the waters, even as the oxygen saturation has increased or stayed invariable, probably as a result of water temperature increase. This has influenced the redox conditions in the system. The mean long-term content of total phosphorus in all areas has not exceeded the norm for a water body of eutrophic status. The ratio defined by $N_{tot}/P_{tot} > 14$ shows that, in these waters, primary production is limited by phosphorus.

Within the eastern Gulf of Finland the eutrophic region is gradually expanding from east to west. Nutrient loading to the Gulf must be reduced. Reserves of nutrients in the sediments, as well as their continued deposition, pose the threat of spreading algal blooms into new areas of the Gulf of Finland. The accumulation of heavy metals promotes toxicity in the water environment, and has negative effects on the biota of the Gulf of Finland.

REFERENCES

City of St. Petersburg. 1999. Review of Environmental Issues and Problems, covering also Nature Management and Environmental Safety Issues. Administration of the City of Saint Petersburg, Russia.

HELCOM. 1974. The Convention on the Protection of the Baltic Sea Marine Environment (Helsinki Convention). Helsinki, Finland.

HELCOM. 1988. Guidelines for the Baltic Monitoring Programme for the third stage. Baltic Marine Environment Protection Commission. 27D. 161 pp.

HELCOM. 1994. Guidelines for the third pollution load compilation (PLC-3). HELCOM Baltic Sea Environment Proceeding, No. 57. Helsinki, Finland.

HELCOM. 1996. Third periodic assessment of the state of the marine environment of the Baltic Sea, 1989-1993, background document. Baltic Sea Environmental Proceedings 64 B. Helsinki Commission, Helsinki, Finland. 252 pp.

HELCOM. 1998. Third Baltic Sea pollution load compilation. Baltic Sea Environmental Proceedings 70. Helsinki Commission, Helsinki, Finland. 33 pp.

Nekrasov, A.V., S.M. Gordeeva, G.I. Bashkina, and B.G. Skakalsky. 2002. Report on the 4th cruise of sailing catamaran Centaurus-II (BFU research expedition in the Eastern Gulf of Finland, July 15-August 9, 2000). BFU Research Bulletin, No 4-5:76-81. Baltic Floating University, RSHU, St. Petersburg, Russia.

Pitkanen, H., T. Tamminen, P. Kangas, T. Huttula, K. Kivi, H. Kuosa, J. Sarkkula, K. Eloheimo, P. Kauppila, and B. Skakalsky. 1993. Late summer trophic conditions in the North-east Gulf of Finland and the river Neva Estuary, Baltic Sea. Estuarine, Coastal and Shelf Science 37:453-474.

Pitkanen, H. and P. Vallipakka. 1997. Extensive deep water oxygen deficit and benthic phosphorus release in the eastern Gulf of Finland in late summer 1996. Proceeding of the Final Seminar of the Gulf of Finland, Year 1996. Helsinki. pp. 51-60.

Savchuk, O. 2002. Eutrophication of the Baltic Sea. BFU Research Bulletin, No 4-5: pp. 25-26. Baltic Floating University, RSHU, St. Petersburg, Russia.

Shiklomanov, I. A., and B.G. Skakalsky. 1991. Dynamics of Water Quality of River Neva-Neva Bay –Eastern part of Gulf of Finland. Journal of Meteorology and Hydrology No 5. Moscow. (in Russian).

Shiklomanov, I. A. and B.G. Skakalsky. 1996. Assessment of hydroecological state of Lake Ladoga – River Neva – eastern part of Gulf of Finland water system and problems of its restoration. Abstracts from the Symposium "The Gulf of Finland-1996." MANEB, St. Petersburg, Russia. (in Russian).

USSR State Committee on Environmental Protection. 1991. Rules on the Protection of Surface Water (Principle Provisions). Annex: Schedule of Maximum Permissible Concentrations of pollutants for waters of fishery, domestic and drinking use. Moscow. (in Russian).

Wulff, F. 1991. Large-scale environmental effects and ecological processes in the Baltic Sea. Research program for the period 1990-1995 and background documents. Swedish Environmental Protection Agency Report N3856. 226 pp.

MODELING OF METAL BINDING ON HUMIC SUBSTANCES USING THE NIST DATABASE: AN *A PRIORI* FUNCTIONAL GROUP APPROACH

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ABSTRACT

Various modeling approaches have been developed for metal binding on humic substances. However, most of these models are curve-fitting exercises. The resulting set of parameters, such as affinity constants (or the distribution of them), depends on pH, ionic strength, and the concentrations of metals and humic substances present. Consequently, these models are not satisfactory to predict metal binding under environmental settings. We developed and present herein an *a priori* model based on the elemental composition and functional group concentrations of humic substances, using the National Institute of Standards and Technology (NIST) database of critically selected stability constants of metal complexes. We tabulated the stability constants of selected metal-organic complexes for organic molecules with selected functional groups, and displayed the corresponding conditional stability constants for many metals of environmental concern at several pH levels. These data show that in addition to oxygen-bearing functional groups, nitrogen-bearing and sulfur-bearing groups are also important for metal complexation and binding. Specifically, the amino acid group plays a significant role for the binding of Cu(II), Hg(II), Cr(III) and Fe(III), whereas the SH-functional group is important for the binding of soft Lewis acid metals, such as Cd(II), Hg(II), and Pb(II). We show that this simple complexation-based model is capable of predicting binding and competitive binding of metals to humic substances when the metal concentration is less than 10^{-5} to 10^{-6} M, the relevant metal concentration range under most environmental settings.

INTRODUCTION

It is well recognized that humic substances react with metals in soils, sediments, and surface waters, and greatly affect the chemical speciation, distribution, transport, bioavailability, toxicity, and ultimate fate of metals in terrestrial and aquatic ecosystems. Numerous laboratory studies of the reactions between metals and humic substances have been reported in the literature, and several review articles have been devoted to this subject (Sposito 1986, Livens 1991, Stevenson 1994, Jenne 1998, Kinniburgh *et al*. 1998). Various modeling approaches have been developed to quantify metal binding on humic substances. Most of these models incorporate a multiligand representation of the important functional groups responsible for metal binding by humic substances. The multiligand models can be divided into two categories: discrete ligand models (Dempsey and O'Melia 1983, Dzombak *et al*. 1986, Fish *et al*. 1986, Cabaniss and Schuman 1988, Gregor and Powell 1988) and continuous distribution models

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(Perdue and Lytle 1983, Perdue *et al.* 1984, Dzombak *et al*. 1986, Buffle *et al.* 1990). The former assumes a discrete distribution of ligands, whereas the latter assumes a continuous distribution of heterogeneous ligands. The random molecular model (Murray and Linder 1983/1984, Woolard and Linder 1999), the mixture model (Mattigod and Sposito 1979, Sposito *et al*. 1982), and the oligoelectrolyte model (De Wit *et al*. 1990, Tipping *et al*. 1990/1991, Bartschat *et al.* 1992, Tipping and Hurley 1992, De Wit *et al*. 1993, Tipping 1993, Milne *et al*. 1995, Bose and Reckhow 1997, Tipping 1998) are examples of discrete ligand models. The Gaussian distribution model (Posner 1966, Perdue and Lytle 1983), the competitive Gaussian model (Dobbs *et al*. 1989, Susetyo *et al*. 1990, Allison and Perdue 1994) and the non-ideal, competitive adsorption model (NICA) (Benedetti *et al.* 1995) are all examples of continuous distribution models. However, most of these models are curve-fitting exercises; the resulting set of parameters, such as affinity constants (or their distribution), depends upon pH, ionic strength, and the concentrations of metals and humic substances present. Therefore, their use for reliable extrapolation from one pH, ionic strength, and concentration range of metals to another is not possible (Bartschat *et al*. 1992). Furthermore, calculations of discrete and continuous affinity constants from experimental data are far from trivial (Borkovek *et al.* 1998). It has been shown that without additional information about the affinity distribution, it is generally impossible to recover the true affinity distribution from realistic experimental data (Cernik *et al.* 1995/1996). In each of the models cited previously, only binding sites composed of major proton-dissociating groups were considered, whereas the presence of a small number of strong binding sites containing nitrogen and sulfur was ignored. At low, environmentally relevant metal concentrations, these strong latter binding sites can contribute significantly to metal binding and control the speciation of metals. An earlier *a priori* predictive metal-organic ligand model was developed (Bartschat *et al*. 1992) in which humic substances were considered as oligoelectrolytes. The humic molecules were represented as impenetrable spheres and the electrostatic interaction effect was calculated using a numerical solution of the Poisson-Boltzmann equation.

In our approach, we tabulated the measured stability constants of complexes formed between many metals of environmental concern and various functional groups present in various aliphatic and aromatic moieties (oxygen-, nitrogen-, and sulfur-bearing ligands). We then developed a mass-balance, competitive adsorption model by separately considering reactions between each metal with each functional group in turn. This approach was similar to that of Bartschat *et al*. (1992) in that the goal was not to obtain an exact fit to experimental data, but rather to develop an *a priori* model for low-metal-concentration environments based on: (1) the elemental composition; and (2) functional group concentrations of available humic substances. To be useful, the model must be capable of predicting the observed trend and major features of adsorption and competitive adsorption of metals over a wide range of pH, metal types, and environmentally relevant concentrations of both metals and humic substances.

A variety of oxygen-bearing functional groups, including COOH, phenolic OH, enolic OH, alcoholic OH, quinone, hydroxyquinone, lactone, and ether, are present in natural humic substances (Stevenson 1994). Using the NIST database of critically selected (measured) stability constants of metal complexes (Smith *et al*. 1997), we extracted and tabulated those constants for metal binding on known organic ligands with various functional groups that exist in humic

substances in soils, sediments, and waters. The ligands we evaluated include: (1) oxygen-bearing groups, including aliphatic dicarboxylic acid separated by either one or two carbons, polycarboxylic acids, diketones, and aromatic carboxylic and phenolic groups on adjacent positions (*i.e.*, phthalic acid, salicylic acid and catechol); (2) nitrogen-bearing groups, including amine, diamine separated by either one or two carbons, and α-amino acid; and (3) sulfur-bearing groups, including sulfhydryl groups (-SH). The metals we considered include: alkaline earths Mg(II) and Ca(II); transition metals Cr(II), Mn(II), Fe(II), Co(II), Ni(II), Cu(II), and Zn(II); heavy metals Cd(II), Hg(II), Pb(II), and Ag(I); and others, such as Cr(III), Fe(III), and Al(III). The selection temperature was 25 ^{o}C and the ionic strength was 0.1 *M*, when they were available from the NIST database.

MODELING APPROACH

Adsorption and Competitive Adsorption of Protons and Metals

Consider a reaction system consisting of a single metal, M, and humic substances with metal binding sites, L. The reaction of M with the ith binding site in humic substances is written as:

$$M + L_i = ML_i \qquad (1)$$

The stability constant is given as:

$$K_{Mi} = \frac{[ML_i]}{[M][L_i]} \qquad (2)$$

where [M] is the equilibrium concentration of the free metal, [ML_i] is the equilibrium concentration of the metal bound on the ith binding site, and [L_i] is the equilibrium free concentration of ith binding site. The ML_i complex is shown as a 1:1 metal-ligand complex, but it can also be a monodentate, bidentate, or multi-dentate complex depending on the functional groups present on these binding sites. Under natural conditions, one might expect that metals are also bound between two macromolecules, or between two sites in the same macromolecule with a favorable conformational arrangement. The binding result is similar to that for two smaller ligands, producing 1:2 metal-ligand complexes (Huheey *et al*. 1996). Because of steric constraints, however, this type of binding does not occur frequently within a macromolecule. Furthermore, since this type of binding is difficult to estimate, we did not consider the formation of these complexes in our modeling approach.

When the L_i particular site cannot be protonated, the metal binding reaction is pH-independent. The Langmuir equation (Dobbs *et al*. 1989) describes pH-independent single-metal binding to such a site as:

$$[ML_i] = \frac{K_{Mi}[M]C_i}{1 + K_{Mi}[M]} \qquad (3)$$

where C_i is the total concentration of i^{th} binding site, given by $C_i = [ML_i] + [L_i]$.

When the L_i binding site can be protonated, a proton and metal ion must compete for the same site. The protonation reaction of the i^{th} binding site can be written as:

$$H + L_i = HL_i \tag{4}$$

The stability constant for the protonated site is given as:

$$K_{1Hi} = \frac{[HL_i]}{[H][L_i]} \tag{5}$$

Dobbs *et al*. (1989) reported the competitive adsorption of a metal can be given as:

$$[ML_i] = \frac{K_{Mi}[M]C_i}{1 + K_{Mi}[M] + K_{1Hi}[H]} \tag{6}$$

whereas that of a proton is described as:

$$[HL_i] = \frac{K_{Hi}[H]C_i}{1 + K_{Mi}[M] + K_{1Hi}[H]} \tag{7}$$

Note that the only difference between the competitive adsorption reaction shown in Eq. (6) and the single-metal, pH-independent adsorption reaction shown in Eq. (3) is the additional term, $K_{1Hi}[H]$, found in the denominator.

Equation (6) can be generalized (Susetyo *et al*. 1990, Allison and Perdue 1994) to express the competitive adsorption of the j^{th} metal (M_j) on the i^{th} protonated binding site involving $j = 1, 2\ldots n$ metals (M_1, M_2, ..., M_n, including the proton) as:

$$[M_j L_i] = \frac{K_{ij}[M_j]C_i}{1 + \sum_{k=1}^{n} K_{ik}[M_k]} \tag{8}$$

Thus, to obtain the total concentration of each $j = 1, 2\ldots n$ metal bound on humic substances, $[M_jL]$, one can sum up concentrations of j^{th} metal bound to each of the m sites (i = 1, 2, ..., m):

$$[M_j L] = \sum_{i=1}^{m} [M_j L_i] = \sum_{i=1}^{m} \frac{K_{ij}[M_j]C_i}{1 + \sum_{k=1}^{n} K_{ik}[M_k]} \tag{9}$$

For pH-dependent metal binding reactions (*i.e.*, for proton stable *i*th type), the effect of pH on metal binding can be conveniently evaluated by using the conditional stability constant.

Combining the metal binding reaction of Eq. (1) and the proton binding reaction of Eq. (4), one gets:

$$M + HL_i = ML_i + H \tag{10}$$

The corresponding equilibrium constant is given as:

$$K = \frac{[ML_i][H]}{[M][HL_i]} = \frac{[ML_i]}{[M][L_i]}\frac{[H][L_i]}{[HL_i]} = \frac{K_{Mi}}{K_{1Hi}} \tag{11}$$

At a constant pH, the conditional stability constant can be written as:

$$K_c = \frac{[ML_i]}{[M][HL_i]} = \frac{K_{Mi}}{K_{1Hi}[H]} \tag{12}$$

When pH << pK_{Hi}, $K_{1Hi}[H]$ >> 1, the *i*th binding site is protonated as HL_i, and Eq. (6) can be written as follows for each metal m (*i.e.*, m_j , j = 1, 2,….n):

$$[ML_i] = \frac{K_{Mi}[M]C_i}{1 + K_{Mi}[M] + K_{1Hi}[H]} \cong \frac{K_c[M]C_i}{1 + K_c[M]} \tag{13}$$

Thus, the competition between a proton and a metal can be treated implicitly by using the conditional stability constant.

The above competitive adsorption equations apply only when the proton/metal stoichiometry is 1:1. In actuality, the number of protons released for each bound metal species (m_j) varies with pH for several functional group types (*i*) due to changes in the degree of protonation of these functional groups. Therefore, the above equations need to be modified. For simplicity, we consider next the case when two protons are released for each metal ion (m_j) bound (*e.g.*, a divalent metal binding on diamine at pH < 6.81 and/or catechol at pH < 8.73). This protonation reaction on the i^{th} binding site can be written as:

$$2H + L_i = H_2L_i \tag{14}$$

and the corresponding stability constant given as:

$$K_{Hi} = \frac{[H_2L_i]}{[H]^2[L_i]} \tag{15}$$

Eq. (6) then becomes:

$$[ML_i] = \frac{K_{Mi}[M]C_i}{1 + K_{Mi}[M] + K_{Hi}[H]^2} \tag{16}$$

The right side of Eq. (12) retains the same form, with the conditional stability constant given as:

$$K_c = \frac{[ML_i]}{[M][H_2L_i]} = \frac{K_{Mi}}{K_{Hi}[H]^2} \qquad (17)$$

Stability and Conditional Stability Constants

The values of the complexation stability constants, their standard deviations, and the number of experimental data entries upon which these values were based were extracted from the NIST database for our selected metals. Functional group combinations are given in Table 1. Values of the corresponding conditional stability constants (log K_c) at pH 4.0, 5.0, 6.0, 7.0 and 8.0 are plotted in Figures 1-9. Based on this information, we make the following observations.

Divalent Metals

For divalent metals, conditional stability constants for each organic ligand follow the extended Irving-Williams series (Irving and Williams 1953; Sigel and McCormick 1970), *i.e.*, they increase from left to right (Ca<Mg< Mn< Fe< Co< Ni< Cu; Cu>Zn).

Ring Size

The formation of a five to six-member ring increases the stability of the complex. For this reason, we only considered dicarboxylic acids and diamines separated by either one or two carbons, and aromatic carboxylic and phenolic groups in adjacent positions.

Table 1. Values of stability constants (log K), their standard deviation (s.d.), and number of experimental data entries (n), compiled from the NIST database.

	L/HL	HL/H2L	Ca2+	Mg2+	Cr2+	Mn2+	Fe2+	Co2+	Ni2+	Cu2+	Zn2+	Cd2+	Hg2+	Pb2+	Cr3+	Fe3+	Al3+	Ag+
Amino Acid																		
log K	9.25	2.36	1.51	1.76	4.47	2.72	3.72	4.40	5.50	8.17	4.68	3.85	7.08	4.77	8.84	9.44	3.72	3.62
s.d.	0.45	0.91	0.44	0.45	0.24	0.54	0.68	0.66	0.75	0.62	0.61	0.48	1.93	0.56	----	2.39	1.20	0.65
N	35	34	11	8	3	18	17	28	29	32	33	23	5	16	1	11	6	11
Diamine																		
log K	9.75	6.81	0.87	0.37	5.48	2.54	4.26	5.16	6.37	9.49	5.27	5.52	13.20	5.52	----	----	---	5.31
s.d.	0.53	1.05	1.07	----	----	0.34	----	0.63	1.15	0.98	0.65	1.18	1.56	0.36	----	----	---	0.59
N	43	43	2	1	1	5	1	14	34	43	19	14	2	6	----	---	---	5
Dicarboxylic Acid																		
log K	5.32	2.65	1.88	2.00	3.89	1.89	2.10	2.40	2.50	4.29	2.58	2.41	----	3.07	8.26	7.18	5.25	1.86
s.d.	0.95	0.63	0.64	0.61	0.04	0.64	0.50	0.48	0.50	0.99	0.43	0.28	----	0.33	----	0.42	1.16	0.76
N	34	33	17	13	2	10	4	19	22	31	25	18	----	8	1	10	6	4
Tetracarboxylic Acid																		
log K	5.60	4.42	5.40	4.23	----	5.32	4.41	4.55	6.21	7.06	6.54	5.10	14.12	7.39	----	11.38	8.41	----
s.d.	0.60	0.55	0.30	0.71	----	0.56	----	----	1.37	1.23	0.82	0.39	0.28	0.35	----	0.66	0.77	----
N	4	4	4	4	----	4	1	1	4	4	4	4	3	3		3	3	----
Phthalic Acid																		
log K	4.66	2.53	1.60	---	2.48	2.68	----	2.74	2.03	2.98	2.86	2.37	----	2.78		----	2.94	---
s.d.	0.48	0.35		---		0.53	----	0.49	0.21	0.39	0.59	0.29	----	----		----		---
N	9	8	1	---	1	4	----	4	9	9	3	2	----	1		---	1	---
Salicylic Acid																		
log K	11.85	2.18	4.08	5.33	8.15	4.86	6.23	5.77	6.32	9.04	6.48	5.13	----	5.60	9.41	15.09	12.89	---
s.d.	2.09	0.82	0.86	0.33	0.77	0.96	0.39	1.37	1.23	1.57	0.46	0.53	----	----	----	2.40	0.59	---
N	18	18	2	2	2	4	2	4	5	12	2	2	----	1	1	13	3	---
Catechol																		
log K	12.77	8.73	5.18	6.47	----	8.06	8.26	9.02	9.35	13.88	9.94	8.44	19.90	13.55	----	19.45	16.23	---
s.d.	0.84	1.16	0.47	0.75	----	0.67	----	0.76	0.74	0.96	0.88	1.08		0.43	----	2.25	1.20	---
N	14	14	6	6	----	9	1	11	11	13	11	9	1	4		9	10	---
Diketone																		
log K	8.61		2.32	3.35	5.96	3.90	5.07	4.74	4.98	7.85	4.35	3.48	11.93	4.57	----	10.60	---	----
s.d.	0.28		----	----	----	----	----	0.48	1.02	0.21	0.49	0.04			----	----	---	----
N	2		1	1	1	1	1	2	2	2	2	2	1	1		1	---	----
R-SH																		
log K	9.94	6.86	3.20	3.12	----	6.15	9.16	8.84	10.68	----	9.71	10.70	16.45	12.52	----	----	---	12.4
s.d.	0.67	1.97	1.47	1.06	----	2.75	3.62	2.70	2.75	----	3.09	3.04	2.21	2.93	----	---	---	----
N	10	10	3	3	----	3	2	8	7	----	8	7	3	5	----	----	---	1

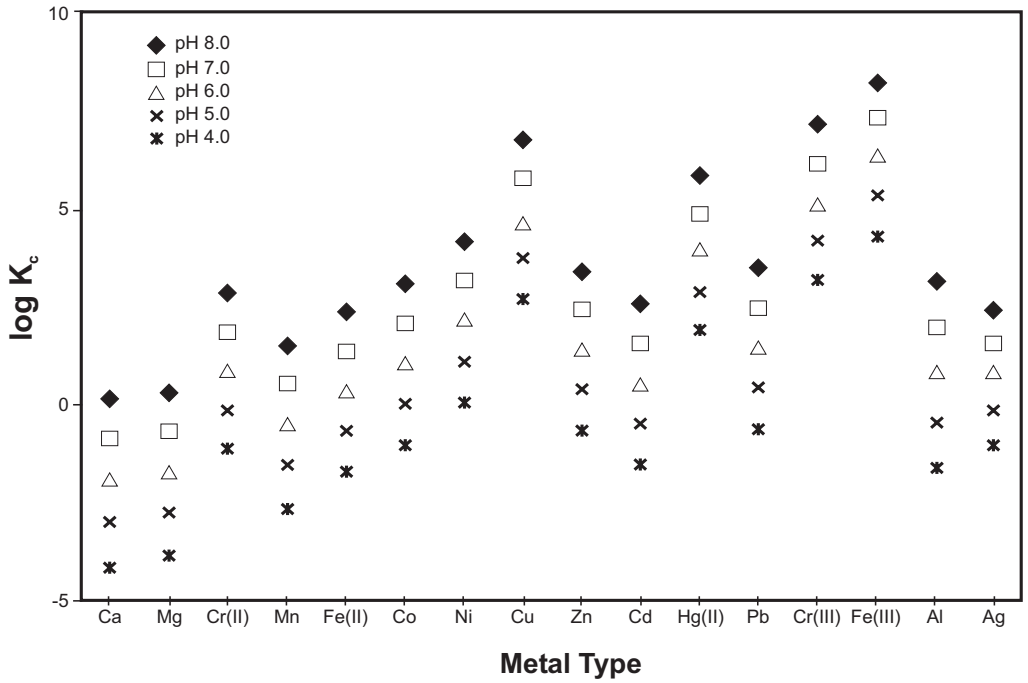

Figure 1. The conditional stability constants (Log K_c) for amino acid complexes of selected metals at pH 4.0, 5.0, 6.0, 7.0 and 8.0.

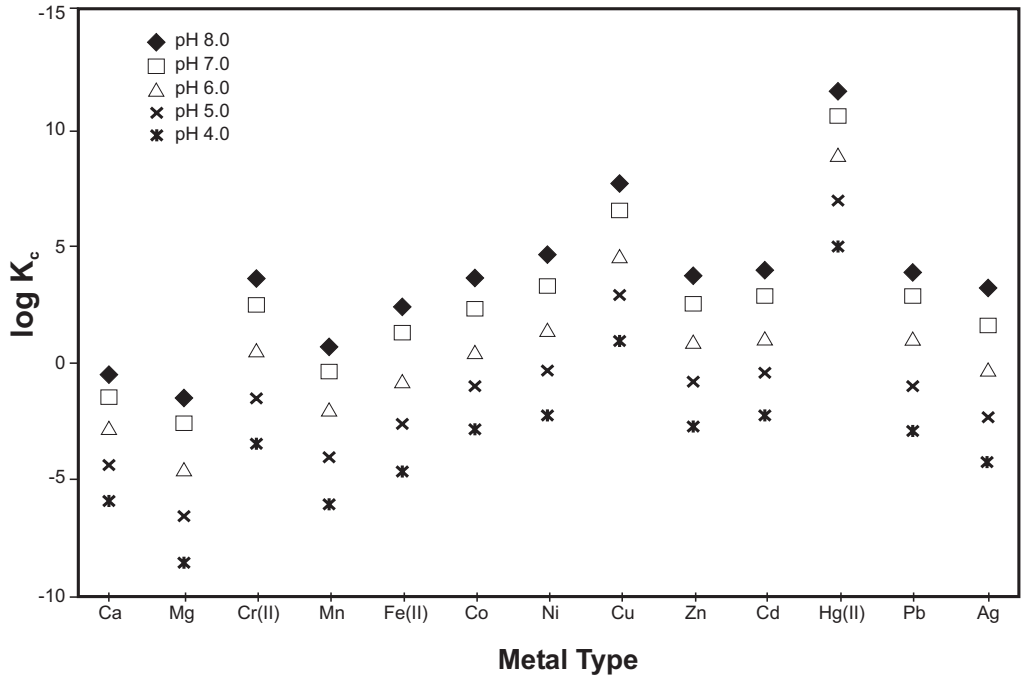

Figure 2. The conditional stability constants (Log K_c) for diamine complexes of selected metals at pH 4.0, 5.0, 6.0, 7.0 and 8.0.

METAL SELECTIVITY

The selectivity of metal-ligand binding obeys the principle of hard and soft acids and bases (HSAB) (Pearson 1963, Klopman 1968, Williams 1971). Briefly, this principle states that hard Lewis acids preferentially react with hard Lewis bases and soft Lewis acids preferentially react with soft Lewis bases (Jin *et al*. 1996). Trivalent metals, such as Fe and Cr, are hard Lewis acids and are preferred by carboxylic sites, whereas heavy metals, such as Cd, Hg, and Pb, are soft Lewis acids and are preferred by soft Lewis base sulfur-containing ligands. Most of the transitional metals are either borderline or soft Lewis acids; therefore, their stability and conditional stability constants relative to N- and S-bearing ligands are generally higher than for oxygen-bearing ligands.

Role of pH

Conditional stability constants for all organic ligand functional groups increase with pH. However, the conditional stability constants of the carboxylic acid groups reach a plateau at pH 5 to 6. Therefore, these carboxylic acid sites are important for metal binding at pH < 6.0 (Figures 3-5). At higher pH levels, diketone, amino acid, diamine, sulfhydryl and catechol groups become more important. Phthalic acid is preferred at lower pH levels, whereas salicylic acid is favored at higher pH levels. However, the catechol site forms the most stable complex at higher pH levels.

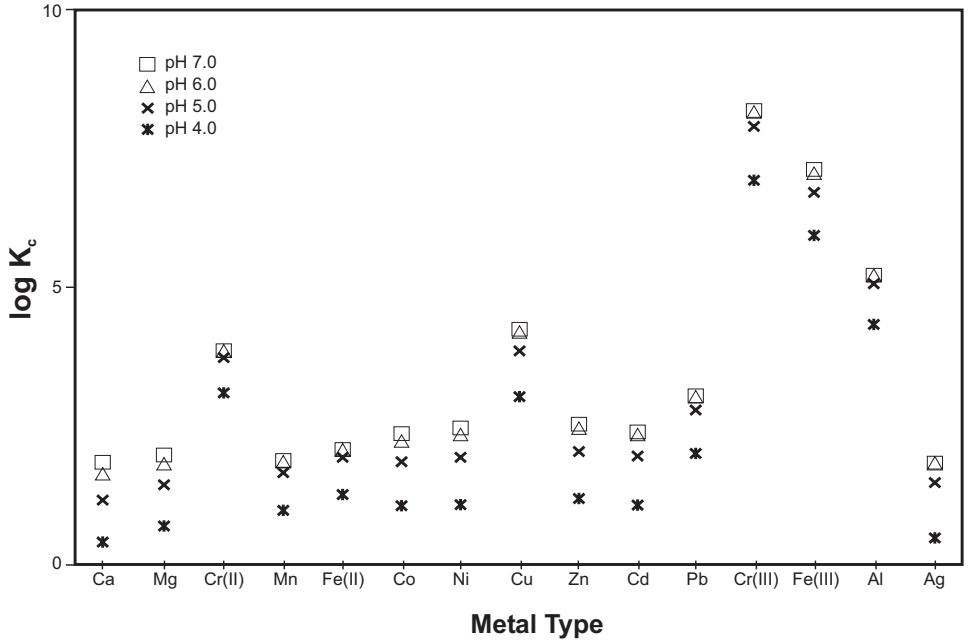

Figure 3. The conditional stability constants (Log K_c) for dicarboxylic acid complexes of selected metals at pH 4.0, 5.0, 6.0, and 7.0.

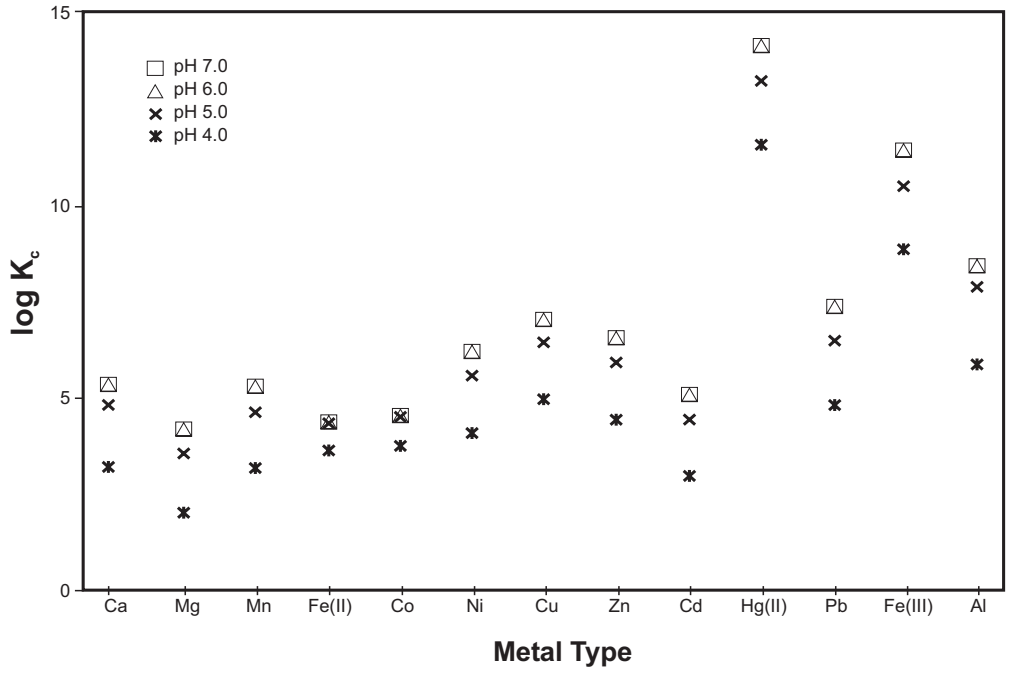

Figure 4. The conditional stability constants (Log K_c) for tetracarboxylic acid complexes of selected metals at pH 4.0, 5.0, 6.0, and 7.0.

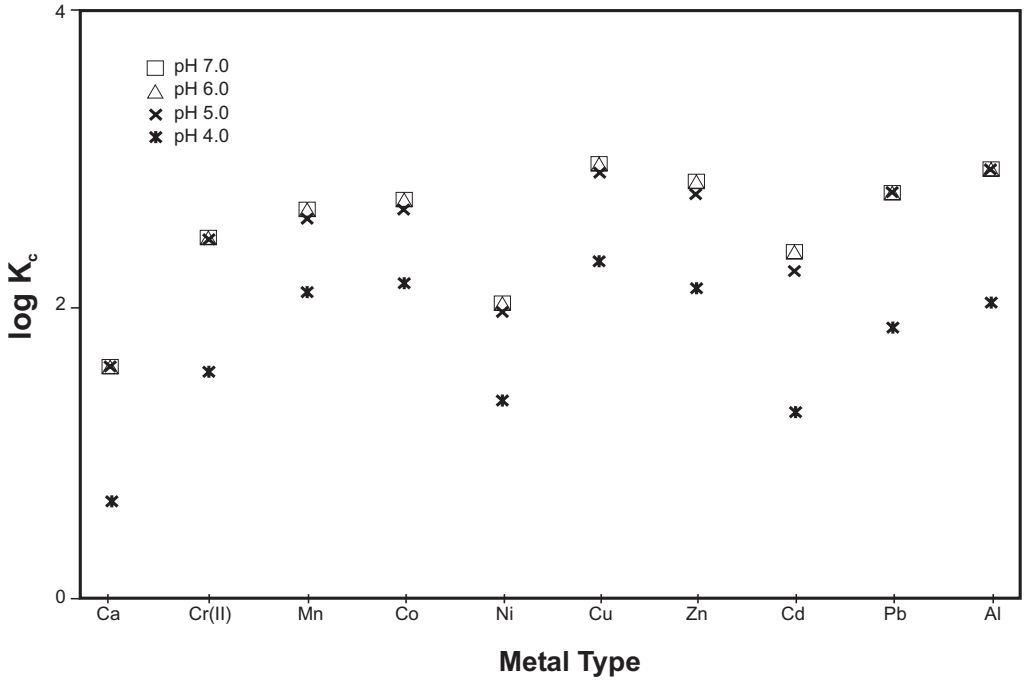

Figure 5. The conditional stability constants (Log K_c) for phthalic acid complexes of selected metals at pH 4.0, 5.0, 6.0, and 7.0.

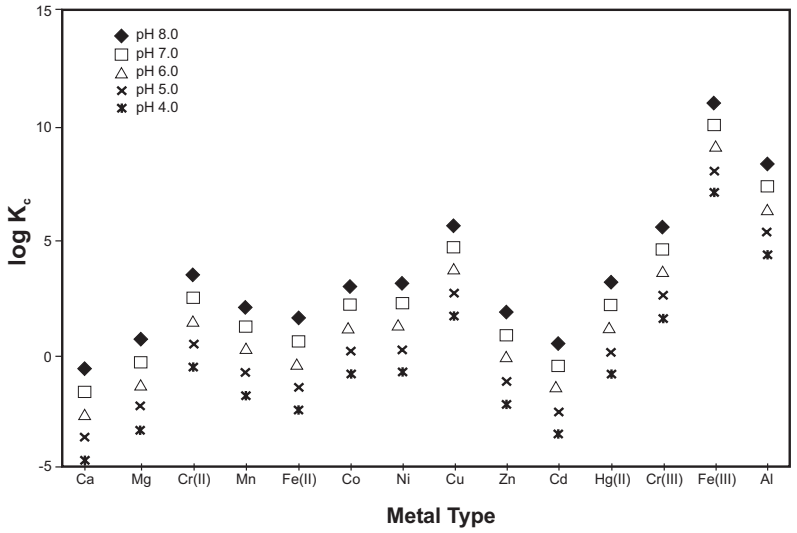

Figure 6. The conditional stability constants (Log K_c) for salicylic acid complexes of selected metals at pH 4.0, 5.0, 6.0, 7.0 and 8.0.

There is also independent experimental evidence that metals bind to nitrogen ligands. Specifically, it is well known that Cu forms many complexes with nitrogen ligands (Nicholls 1974); therefore, Cu is expected to bind to nitrogen-bearing functional groups in humic substances. Indeed, Keefer *et al.* (1984) extracted and fractionated organic matter from sludge-amended soil and found Cu was most often bound by hydrophilic bases (amino acids).

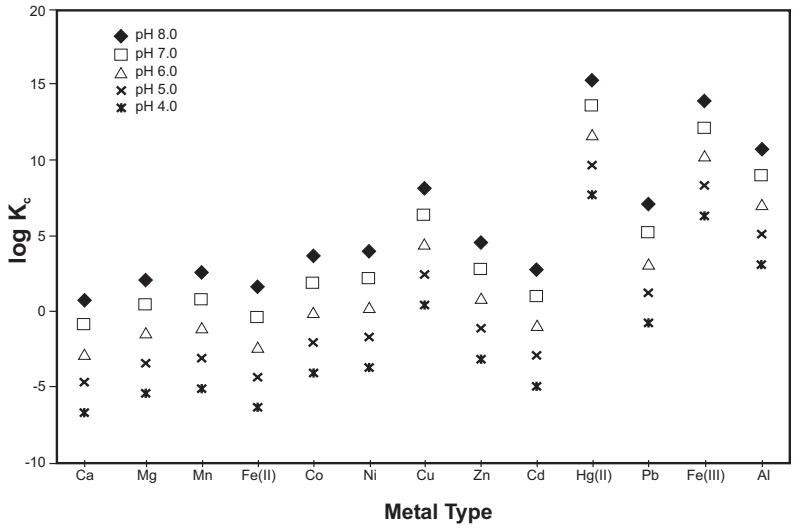

Figure 7. The conditional stability constants (Log K_c) for catechol complexes of selected metals at pH 4.0, 5.0, 6.0, 7.0 and 8.0.

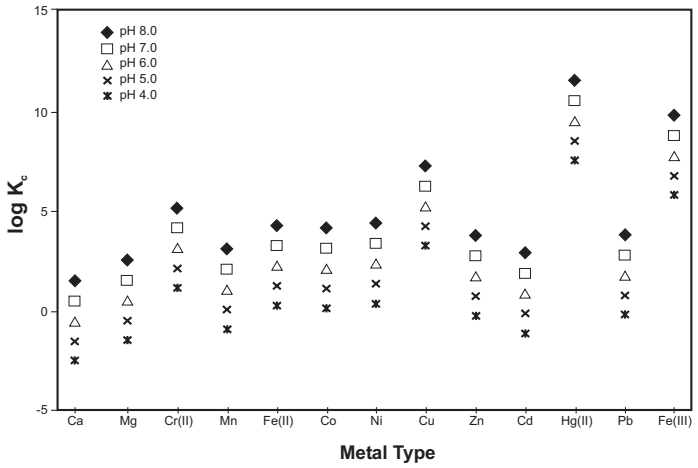

Figure 8. The conditional stability constants (Log K_c) for diketone complexes of selected metals at pH 4.0, 5.0, 6.0, 7.0 and 8.0.

Aliphatic Versus Aromatic Effect

For most metals, the conditional stability constants for aliphatic dicarboxylic acid are greater than for aromatic dicarboxylic acid (*e.g.*, phthalic acid).

Number of Functional Groups

Single amine groups and single carboxylic acid groups are not important for metal binding. Similarly, alcoholic OH groups and S bound to two carbons (R1-S-R2) do not contribute significantly to metal binding (data not shown).

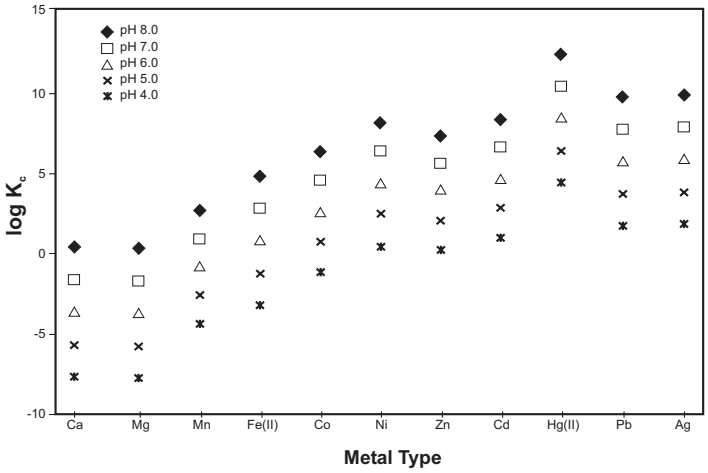

Figure 9. The conditional stability constants (Log K_c) for sulfur-bearing group complexes of selected metals at pH 4.0, 5.0, 6.0, 7.0 and 8.0.

MODEL PARAMETERS

We can qualitatively describe binding preferences between metals and organic ligands using our tabulated data (Table 1). However, to quantitatively describe the distribution of metals adsorbed on these ligand sites, we need to know the pH, ionic strength, equilibrium concentrations of metals, and total concentrations of different types of binding site functional groups on the humic substances. The effects of these various parameters are discussed in the following sections.

Effect of pH on Metal Binding

pH is generally considered one of the most important factors affecting the behavior of metals in the environment. The speciation of hydrolyzable metal ions and the precipitation of metal hydroxides depend directly on pH. The pH also affects the protonation and deprotonation of various functional groups in humic substances, thus directly affecting metal binding. We have shown that the competition between metals and protons can be either treated explicitly using Eq. (6) or Eq. (16), or implicitly by using the conditional stability constant and Eq. (12) or Eq. (17). In our model, we used the latter approach. There are two advantages to using this approach: (1) the pH-dependence of metal binding and the relative stability of metal complexes with various functional groups are clearly shown; and (2) when changes in pH alter the number of protons released for each metal bound, Eq. (6) requires modification, whereas the right side of Eq. (12) retains the same form. The disadvantage of this approach is that one has to precalculate the conditional stability constants for these functional groups at various pH levels, which is inconvenient for general modeling.

Ionic Strength

It is well known that ionic strength affects the activity coefficients of electrolytes. Ionic strength also affects the physical and chemical properties of humic substances. Properties such as molecular shape, charge, and conformational characteristics are all affected to some degree by ionic strength (Ghosh and Schnitzer 1980). In our model, the assumed ionic strength is 0.1 M.

Equilibrium Metal Concentration

The binding of metals on humic substances is affected by equilibrium free metal ion concentrations due to the mass action law. Furthermore, humic substances have a much higher affinity for metals at high ligand-to-metal ratios. Specifically, the relative concentration of metals to humic substances determines whether they are bound only to high-affinity-low-concentration sites or are bound to low-affinity-high-concentration sites after the former sites are saturated.

Total Concentrations of Binding Sites

The total concentration of oxygen-bearing functional groups can be estimated based on the total acidity of the humic substances. However, concentrations of nitrogen- and sulfur-bearing metal binding functional groups are best estimated based on the elemental composition of the humic substances as illustrated in subsequent sections.

FUNCTIONAL GROUP CONCENTRATION

The major elemental composition and functional group content of soil, aquatic, and sedimentary humic and fulvic acids are given in Table 2 (Senesi 1992). We will examine each of these three types of functional groups separately**.**

Table 2. Ranges of major elemental composition (%) and functional group content ($mmol_c\ g^{-1}$) of soil, aquatic, and sedimentary humic acids (HA) and fulvic acids (FA).

	Soil		Freshwater		Seawater	Sedimentary	
Parameter	HA	FA	HA	FA	FA	HA	FA
C	53.8-58.7	40.7-53.1	50.2-62.1	41.6-59.7	50.0	48.4-53.7	41.1-48.9
O	32.7-38.7	39.7-49.8	23.5-44.8	31.6-51.6	36.4	32.3-40.8	37.6-47.0
H	3.2-6.2	3.2-7.0	3.1-5.1	2.7-5.9	6.8	5.0-6.3	3.9-6.4
N	0.8-5.5	0.9-3.3	0.5-3.2	0.5-2.2	6.4	4.6-6.7	7.1-8.2
S	0.1-1.5	0.1-3.6	0.6-1.0	0.4-4.3	0.5	-----	-----
Total acidity	5.6-7.7	6.4-14.2	7.1-8.9	9.6-16.6	-----	3.0-5.5	2.0-5.5
COOH	1.5-5.7	6.1-11.2	4.0-5.9	4.0-8.9	5.5	2.0-4.0	1.0-4.0
Phenolic OH	2.1-5.7	0.3-5.7	2.0-3.0	0.8-3.0	-----	0.5-2.5	0.0-1.5
Alcoholic OH	0.2-4.9	2.6-9.5	-----	-----	-----	0.0-3.0	-----
Quinonic CO	1.4-2.6	0.3-2.0	4.3-5.1[a]	4.3-7.4[a]	-----	3.0-5.0[a]	5.0-6.0[a]
Ketonic CO	0.3-1.7	1.6-2.7	-----	-----	-----	-----	-----
OCH_3	0.3-0.8	0.3-1.2	-----	-----	-----	-----	-----

[a] Sum of the quinonic and ketonic CO.

TOTAL CONCENTRATION OF OXYGEN-BEARING GROUPS

The carboxylic acid group of organic matter consists of two fractions: an aliphatic fraction (f_{ali}) and an aromatic fraction (f_{aro}). Leenheer *et al*. (1995ab) found that 78% of the total carboxyl functional group of fulvic acid (FA) from the Suwannee River was aliphatic, with the remaining 22% aromatic. However, it is likely that the aromatic fraction is higher for humic acids (HA). Thus, we have assigned the following default values: $f_{ali} = 0.6$ and $f_{aro} = 0.4$ for HA. The user has the option to change these default values. The aliphatic and aromatic fractions can be further divided into single carboxylic acid (our assigned default value: $f_{aliacid} = f_{aroacid} = 0.5$), dicarboxylic acid ($f_{alidiacid} = f_{arodiacid} = 0.3$), and polycarboxylic acid ($f_{alipolyacid} = f_{aropolyacid} = 0.2$)

fractions. We further assume that: (1) all polyacids can be represented by using tetra-acid; (2) in 50% of benzoic acid groups, the carboxylic group is adjacent to a phenolic OH ($f_{aroacidOH} = 0.5$); and (3) in 50% of aromatic dicarboxylic acid sites the two carboxylic groups are in the adjacent position ($f_{arodiacid12} = 0.5$). Again, the user can change these assigned default values.

Thus, the concentrations of the various aliphatic acids can be estimated as:

$$C_{alidiacid} = f_{alidiacid}\, f_{ali}\, C_{COOH}\, C_{HS} /1000$$
$$C_{alitetraacid} = f_{alitetraacid}\, f_{ali}\, C_{COOH}\, C_{HS} /1000$$

Similarly, the concentrations of aromatic carboxylic acid sites can be estimated as follows:

$$C_{salicylic} = f_{aroacidOH}\, f_{aroacid}\, f_{aro}\, C_{COOH}\, C_{HS} /1000$$
$$C_{phthalic} = f_{arodiacid12}\, f_{arodiacid}\, f_{aro}\, C_{COOH}\, C_{HS} /1000$$

where C_{COOH} is the total concentration of the COOH group in the humic substances (mmol g^{-1}), C_{HS} is the total concentration of humic substance in solution (g L^{-1}) and the factor 1000 is used to convert concentration into mol L^{-1}.

The concentration of diketone group sites is estimated by:

$$C_{diketone} = f_{diketone}\, C_{CO}\, C_{HS} /1000$$

where $f_{diketone}$ is the fraction of diketone, and C_{CO} is the total concentration (mmol g^{-1}) of the ketone group in the humic substance. We assumed that $f_{diketone} = 0.3$.

The concentration of phenolic group sites can be estimated as follows. We assume the phenol group content of humic substances consists of single phenol ($f_{phenol} = 0.5$), diphenol ($f_{diphenol} = 0.3$) and polyphenol ($f_{polyphenol} = 0.2$). We further assume that in 50% of the diphenol sites the two phenolic OH groups are in adjacent positions ($f_{diphenol12} = 0.5$). In our model, single phenol and polyphenol are not considered. Thus, the concentration of catechol is given as:

$$C_{catechol} = f_{diphenol12}\, f_{diphenol}\, C_{phenol}\, C_{HS} /1000$$

where C_{phenol} is the total phenolic OH concentration (mmol g^{-1}) in the humic substance.

TOTAL CONCENTRATION OF NITROGEN-BEARING GROUPS

Greater than 90% of N in the surface layer of most soils exists in an organic form (Stevenson 1994). In humic and fulvic acids, 20 to 45% of the N occurs as amino acid, 33 to 59% as acid insoluble, 8 to 14% as NH_3, 2 to 5% as amino sugars and the rest (5 to 22%) as hydrolyzable unknown-N (HUN). Thus, the concentration of amino acid adsorption sites can be estimated as:

$$C_{aminoacid} = f_{aminoacid} (N\%/1.4) C_{HS} /1000$$

where N% is the total percent of N by weight in the humic substances. The default value of $f_{aminoacid}$ is 0.33.

The concentrations of diamine groups in HA and FA are unknown. According to Stevenson (1994), the N in the hydrolyzable unknown (N HUN) can exist in the following types of linkages: (1) as a free amino ($-NH_2$) group; (2) as an open chain (-NH-, =NH-) group; (3) as part of a heterocylic ring; (4) as a bridge constituent linking quinone groups together; and (5) as an amino acid attached to an aromatic ring such that it is not released by acid hydrolysis. The first two types of linkages are essentially primary, secondary, and tertiary amine groups. As a default value we assume that 3% of the organic N content of humic substance exists as diamines where the amino groups are separated by either one or two carbons ($f_{diamine} = 0.03$). Thus, the concentration of diamine group sites for adsorption can be estimated as follows:

$$C_{diamine} = f_{diamine} (N\%/1.4) C_{HS} /1000$$

TOTAL CONCENTRATION OF SULFUR-BEARING GROUPS

Freney (1967) found the average distribution of S in 24 Australian soils was as follows: ester sulfate-S (52%); carbon-bound (41%); and inorganic (7%). The carbon-bound S was directly attached to either one carbon (R-SH, such as cysteine) or two carbons (R1-S-R2, such as methionine). We assume, as a default value, that 60% of the carbon-bound S is attached to one carbon ($f_{S1C} = 0.6$). The single carbon-bound S includes cysteine, cysteine types of amino acids, amines and carboxylic acids with a sulphydryl group (-SH). The concentration of the SH-functional group sorption sites can be estimated as follows:

$$C_{SH} = f_{S1C} f_{SC} (S\%/3.2) C_{HS} /1000$$

where S% is the total weight percent of S in the humic substances and f_{SC} is the fraction of total sulfur carbon-bound. Our assigned default values of f_{SC} and S% are 0.45 and 1.5%, respectively.

CASE STUDIES

Fish and Morel (1985) reported experimental data for Cu(II) binding on Grassy Pond FA determined by an ion-selective electrode (ISE) and by fluorescence quenching (FQ). These data were used to assign the final default parameter values described previously. As a model test exercise, this self-consistent set of parameters was then used to model the experimental data of several other metals on humic substances of various origins. The assigned parameter values described previously were not further adjusted in this test (see Figures 11-17). The experimental data of Fish and Morel (1986), along with one model "calibration" result, are presented in Figure 10. Our goal was not to obtain an exact fit of experimental data; rather we wished to determine if our simple *a priori* model can be used to reliably predict general metal binding behavior under various environmental conditions.

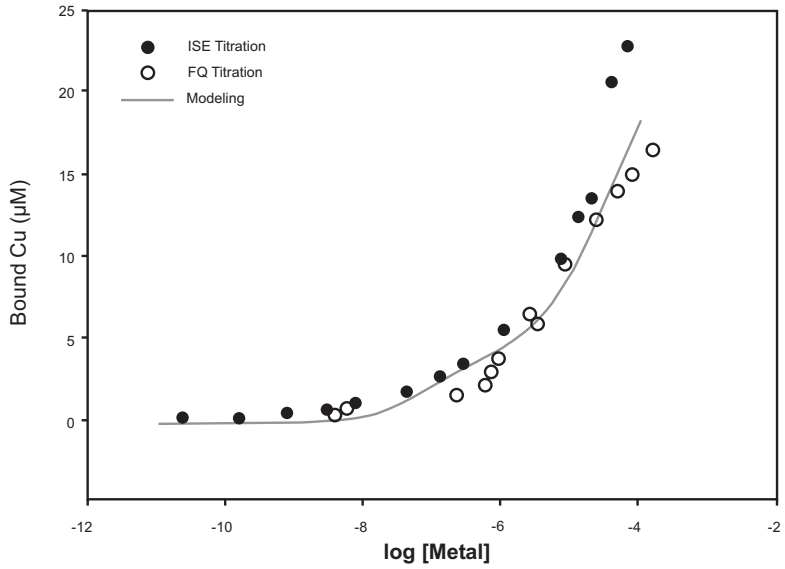

Figure 10. Comparison of model prediction of Cu binding with experimental data (ion-selective electrode titration and fluorescence titration) from Fish and Morel (1985) on Grassy Pond FA.

The experimental data of Cu(II) binding on purified peat HA (Benedetti *et al.* 1995) were modeled and the results are presented in Figure 11. Our simple model over-estimated the binding of Cu(II) at concentrations above 10^{-5} M. However, at lower concentrations, our model quantitatively predicted the Cu(II) binding.

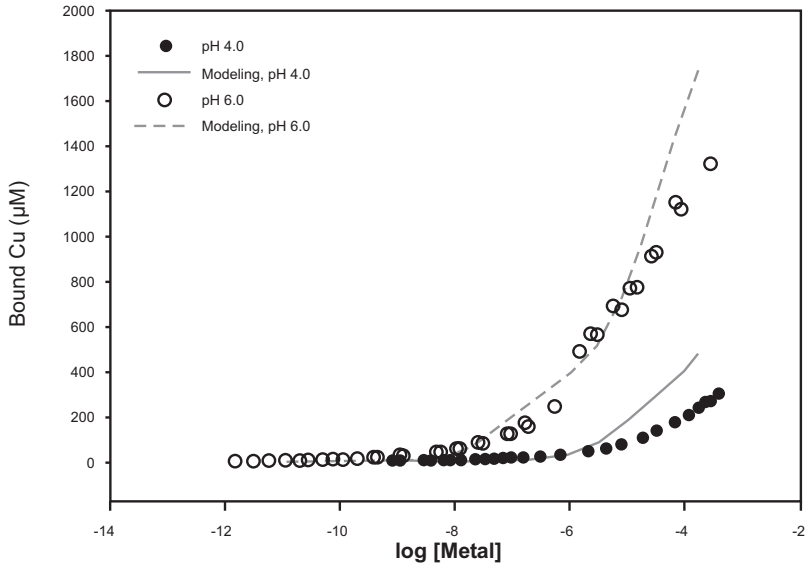

Figure 11. Comparison of model prediction of Cu binding with experimental data from Benedetti *et al.* (1995) on purified peat HA.

The experimental data of Cd(II) binding on purified peat HA (Benedetti *et al*. 1995) were also modeled and the results compared to data are presented in Figure 12. Similar to the case for Cu(II), the model quantitatively predicted Cd(II) binding at metal concentrations up to 10^{-4} M. At higher concentrations, however, the model underestimated Cd(II) binding.

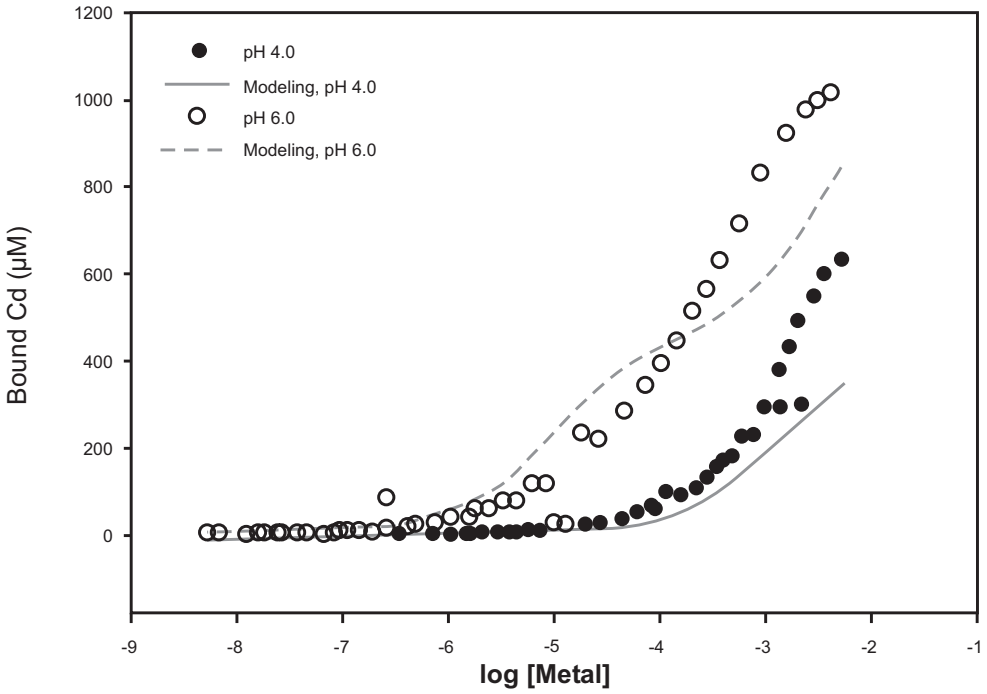

Figure 12. Comparison of model prediction of Cd binding with experimental data of Benedetti *et al*. (1995) on purified peat HA.

The experimental data of Pb(II) binding on purified peat HA (Kinniburgh *et al*. 1996) and on sludge FA (Sposito *et al.* 1981) were modeled and the results compared to the data are presented in Figure 13. Once again, we were able to quantitatively predict Pb(II) binding at metal concentrations below 10^{-6} M. At higher metal concentrations, however, the model greatly underestimated Pb(II) binding.

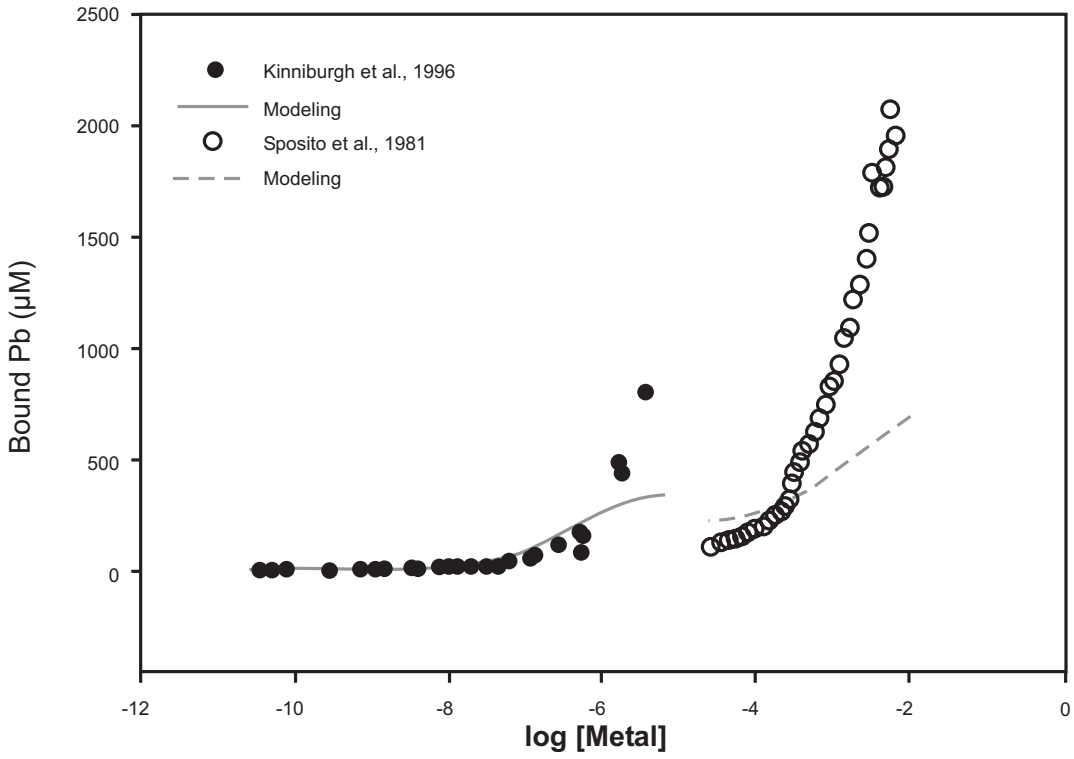

Figure 13. Comparison of model prediction of Pb binding with experimental data of Kinniburgh *et al*. (1998) on purified peat HA and with those of Sposito *et al.* (1981) on sludge FA.

Experimental data of Jin (unpublished data) of single metal, as well as multiple metal, adsorption on HA from a flood plain of the Altamaha River in south Georgia were modeled. Results were compared to the experimental data and are presented in Figures 14 - 17. For single metal adsorption, the model quantitatively described Al(III) sorption, but underestimated most other metals, with the exception of Ni(II) and Co(II) (Figures 14 and 15). However, for most of the metals, our modeling results only differed from the experimental data by a factor of two. The underestimated adsorption of Cr(III) was likely caused by the unavailability of measured stability constants for organic ligands and Cr(III) in the NIST database. Specifically, we had stability constants for only three of the nine ligands for Cr(III) used in the model. The underestimated adsorption of Pb(II) was probably due to one of two reasons. Either Pb(II) forms multiple bonds with some of the ligands, resulting in higher stability, or Pb(II) is bound to other unknown and unaccounted for ligands. For multiple metal adsorption, the model correctly predicted that Cr(III), Al(III), Cu(II) and Pb(II) are significantly adsorbed, whereas other metals are essentially not adsorbed (Figures 15 and 16).

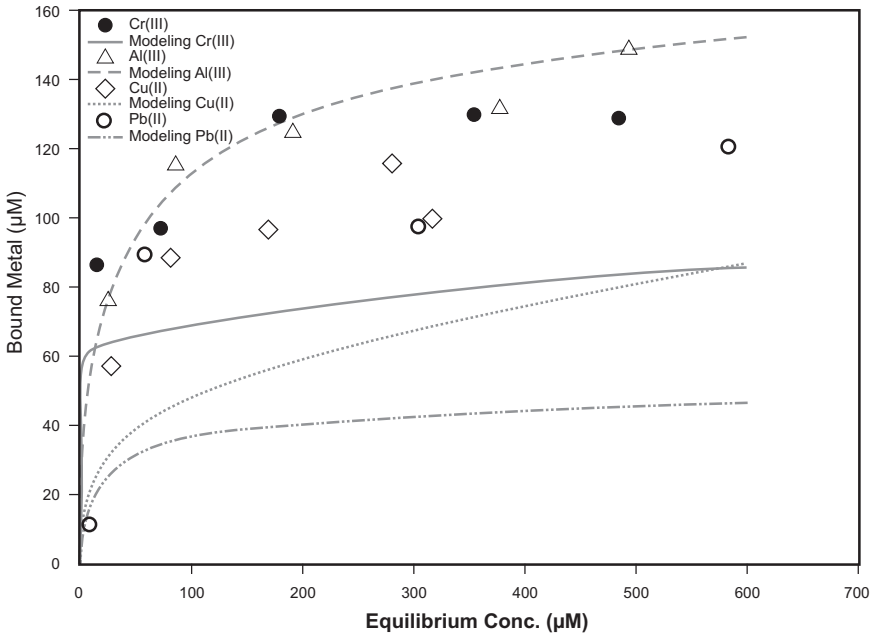

Figure 14. Comparison of model prediction of single ion adsorption for Cr(III), Al(III), Cu(II), and Pb(II) on HA from a flood plain of the Altamaha River in south Georgia. Experimental data were obtained from Jin *et al*. (unpublished data, pH 4.00).

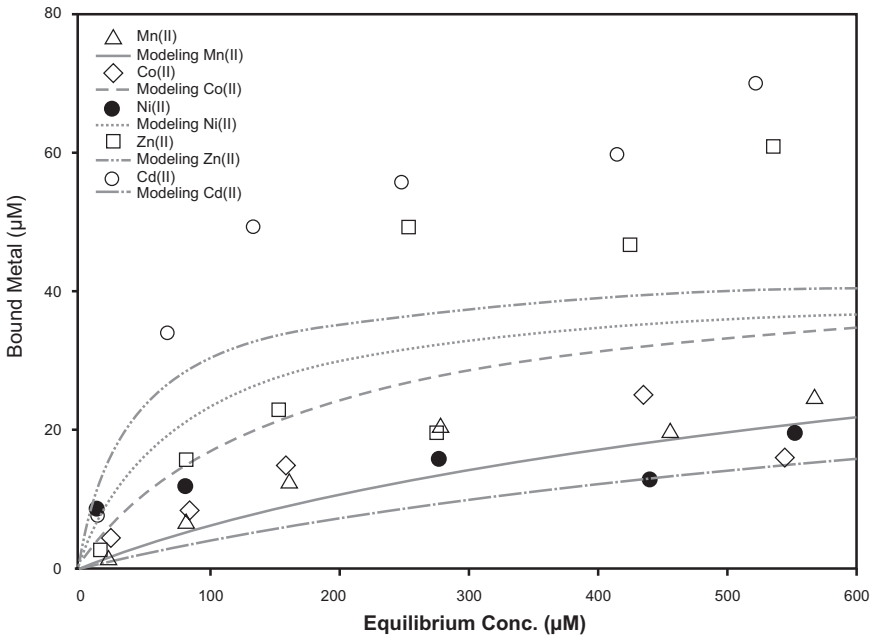

Figure 15. Comparison of model prediction of single metal adsorption for Mn(II), Co(II), Ni(II), Zn(II) and Cd(II) on HA from a flood plain of the Altamaha River in south Georgia. Experimental data were obtained from Jin *et al*. (unpublished data, pH 4.00).

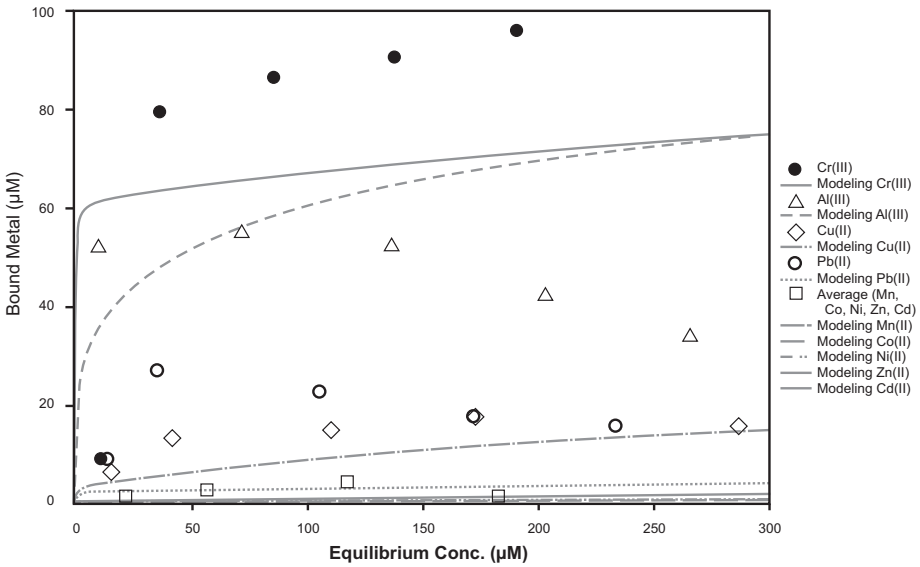

Figure 16. Comparison of model prediction of competitive ion adsorption from solutions containing thirteen metals at the same concentration. Experimental data were obtained from Jin *et al*. (unpublished data, pH 4.00) on HA from a flood plain of the Altamaha River in south Georgia.

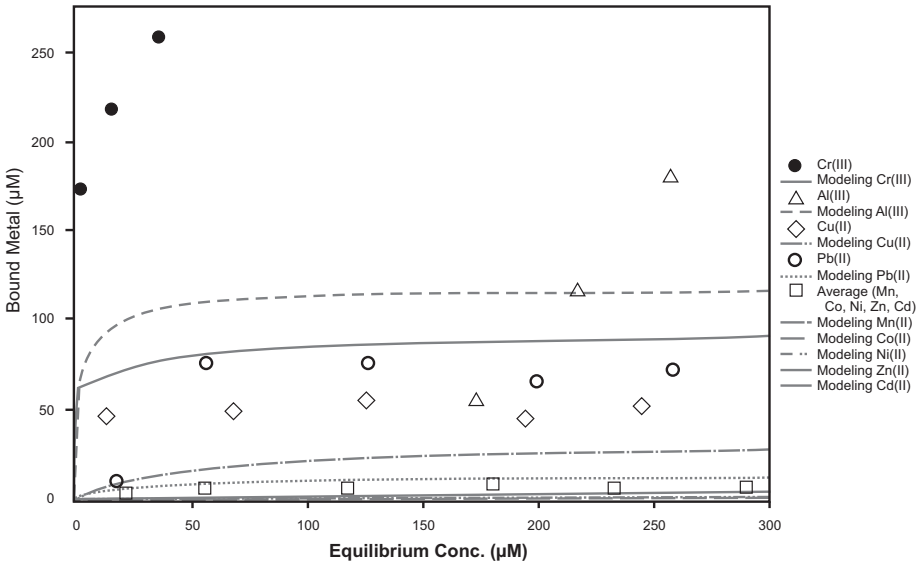

Figure 17. Comparison of model prediction of competitive ion adsorption from solutions containing thirteen metals at the same concentration. Experimental data were obtained from Jin *et al.* (unpublished data, pH 4.98) on HA from a flood plain of the Altamaha River in South Georgia.

As stated earlier, our goal was not to obtain an exact fit to the experimental data. Rather our interest was to develop an *a priori* model that was capable of predicting the trend and major

features of adsorption and competitive adsorption of metals by organic matter under a wide range of environmental conditions. As noted, we did not change the ligand parameter values once the model was calibrated (see Figure 10). However, if the actual elemental and chemical analysis data for individual humic substances had been available to establish a more accurate estimate of the model ligand parameters, the difference between the experimental data and model predictions would likely have been reduced and the concentration range of quantitative predictability increased.

MODEL DEFICIENCIES

The model presented herein clearly has some deficiencies. Specifically, it underestimates the adsorption for several metals. This may result from: (1) the unavailability of stability constants for the metal-ligand complexes; (2) the formation of 1:2 type metal-ligand complexes with functional groups within or between macromolecules of the humic substances; and (3) the binding of metals to other functional groups in the humic substances that the model does not consider. In addition, our model does not consider hydrolysis and the speciation of free metal ions and their effect on metal binding with humic substances. Thus, our simple model can only handle adsorption of metals at relatively low pH levels. We hope that this simple model will be linked to a suitable metal speciation model, such as MINTEQA2 (Allison *et al.* 1991), that will allow one to compute the hydrolysis and speciation of metals with inorganic species and the complexation with organic ligands simultaneously.

SUMMARY

We developed an *a priori* metal-organic matter predictive adsorption model based on the elemental composition and functional group concentrations of humic substances, using the NIST database of critically selected stability constants of metal complexes. We extracted and tabulated the stability constants for metal complexes with selected functional groups (organic ligands) for metals of environmental concern. We also displayed the corresponding conditional stability constants for these metals at several pH levels. Our data showed that in addition to oxygen-bearing functional groups, the nitrogen-bearing and sulfur-bearing groups are also important for metal binding. Specifically, the amino acid group plays a significant role for binding of Cu(II), Hg(II), Cr(III) and Fe(III), whereas the SH-functional group is important in the binding of soft Lewis acid metals, such as Cd(II), Hg(II), and Pb(II). We have shown that our simple model is capable of quantitatively predicting adsorption and competitive adsorption of metals when the concentration of metals is less than 10^{-5} to 10^{-6} M, the relevant metal concentration range under most environmental settings.

We did not consider hydrolysis and the speciation of free metals and their effect on overall binding to humic substances. Thus, we have only modeled the adsorption of metals at relatively low pH. We hope that this model will be linked to an appropriate metal speciation model, such as MINTEQA2 (Allison *et al*. 1991), that will allow computation of hydrolysis and

speciation of metals with inorganic species and the complexation with organic ligands, simultaneously.

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REFERENCES

Allison, J.D. and E.M. Perdue. 1994. Modeling Metal-Humic Interactions with MINTEQA22. *In:* Humic Substances. *In:* The Global Environment and Implications On Human Health, N. Senesi and T.M. Miano (eds). Elsevier Science. New York. Pp. 927-94.

Allison, J.D., D.S. Brown, and K.J. Novo-Gradac. 1991. MINTEQA2/PRODEFA2, A geochemical assessment model for environmental systems: Version 3.0 User's Manual. U.S. Environmental Protection Agency. EPA/600/3-91/021. Athens, GA.

Bartschat, B.M., S.E. Cabiniss, and F.M.M. Morel. 1992. Oligoelectrolyte model for cation binding by humic substances. Environmental Science & Technology 26:284-294.

Benedetti, M.F., C.J. Milne, D.G. Kinniburgh, W.H. Van Riemsdijk, and L.K. Koopal. 1995. Metal ion binding to humic substances: application of the non-ideal competitive adsorption model. Environmental Science & Technology 29:446-457.

Borkovek, M., U. Rusch, and J.C. Westall. 1998. Modeling of Competitive Ion Binding to Heterogeneous Materials with Affinity Distributions. *In:* Adsorption of Metals by Geomedia, E.A. Jenne (ed). Lewis Publishers. Boca Raton, Florida. Pp 467-520.

Bose, P. and D.A Reckhow. 1997. Modeling pH and ionic strength effects on proton and calcium complexation of fulvic acid: A tool for drinking water-NOM studies. Environmental Science & Technology 31:765-770.

Buffle, J., R.S. Altman, and M. Fillea. 1990. Effect of physico-chemical heterogeneity of natural complexants Part II. Buffering action and role of their background sites. Geochimica et Cosmochimica Acta 54:225-237.

Cabannis, S.E. and M.E. Shuman. 1988. Copper binding by dissolved organic matter. I. Suwannee River fulvic acid equilibria. Geochimica et Cosmochimica Acta 52:185-193.

Cernik, M., M. Borkovec, and J.C. Westall. 1995. Regularized least-squares methods for the calculation of discrete and continuous affinity distributions for heterogeneous sorbents. Environmental Science & Technology 29:413-425.

Cernik, M., M. Borkovec, and J.C. Westall. 1996. Affinity distribution description of competitive ion-binding to heterogeneous materials. Langmuir 12:6127-6137.

Dempsey, B.A. and C.R. O'Melia. 1983. Proton and calcium complexation of four fulvic acid fractions. *In:* Aquatic and Terrestrial Materials, R.F. Christman and E.T. Gjessing (eds). Ann Arbor Science Publishers. Ann Arbor, Michigan. Pp 239-273.

De Wit, J.C.M., W.H. Van Riemsdijk, M.M, Nederlof, D.G. Kinniburgh, and L.K. Koopal. 1990. Analysis of ion binding on humic substances and the determination of intrinsic affinity distribution. Analytical Chimica Acta 232:189-207.

De Wit, J.C.M., W.H. Van Riemsdijk, and L.K. Koopal. 1993. Proton binding to humic substances. 1. Electrostatic effects. Environmental Science & Technology 27:2005-2014

Dobbs, J.C., W. Susetyo, L.A. Carreira, and L.V. Azarraga. 1989. Competitive binding of protons and metal ions in humic substances by lanthanide ion probe spectroscopy. Analytical Chemistry 61:1519-1524.

Dzombak, D.A., W. Fish, and F.M.M. Morel. 1986. Metal-humic interactions. 1. Discrete ligand and continuous distribution models. Environmental Science & Technology 20:669-676.

Fish, W. and F.M.M. Morel. 1985. Propagation of error in fulvic acid titration data: A comparison of three analytical methods. Canadian Journal of Chemistry 63:1185-1193.

Fish, W., D.A. Dzombak, and F.M.M. Morel. 1986. Metal-humic interactions. 2. Application and comparison of models. Environmental Science & Technology 20:676-683.

Freney, J.F. 1967. Sulfur-coated organics. *In:* Soil Biochemistry, A.D. McLaren and G.H. Peterson (eds). Marcel Dekker. New York. Volume 1:229-259.

Ghosh, K. and M. Schnitzer. 1980. Macromolecular structures of humic substances. Soil Science 129:266-276.

Gregor, J.E. and H.K.J. Powell. 1988. Protonation reactions of fulvic acids. Journal of Soil Science 39:243-252.

Huheey, J.E., E.A. Keiter, and P.L. Keiter. 1996. Inorganic chemistry: Principles of Structure and Reactivity, Fourth Edition. Harper Collins College Publishers. New York.

Irving, H and R.J.P. Williams. 1953. The stability of transition-meal complexes. Journal of the Chemical Society 85:3192-3220.

Jenne, E. A. 1998. Adsorption of metals by geomedia: Data Analysis, modeling, controlling factors and related issues. *In:* Adsorption of Metals by Geomedia, E.A. Jenne (ed). Lewis Publishers. Boca Raton, Florida. Pp 2-74.

Jin, X., G.W. Bailey, Y.S. Yu, and A.T. Lynch. 1996. Kinetics of single and multiple metal ion sorption processes on humic substances. Soil Science 161:509-520.

Keefer, R.F., E.E. Codling, and R.N. Singh. 1984. Fractionation of metal-organic components extracted from a sludge-amended soil. Soil Science Society of America Journal 48:1054-1059.

Kinniburgh, D.G., C.J. Milne, M.F. Benedetti, J.P. Pinheiro, J. Filius, L. K. Koopal, and W.H. Van Riemsdijk. 1996. Metal ion binding by humic acid: application of the NICA-Donnan model. Environmental Science & Technology 30:1687-1698.

Kinniburgh, D.G., W.H. Van Riemsdijk, M.F. Benedetti, and L. K. Koopal. 1998. Ion binding to humic substances: Measurements, models and mechanisms. *In:* Adsorption of Metals by Geomedia, E.A. Jenne (ed). Lewis Publishers. Boca Raton, Florida. Pp 486-522.

Klopman, G. J. 1968. Chemical reactivity and the concept of charge-and-frontier-controlled reactions. Journal of American Chemical Society 90:223-234.

Leenheer, J.A., R.L. Wershaw, and M.M. Reddy. 1995. Strong acids, carboxylic-group structures in fulvic acid from the Suwannee River, Georgia. 1. Minor structures. Environmental Science & Technology 29:393-398.

Leenheer, J.A., R.L. Wershaw, and M.M. Reddy. 1995. Strong acids, carboxylic-group structures in fulvic acid from the Suwannee River, Georgia. 2. Major structures. Environmental Science & Technology 29:399-405.

Livens, F.R. 1991. Chemical reactions of metals with humic material. Environmental Pollution 70:183-208.

Mattigod, S.V. and G. Sposito. 1979. Chemical modeling of trace metal equilibria in contaminated soil solutions using the computer program GEOCHEM. *In:* Chemical Modeling in Aqueous Systems, E.A. Jenne (ed.). American Chemical Society. Washington DC. Pp. 837-856.

Milne, C.J., D.G.Kinniburgh, J.C.M. De Wit, W.H. Van Riemsdijk, and L.K Koopal. 1995. Analysis of proton binding by a peat humic acid using a simple electrostatic model. Geochimica Et Cosmochimica Acta 59:1101-1112

Murray, K. and P.W. Linder. 1983. Fulvic acids: structure and metal bindings. I. A random molecular model. Journal of Soil Science 34:511-523.

Murray, K. and P.W. Linder. 1984. Fulvic acids: structure and metal bindings. II. Predominant metal binding sites. Journal of Soil Science 35:217-222.

Nicholls, D. 1974. Complexes and First Row Transition Elements. Macmillan, London.

Pearson, R.G.J. 1963. Hard and soft acids and bases. Journal of American Chemical Society 85:3533-3539.

Perdue, E.M. and C.R. Lytle. 1983. Distribution model for binding of protons and metal ions by humic substances. Environmental Science & Technology 17:654-680.

Perdue, E.M., J.H. Reuter, and R.S. Parrish. 1984. A statistical model of proton binding by humus. Geochimica et Cosmochimica Acta 48:1257-1263.

Posner, A.M. 1966. The humic acids extracted by various reagents from a soil. Part I. Yield, inorganic components, and titration curves. Journal of Soil Science 17:65-78.

Senesi, N. 1992. Metal-humic substance complexes in the environment. *In:* Biogeochemistry of trace metals, D.C. Adriano (ed.). Lewis Publishers. Boca Raton, Florida. Pp. 429-496

Sigel, H. and D.B. McCormick. 1970. On discriminating behavior of metal ions and ligands with regards to biological significance. Accounts of Chemical Research 3:201-220.

Smith, R.M. and A.E. Martell. 1997. Critically selected stability constants of metal complexes NIST Database 46 Version 5.0 Users' Guide. NIST, U.S. Department of Commerce. Gaithersburg, MD.

Sposito, G. 1986. Sorption of trace metals by humic materials in soils and natural waters. Critical Reviews of Environmental Controls 16:193-229. CRC Press. Boca Raton, Florida.

Sposito, G., F.T. Bingham, S.S. Yadav, and C.A. Inouye. 1982. Trace metal complexation by fulvic acid extracted from sludge: II. Development of chemical models. Soil Science Society of America Journal 46:51-56.

Sposito, G., K.M. Holtzclaw, and C.S. LeVesque-Madore. 1981. Trace metal ion complexation by fulvic acid extracted from sewage sludge: I. Determination of stability constants and linear correction analysis. Soil Science Society of America Journal 45:465-468.

Stevenson, F.J. 1994. Humus Chemistry: Genesis, Composition, and Reaction. Second Edition, John Wiley & Sons. New York.

Susetyo, W., J.C. Dobbs, L.A. Carreira, L.V. Azarraga, and D.M. Grimm. 1990. Development of a statistical model for metal-humic interactions. Analytical Chemistry 62:1215-1221.

Tipping, E., M.M. Reddy, and M.A. Hurley. 1990. Modeling electrostatic and heterogeneity effects on proton dissociation from humic substances. Environmental Science & Technology 24:1700-1705.

Tipping, E., C. Woof, and M.A. Hurley. 1991. Humic substances in acid surface waters: modeling aluminum binding, contribution to ionic charge-balance, and control of pH. Water Research 25:425-435.

Tipping, E. and M.A. Hurley. 1992. A unifying model of cation binding by humic substances. Geochimica et Cosmochimica Acta. 56:3627-3641.

Tipping, E. 1993. Modeling the competition between alkaline earth cations and trace metal species for binding by humic substances. Environmental Science & Technology 27:520-529.

Tipping, E. 1998. Humic ion-binding Model VI: An improved description of the interactions of protons and metal ions with humic substances. Aquatic Geochemistry 4:3-48.

William, D.R. 1971. The Metals of the Life. The Solution Chemistry of Metal Ions in Biological Systems. Van Nostrand Reinhold. London.

Woolard, C.D. and P.W. Linder. 1999. Modeling of the cation binding properties of fulvic acid: An extension of the random algorithm to include nitrogen and sulfur donor sites. Science of the Total Environment 226:35-46.

LOUISIANA ENVIRONMENTAL MODELING SYSTEM FOR HYPOXIA RELATED ISSUES

Robert F. Carousel and Rosemarie C. Russo[1]

ABSTRACT

An environmental assessment tool to evaluate the potential impacts of nonpoint source (NPS) pollutants discharged from the Mississippi River basin into the Gulf of Mexico, and to assess pollutant effects on receiving water quality, is described. This tool, the Louisiana Environmental Modeling System (LEMS), builds upon a joint effort by the U.S. Navy and EPA called the Northern Gulf Littoral Initiative (NGLI). LEMS will expand the state-of-the-art monitoring and coastal forecasting system developed for the Northern Gulf of Mexico. The modeling domain will focus on the western side of Louisiana to encompass the Gulf hypoxic zone and the near coastal region. LEMS will include appropriate loading algorithms to form a comprehensive model for evaluating NPS discharges. The project should provide an assessment technology that does not currently exist for the Gulf hypoxic zone, one that combines a three-media pollutant loading model with an advanced coastal hydrodynamic and water quality model. Technology to evaluate NPS discharges is important because of their impact on water quality, including the formation of a hypoxic (dead) zone in the Gulf of Mexico off the Louisiana coast line. The selected model components will be refined as necessary and coupled to produce the LEMS system, then applied to assess the water quality effects associated with NPS discharges on a large scale in the Louisiana coastal regions of the Gulf of Mexico. LEMS will provide an innovative and useful approach to evaluate the impact of NPS discharges as they are transported and transformed through land runoff, surface stream networks, groundwater pathways and coastal tidal processes. Application of this modeling system will help to define the magnitude of reduction in the various NPS discharges to the Mississippi River basin and directly into the Gulf of Mexico that would be required to obtain a long-term decrease in the extent of hypoxia formation in the coastal zone of the Gulf of Mexico.

BACKGROUND

A unique environmental assessment tool is proposed to evaluate the potential impacts of nonpoint source (NPS) pollutants discharged from the Lower Mississippi River basin, and to assess their effects on receiving water quality in the coastal zone of the Gulf of Mexico. In order to build upon a joint effort by the U.S. Navy and EPA called the Northern Gulf Littoral Initiative (NGLI), this work will extend the monitoring and coastal forecasting system developed for the Northern Gulf of Mexico. In addition, the EPA has funded an effort to develop a state-of-the-art watershed NPS pollutant loading modeling system that links hydrology, surface and groundwater

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components together in one combined system using a distributed-parameter approach rather than a lumped-parameter approach. These two modeling efforts will be linked together to form a comprehensive modeling system called LEMS for evaluating NPS discharges. The proposed model linkage is unique and innovative in that no technology exists that combines a three-media, watershed pollutant loading model with an advanced coastal hydrodynamic and water quality model. This new model will enable evaluation of the various NPS component discharges, which is important because of the potential impacts on Gulf water quality, including the formation of a hypoxic (dead) zone off the Louisiana coast line.

The mathematical model components proposed for this new assessment methodology (LEMS) include developmental, advanced watershed pollutant loading models coupled to an existing advanced coastal transport model. This in turn will be coupled to an existing, advanced water quality model to augment environmental impact assessment capabilities for coastal systems.

The EPA has already funded an effort to develop the state-of-the-art watershed pollutant loading modeling system component that links surface, instream and groundwater components together in one combined system. Once the coupling to form the LEMS has been accomplished, assessments will be aided through the use of quantified estimates of uncertainty associated with both the models and the supporting databases. This work is unique and innovative in that no existing model technology or development and application at this large of a scale has been conducted. This research and development effort is critical to provide comprehensive assessment approaches for large scale watershed management. The NGLI and EPA efforts to date have been successful in development of the advanced coastal zone hydrodynamic component of this modeling system, ECOMSED, and the advanced water quality model component, RCA.

Model coupling is achieved by extracting required data from one model component output (*e.g.*, circulation) to serve as input for the next model component (*e.g.*, water quality). Once the linked system (LEMS) is complete, it will provide a unique capability for NPS management organizations to evaluate the impact of nonpoint source pollutant discharges within a watershed and its tributaries and the subsequent migration of these discharges to the Gulf of Mexico. Because the model components developed in this project are physically-based, they will be readily transportable to other watersheds and estuaries/coastal zone waters.

The Louisiana Gulf coast modeling domain will be used to illustrate our approach to refine and couple mathematical simulation models to assist in the assessment of NPS pollutant loading impacts from the Lower Mississippi River Basin/Region. This effort will involve extending the NGLI modeling domain west of the Mississippi River along the Louisiana and Texas coastlines. The existing NGLI modeling domain is presented in Figure 1, extending east of the Mississippi River along the Mississippi and Alabama coastlines, including Mobile Bay. The Lower Mississippi Region consists of the tributary drainage of the Mississippi River below its confluence with the Ohio River, with the exception of major portions of the Arkansas, Red, and White Rivers, and coastal rivers and streams from the western boundary of the Pearl River Basin in Mississippi to the eastern edge of the Sabine River Basin in western Louisiana.

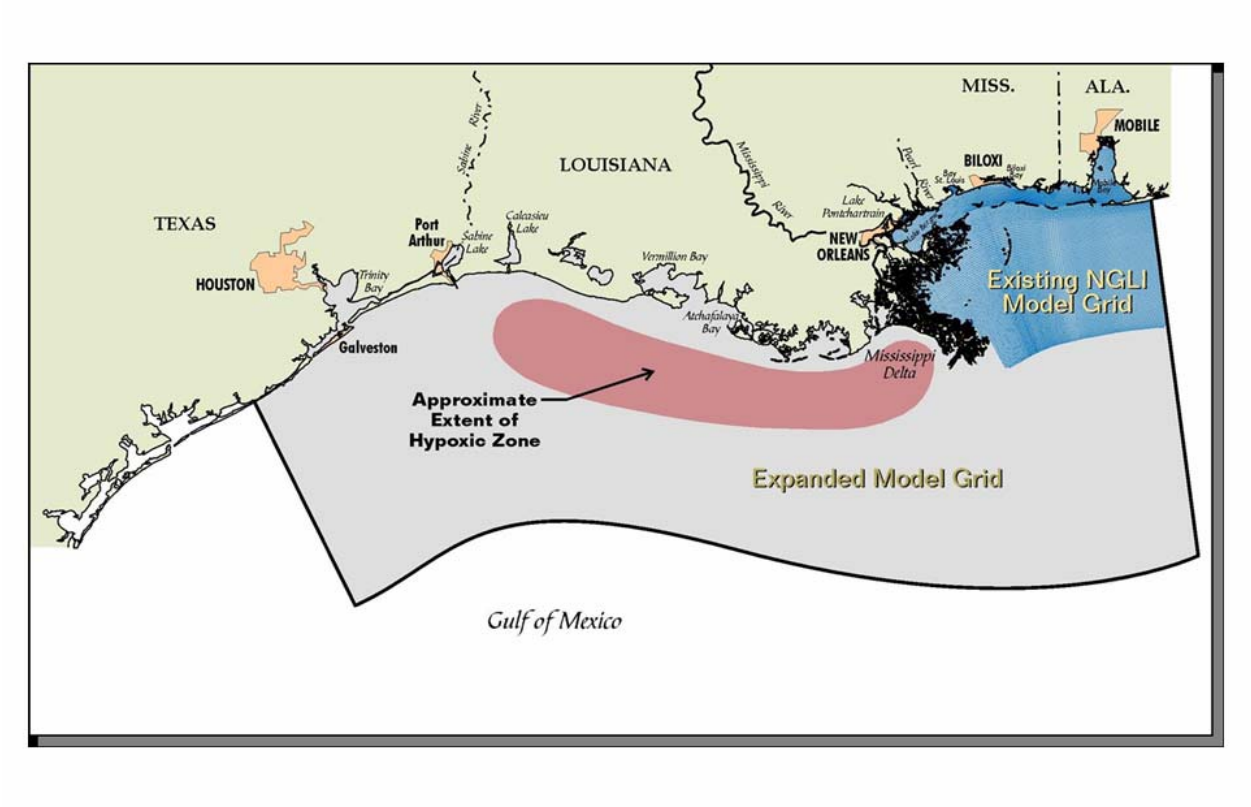

Figure 1. Proposed Louisiana modeling domain.

The modeling strategy presented will develop watershed hydrologic and water quality simulation models, initially for the Lower Mississippi Region, that will serve as templates for expansion to the whole Mississippi Basin. The proposed LEMS model(s) components consist of developmental watershed hydrologic and water quality components (*i.e.*, MODHMS) and existing estuarine hydrodynamic and water quality components (*i.e.*, ECOMSED/RCA). This paper describes the watershed and water quality component models, including models of the input loadings to the tidal Gulf of Mexico and Mississippi River, as well as water quality models of the interior river basins, for estimating local impacts of land management activities and practices.

The modeling system proposed for the Gulf program will be useful for evaluation of both local water quality (*i.e.*, in upstream areas) and regional water quality (*i.e.*, in the lower Mississippi River and Gulf of Mexico). It will also provide an aquatic ecosystem model whose purpose is to simulate the fate of common pollutants (nutrients, sediment and toxic contaminants) and their effect on aquatic biota. There is a decided emphasis and need for assessing the impact of trace contaminants on the aquatic ecosystem.

Nonpoint source (NPS) pollutant discharges (*e.g.*, nutrients, sediments, and toxics) into the Gulf of Mexico, largely to and through the Mississippi River Basin, are contributing to the formation of a zone of low dissolved oxygen (hypoxia) along the Gulf coast of Louisiana and Texas. It has been estimated that over 90% of the NPS pollutant loads to the Gulf originate from the Mississippi River Basin. Activities on the landscape impact vegetation, habitat, soil structure and certain ecological endpoints, as well as determine the NPS pollutants exported and their amounts.

An integral part of managing a watershed is land management. Therefore, our goal is to develop modeling tools to assess the environmental/ecological benefit of management alternatives. The work described herein focuses on the analysis of sediment, nutrient and chemical removal efficiencies using different vegetation species and layout landscaping/buffer zone designs. Ultimately, these models will provide a tool for determining if well-chosen land management practices can solve problems like the hypoxic zone in the Gulf of Mexico, and to what degree the management options must be maintained or even expanded to protect future water quality.

MODEL DEVELOPMENT

In the terrestrial environment, physical processes will be simulated by the model MODHMS. MODHMS is a fully integrated and comprehensive hydrologic and groundwater modeling system. It was developed by incorporating additional modules into the popular MODFLOW code to provide a physically-based, spatially-distributed, conjunctive surface/subsurface modeling framework that includes: three-dimensional (3D), variably-saturated subsurface flow (Richards equation); two-dimensional (2D) areal overland flow (diffusion wave approximation); and flow through a network of one-dimensional (1D) channels or pipes (diffusion wave approximation, with Priesmann Slot conceptualization for pressurized flow in pipes).

MODHMS addresses a variety of hydrologic modeling situations. The hydrologic cycle is viewed as a fully integrated system with dynamic interactions between all regimes of flow. Various surface and subsurface features are addressed by the model to provide the flexibility of implementation in both rural and urban settings, with or without flow controls. Comprehensive interception and evapotranspiration calculations are also included, with various Potential Evapotranspiration formulas (ET from bare ground or vegetated surfaces) that can be applied in different regions of the model domain. Analytic infiltration formulas can also be applied in different regions of the model domain where the subsurface is not explicitly modeled. Hydraulic structures, withdrawals, and flow regulation schemes are also simulated to provide a tool for analysis as well as management of water budgets.

Computation burdens are alleviated by treating one or more flow domains as 'inactive' in portions of a simulated region. A curvilinear grid option discretizes the equations using integrated, finite differences to allow for flexible gridding around geometric, topographic, or

lithologic features. Sequential coupling of flow domains is also provided as an option for efficient simulation of low interaction systems. Newton-Raphson linearization, with backtracking and under-relaxation formulas, provides robustness for highly non-linear situations.

Due to the underlying MODFLOW structure, MODHMS interfaces with several GUIs for subsurface analyses and with ArcView GIS products to provide a state-of-the-art system for comprehensive watershed analyses.

A three-dimensional, time-variable, coupled hydrodynamic and water quality model (Environmental Modeling System - EMS) will be linked to the hydrologic and groundwater model MODHMS to produce the LEMS (Figure 2). The proposed EMS model will be used to simulate processes in receiving waters, chiefly estuarine waters. It consists of two primary modeling components:

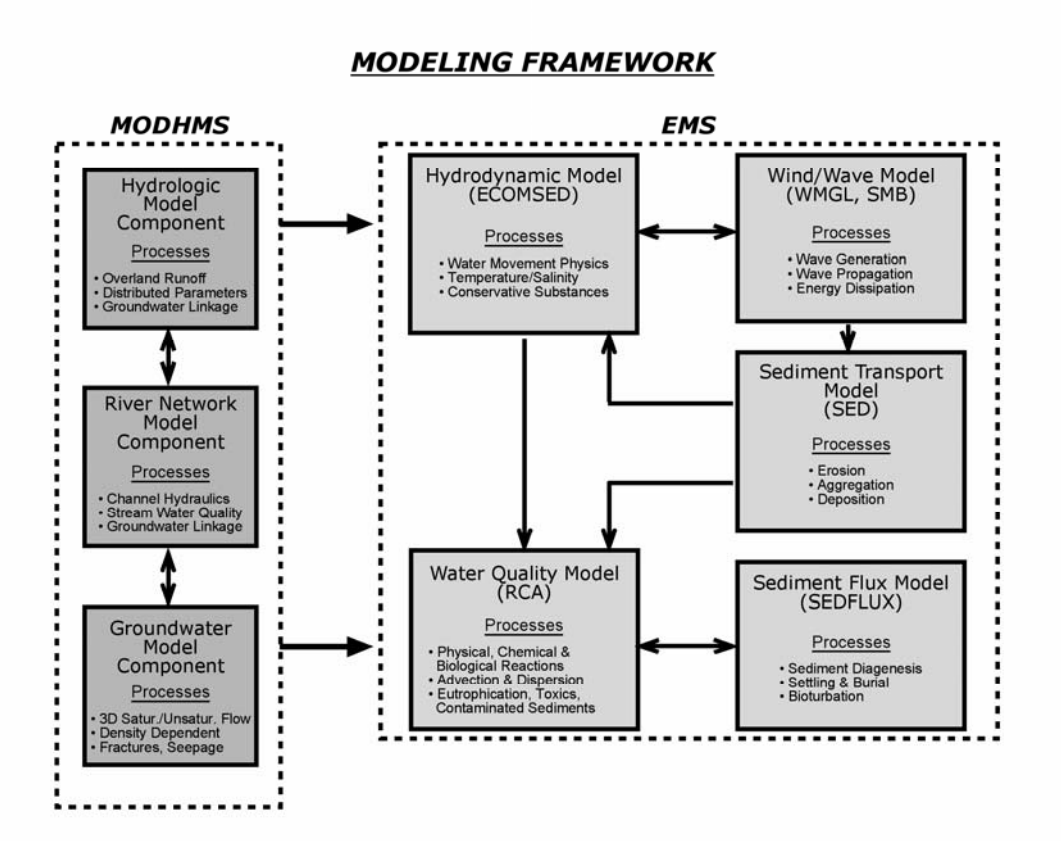

Figure 2. Modeling framework.

1. The hydrodynamic/pollutant transport model ECOMSED, a multi-dimensional, time-dependent, estuarine and coastal water circulation model (linked to a Wind/Wave Model and a Sediment Transport Model); and
2. The water quality model, RCA, that is capable of analyzing various water quality processes (*e.g.*, eutrophication, toxic fate and transport, and contaminated sediment transport), also linked to the Sediment Transport Model and a Sediment Flux Model.

The ECOMSED model incorporates state-of-the-art equations that describe the motion of water due to freshwater inflow, density-driven currents, and meteorology confined by a realistic representation of the system's bathymetry. A system of curvilinear coordinates is used in the horizontal direction, allowing for a smooth and accurate representation of variable shoreline geometry. The curvilinear coordinate system also allows multiple branches for application to geometrically complex water bodies such as dendritic reservoirs or estuaries. Variable grid spacing allows higher resolution in areas of interest. In the vertical scale, the model can use either a transformed coordinate system, known as the sigma-coordinate (σ) transformation, to permit better representation of bottom topography or a z-level coordinate system for better representation of channels. Water surface elevation, water velocity, temperature, salinity, and water turbulence are calculated in concert with the Wind/Wave Model in response to meteorological conditions (wind and incident solar radiation), freshwater inflow, tides, temperature and salinity (density) at the model boundaries. In this fashion, the model can also be applied to estuaries, rivers, or portions of a waterbody by specifying upstream and/or downstream head boundary conditions, all of which can be time-varying.

The three-dimensional, time-variable water quality model, RCA, is a generalized modeling framework. It is made problem-specific via the development or inclusion of a FORTRAN-based kinetic subroutine that describes the physical, chemical and biological processes of the ecosystem under investigation. The RCA computer code is the direct descendant of the WASP model. RCA differs from WASP in that the computational grids used by RCA are structured grids, while WASP uses a pointer schematization. The use of a structured grid system permits the RCA code to take advantage of computers that are capable of parallel processing, thus reducing program execution times. The RCA computer code uses finite-difference techniques to simulate the time-varying processes of advection and dispersion, while considering point and nonpoint source pollutant mass loading, boundary exchange, and linear and non-linear losses and production. Information concerning the advective and dispersive transport fields is provided to RCA by MODHMS or ECOMSED.

Model Linkage

There are multiple connections to be considered when developing the linkage strategies. Three models are used as an example: MODHMS (land), MODHMS (stream), and EMS for estuaries. These three models simulate different processes/constituents at different time and space scales. Their physical domains (discussed previously) and resulting transfer of information must be appropriately integrated to allow efficient operation and effective representation of the sub-basins of the Lower Mississippi Region system. These process/constituent, space, and time linkage issues and approaches for the three possible example interfaces (*i.e.*, MODHMS (land)–EMS, MODHMS (stream)–EMS, and MODHMS (land)–MODHMS (stream)) are discussed in the following. For modeling large basins, with the likelihood of using MODHMS for many watersheds and the resulting linkage issues, model organization and efficiency would be greatly improved if EMS were directly interfaced with the MODHMS system that it uses for time series input data. This would allow EMS to directly utilize all of the meteorological and calibration data assembled for the MODHMS watershed

modeling, as well as provide the direct linkage of flows and environmental constituents between the models. The final linked models (LEMS) will be contained within one model GUI that will allow use of the models both in a stand-alone manner and in the linked version. The following model linkages will be completed as part of this effort.

MODHMS (land)–MODHMS (stream) Linkage: MODHMS (land) produces loadings of flow, sediment, heat, and environmental constituents as input to the tributary water bodies. In this example, the receiving water body is the MODHMS (stream) module. This linkage is well established and relatively straightforward, since the two components of MODHMS are generally used together, and they are combined in a single, integrated software system. Within the MODHMS input structure, all land area that drains to a stream reach is easily connected to the reach. Correspondence between state variables in the two components is built into the system, and remaining issues have been resolved in numerous previous applications of the program.

MODHMS (land)–EMS Linkage: In this linkage, nonpoint source loadings of flow, sediment, heat, and environmental constituents are generated by MODHMS and transferred to water bodies (*i.e.*, lakes, tidal rivers or estuaries) simulated by EMS. For modeling applications of this scale, an appropriate spatial linkage scheme consists of allocating the total load generated from the area tributary to the water body EMS "model segments" by prorating based on the length of shoreline in each segment. A time step of one hour for the simulation (*i.e.*, the interval of the internal model calculations) of most watersheds is normally used. In addition to watershed size and hydrologic response times, the appropriate time step is also controlled by the availability of representative precipitation data. For most watersheds, there are sufficient hourly precipitation stations in and near the watershed to allow an hourly simulation interval. EMS will read the hourly outputs from MODHMS and interpolate/adjust the values to match the internal time step. Since the water quality state variables in EMS are very similar to those in MODHMS, the correspondence used for the MODHMS (land)–MODHMS (stream) linkage will be used to complete this linkage.

MODHMS (stream)–EMS Linkage: In most situations, this linkage will be implemented in one direction since MODHMS (stream) will provide the upstream inflows (and sometimes tributary inflows) to EMS at locations of minimal tidal influence. However, in certain circumstances, MODHMS may receive information from the EMS output. The spatial and temporal linkages are relatively straightforward, but development will be needed to link the water quality constituents, and this is not reflected in Figure 2. These situations can arise when assessing groundwater interactions in coastal areas for quantifying groundwater loading of pollutants, such as nitrite plus nitrate nitrogen, bacteria and/or toxic contaminants. While the constituents in the two water quality models are similar, there are some differences in the definition of organic material.

DATA REQUIREMENTS AND DEVELOPMENT PROCEDURES

Model data requirements for MODHMS are required in both spatial and temporal detail, especially for a basin the size and complexity of the Lower Mississippi. Table 1 lists the typical data requirements for a MODHMS application to NPS pollution in which chemicals and managements practices will be modeled. The following sections provide some details on the principal data types and procedures needed for a large, regional modeling effort such as this. Data for EMS are required in both spatial and temporal detail, and potentially in vertical detail in receiving waterbodies where vertical stratification can occur. Table 2 lists the typical data requirements for an EMS application for both the hydrodynamic model (ECOMSED) and the water quality model (RCA). Additional detail on the principal data requirements for tidal model applications is presented in the following sections.

It should be noted that while the data requirements are large, the data are readily available, and for the most part exist in electronic form.

Table 1. Data Requirements for MODHMS Model Application

	Item	Source
1.	Precipitation and meteorological data (for simulation period)	NOAA
2.	Watershed land use/land cover characteristics	EPA BASINS
3.	Hydrography and channel characterization	EPA BASINS
4.	Monitoring program observations	Federal/State data sets
5.	Other useful information	
	a. Description/quantification of other contaminant/pollutant sources (*e.g.* point sources, feedlots)	
	b. Technical reports or articles that analyze and/or summarize the available monitoring data	
	c. Soils characterization information for estimating model parameters	
	d. Agronomic data, such as expected residue amounts and crop cover, expected uptake of N and P, manure applications and nutrient content, and expected crop moisture requirements	

Table 2. Data Requirements for EMS and RCA Model Application

ECOMSED

	Item	Source
1.	Meteorological data (solar radiation, wind speed and direction)	NOAA
2.	Bathymetric data	USGS, NOAA
3.	Tidal stage at model boundaries	NOAA
4.	Freshwater river flow	USGS
5.	Monitoring program observations (salinity, temperature, current velocities)	NOAA, Navy
6.	Other useful information	
	a. Shipping channel geometry	
	b. Bottom roughness estimates	
	c. Off-shore coastal conditions	

RCA

	Item	Source
1.	Meteorological data (solar radiation, wind speed and direction, light extinction)	NOAA
2.	PS, NPS and atmospheric pollutant loadings estimates	EPA, NOAA
3.	Monitoring program observations (BOD, DO, nutrients, sediment, metals, toxic contaminants, biological endpoints, cations/anions)	EPA, USGS
4.	Reaeration estimates (based on wind speed or surface water currents)	Literature
5.	Biological species diversity and abundance	State Agencies

Watershed Characterization Data

Basin characterization data are defined as spatial data (generally in GIS format) that are used to:

- Segment the basin based on drainage, topography, land use and cover, and soils;
- Segment the stream/river network; and
- Estimate various model inputs such as slope, elevation, drainage, sediment, and soil nutrient parameters.

The specific data needed include basin boundaries, DEM (Digital Elevation Model) output, land use, soils data, and stream/river network coverage (hydrography).

A uniform database, containing much of these data for the U.S., had been assembled by EPA in the BASINS software package. This database is organized by EPA Region, and the data for the Lower Mississippi Basin are contained primarily within Regions 4 and 6, with a small portion (within the state of Missouri) in Region 7. The BASINS database contains the following products or coverages that will be useful for this effort: Hydrologic Unit Boundaries, State and County Boundaries, Digital Elevation Model, Land Use and Land Cover, State Soil and Geographic Database (STATSGO), Reach File Versions 1 and 3 (RF1, RF3), and Dam Location.

The bulk of these data and coverages will be used to perform the watershed segmentation for each river basin/subbasin simulated. Stream segmentation utilizes the RF1 and DEM data, as well as the locations of monitoring stations and dams to sub-divide the land segments to derive smaller stream segments from the RF1 segments. The shorter stream segments are used to reduce numerical dispersion and allow better evaluation of local water quality by more accurately representing the location of point and nonpoint pollution sources.

River, Lake and Estuary Physical Characterization

Once the model watershed and river network segmentation has been completed, each river, lake or estuary segment (reach) must be analyzed to develop its hydraulic characteristics. These data can be developed from a rating table applicable to the reach, or by using Manning's equation along with channel (and flood plain) cross section and roughness data. These latter types of data are available from USGS District offices, U.S. Army Corps of Engineers, previous modeling studies, or in some cases adequate data are available in the EPA River Reach File (RF1) database.

For estuarine applications, waterbody bathymetric data also must be obtained (typically from NOAA), along with digital shoreline information for the development of the model grid. The shoreline data are needed to develop the orthogonal, curvilinear spatial grid using existing software within an ArcView platform. The bathymetric data are then used to develop the average water depth in each model segment at some reference tidal condition (*e.g.*, mean sea level, etc). This step in model development is extremely important for the success of the subsequent model application, in that shoreline and shipping channel features must be considered when developing the model grid for an accurate representation of the system bathymetry.

Climatic Data

MODHMS requires hourly rainfall data, with as much spatial resolution as possible, for adequate calibration because rainfall is the primary driving force for the model. Development of an adequate rainfall database will require significant effort to obtain the hourly and daily data, fill-in missing data, disaggregate daily rainfall data to hourly, and perform the spatial averaging and related GIS tasks to assign representative rainfall records to the model segments. Good spatial description of rainfall is one of the most important determinants of watershed model accuracy relative to reproducing observed flow and contaminant/pollutant delivery at watershed outlets.

Loading Data

Point sources of pollution (municipal and industrial discharges) often represent significant loadings of nutrients and toxic materials into rivers and lakes. However, with the exception of the industrial areas between Baton Rouge and New Orleans (LA), point source impacts in most watersheds of the Lower Mississippi Region are expected to be small. This is a result of the relatively low (rural) population density and the predominance of agriculture in the Region. However, point sources will be located, characterized, and input to the model of each basin in order to develop a complete model. Adequate government databases to identify and characterize point sources are readily available: The EPA Permit Compliance System (PCS); the Industrial Facilities Discharge database; the NPDES discharge monitoring reports (DMRs); and/or specific facility effluent records provide relatively complete information on the identification, location, type, quantity and composition of the discharges. In this phase of the modeling, atmospheric loadings will be estimated from the literature for the environmental constituents under evaluation, and assigned areally on a constant or time-varying basis. The nonpoint and groundwater sources of environmental constituents and pollutants will be derived from the application of the MODHMS model for the watersheds under study. Application of the MODHMS model and subsequent linkage to EMS will provide NPS and groundwater pollutant/environmental constituent loadings based on land use, soil types and surface/groundwater interactions specific to the site under investigation.

Land Use and Cover

Land use and cover are major determinants of the hydrologic and water quality response of a watershed to precipitation. Therefore, within each watershed segment, a set of land uses will be modeled explicitly. Forest and agriculture comprise the majority (79%) of the area, with wetland (11%) making up much of the rest, in the lower Mississippi Region. Urban land is only significant in a few basins, constituting up to 33% of one cataloging unit, and 10% of several others. While an urban land category is needed in the model, most effort in model development will be expended in modeling cropland, forest and wetlands.

MODEL CALIBRATION AND VALIDATION

The completion of the LEMS model development effort (MODHMS and EMS) will require the application of numerous, technically complex tasks. These include:

- The assessment of current conditions and estimation of existing pollutant loads from various sources;
- Reproducing existing or past watershed conditions (model calibration/validation);
- Projecting future conditions due to watershed pollutant load reduction measures;
- Evaluating alternative pollutant loading scenarios and assessing the effectiveness of best management practices (BMPs); and
- Providing estimates of the uncertainty associated with the various models/model components and their supporting databases.

The proposed LEMS quantitative modeling framework can be a very useful tool for assessing the instream environmental impacts due to point and nonpoint source pollutant discharges, and for assessing the role and relative effectiveness of alternative remedial programs aimed at correcting environmental pollution problems. However, models are of only limited use if not properly calibrated and validated to observed conditions over a range of environmental forcing conditions (*e.g.*, freshwater flow, tidal variations, meteorology, pollutant loading, etc). The following sections describe these two processes in more detail.

Observed Water Quality Data for Calibration

Calibration of watershed and receiving water quality models requires observed values of the model state variables over a range of environmental conditions. Observed values are compared with model calculations and the model parameters adjusted within generally acceptable ranges to improve agreement between the model output(s) and observed data. For a model of flow, sediment, nutrients, dissolved oxygen/BOD dynamics, and toxics, such as the LEMS, the following measurements and special studies are needed in the receiving waterbody(ies):

- Water flow and velocity; temperature; and concentrations of suspended sediments (TSS); dissolved oxygen (DO); biochemical oxygen demand (BOD); total organic carbon (TOC); nitrogen species (*i.e.*, dissolved and particulate organic, ammonia, and nitrite plus nitrate nitrogen); phosphorus species (*i.e.*, dissolved and particulate organic and orthophosphate); chlorophyll-a; and toxic constituents of interest (*e.g.*, metals, PCBs, PAHs, etc.); and
- Light extinction studies; sediment oxygen demand studies (SOD); reaeration studies; and light and dark bottle studies.

Primary sources of these data are USGS-operated or cooperative monitoring stations. Other organizations, such as EPA and the Corps of Engineers, also conduct relevant studies and collect and catalog such data. While there are a large number of monitoring stations in the Lower Mississippi Region, the actual number with useful data may be relatively small. Many

stations collect only flow, or collect concentration data for only a limited set of constituents, or have a short period of record, or collect data too infrequently to be of much use. The available data must therefore be obtained, evaluated, cataloged, and formatted to make them accessible by the LEMS software to facilitate its calibration and presentation of model results.

Geographic Information Systems (GIS) have many applications in water quality protection and management. GIS provide the ability to create computer maps, linked to highly compact databases, that represent all of the important features within a watershed. For LEMS, we propose to maintain the modeling databases in a GIS-relatable format in order to be able to create digital maps of soils, geological features, groundwater table contours, surface terrain, population patterns, stream and transportation networks, and land use. With GIS, all of this cartographic information can be seamlessly connected and integrated, regardless of the original scale or source of the data. The GIS is also a powerful tool for conducting planning exercises, storing field-gathered data, and producing highly effective maps for analytical work or public communication.

MODHMS (Landside) Calibration

It is expected that the calibration and verification analyses will consist of time-variable model runs for wet, dry and/or average hydrologic years. The data requirements for this process will include: meteorology, topography, land use and land cover, soil hydrology and nonpoint pollutant build-up and wash-off characteristics. Calibration of the MODHMS model may be limited due to the availability of the necessary data and, therefore, may be based on literature or similar (paired) watershed-derived runoff concentrations, coefficients and various model parameters. Site-specific water quality data for runoff will most likely be limited or not available and, therefore, these data will have to be obtained or inferred from other sources such as Doppler radar data for local or county storm databases, NPDES-Municipal Stormwater Discharge Permit Reports and other local studies, if available.

As indicated, a paired or surrogate watershed approach may be called for if the required water quality and flow model calibration data are not available for the watershed under study. If these data are unavailable, they may be imported from a surrogate watershed that has similar characteristics to the watershed under study and that contains the needed data to complete model calibration, and possibly validation analyses. The characteristics to be matched include percent impervious/pervious land cover/land use, agricultural activities, watershed size, rainfall amount and patterns, and stream characteristics. After a suitable surrogate watershed is selected, the watershed model will be calibrated, and possibly validated, to the available data. The resulting modeling coefficients and parameters will then be assumed to apply to the watershed under study and used for all subsequent modeling analyses. Although this is not the ideal method of MODHMS application for the watershed under study, proper identification and use of a surrogate watershed will minimize potential sources of error.

MODHMS will be calibrated to the available data pertinent to each selected watershed, and will include at a minimum stream flow and the water quality parameter(s) of concern. Calibrated parameter values will be constrained to a set of model coefficients consistent with previous modeling studies, literature values, special field studies, and general modeling experience.

EMS (Receiving Water) Calibration

The receiving water quality models in EMS will also be calibrated to the available data pertinent to each assessment selected watershed. The calibration could include dissolved oxygen, BOD, the various nutrient forms of nitrogen and phosphorus, phytoplankton (chlorophyll-a), total suspended solids, fecal coliforms, metals and toxic organic contaminants. Calibration of the EMS will also develop a set of model coefficients consistent with previous modeling studies, literature values, special field studies, and general modeling experience.

Comparative statistical approaches will be used to assess the level of calibration. These approaches will include a comparison of the observed and modeled values and their probability distributions, regression analyses (Student's t-test), and calculation of the relative error. The results of these analyses will offer support for the credibility of the model's ability to accurately predict the concentrations of the simulated water quality parameters. When used in conjunction with the qualitative comparison of the stream flow and specific parameter temporal distributions of the data versus the EMS output, the statistical evaluations will also provide insight into the inherent overall capability of the EMS to predict water quality. The statistical analyses should confirm that the EMS achieves its main objectives: to defensibly represent the factors controlling the water quality of the receiving stream; and to provide a useful tool for managing that water quality. The objective is not to simply curve-fit model to data, but to describe the behavior of the data with a modeling framework that captures the principal mechanisms relevant to the problem.

Model Validation

After completion of the calibration of MODHMS and EMS, the LEMS will be validated against another data set, if available, to test the model under different ambient forcing conditions. A new hydrologic period will be used for the LEMS validation to test the model under a different annual or seasonal rainfall cycle. It is expected that the validation process will result in the fine tuning of certain model parameters to optimally simulate both the calibration and validation conditions. The final end-product of the calibration and validation analyses will be a robust set of LEMS parameters that characterize the system such that both data sets are adequately simulated with changes only to ambient model inputs, *i.e.* river flow, temperature, meteorology, and stream geometric characteristics. The completed calibrated and validated models will be used for the investigation of remedial alternatives.

Quantifying Uncertainty

Uncertainty associated with LEMS application will be quantified based on covariance determinations. The methodology will include a means of understanding the relative uncertainties contributed by the model constructs themselves and those attributable to the data employed, and how the uncertainties propagate through the modeling system.

INVESTIGATION OF REMEDIAL ALTERNATIVES

The ultimate purpose of developing numerical models of the environment is to assess the potential impacts of various pollutant or stressor loadings to receiving waterbodies, and to investigate the effectiveness of proposed remedial alternatives. This concept is built into the determination of Total Maximum Daily Loads (TMDLs), and requires an iterative approach. That is to say, after the models are calibrated/validated, they are used to determine the total waste assimilative capacity of the receiving waters, and subsequently the point and nonpoint source pollutant load allocations that are required to reach or maintain water quality standards. This process can also be used to evaluate existing regulation of both point and nonpoint pollutant sources relative to establishing equitable future waste load allocations (WLAs) and load allocation (LA) scenarios. The TMDL regulations require the analyses to include a margin of safety (MOS) that may take one of the following forms:

1. Setting the water quality target lower than the water quality criterion;
2. Allowing for an additional unspecified pollutant allocation;
3. Relating the MOS to the level of conservatism built into the modeling analysis (implicit); and/or
4. Basing the MOS on the estimated TMDL procedure accuracy (explicit).

The MOS to be applied for the LEMS framework will be in the form of the explicit MOS: that is, based on estimated model accuracy/uncertainty.

The determination of TMDLs requires the adjustment of model pollutant loadings, both point and nonpoint, to establish those that are necessary to reach/maintain the applicable water quality standards or targets. Inherent in this process is the determination of a baseline water quality condition that implies an associated critical condition for analysis. This baseline condition in a traditional riverine WLA is typically selected at a steady-state, low-flow, high temperature condition when the PS pollutant load would receive minimal dilution. Typically, the low-flow is defined as the 7Q10 flow (minimum 7-day average flow with a recurrence interval of once in ten years). In TMDL settings, where both PS and NPS pollutant loadings must be considered, baseline conditions need to include the effects of NPS runoff that typically occurs during wet weather conditions. Therefore, for TMDL analyses a time-variable annual cycle is selected as the baseline condition that represents wet, dry and average rainfall-runoff conditions.

CONSEQUENCES OF CATCHMENT PROCESSES AND CLIMATE CHANGES ON THE ECOSYSTEMS OF LARGE SHALLOW TEMPERATE LAKES

Tiina Nõges & Peeter Nõges[1]

ABSTRACT

Located on the border of Estonia and Russia, Lake Peipsi (3,555 km^2, mean depth 7.1 m) is a transboundary waterbody and the largest international lake in Europe. Lake Võrtsjärv (270 km^2, mean depth 2.8 m) is the largest lake belonging entirely to Estonia. The watershed of Lake Võrtsjärv (3,104 km^2) lies within the basin of Lake Peipsi (47,800 km^2). Regular investigation of these large, shallow, nonstratified lakes began in the 1960s. Large interannual fluctuations of water levels (WL) and changes in anthropogenic eutrophication stress determine most of the functional variability of these ecosystems. Anthropogenic stress resulted in intense agricultural eutrophication of these lakes between the 1950s and the 1990s; a decrease in loadings occurred during the 1990s following the collapse of agriculture. The average 30-year periodicity in these lakes is explained by variability in large-scale atmospheric circulations and changes in winter North Atlantic Oscillation indices (NAO). The maximum range of water level in Lake Võrtsjärv is 3.2 m, corresponding to a 140% change in lake area, a 250% change in mean depth, and a 3.5 times difference in volume. The ecosystem of Lake Võrtsjärv is very sensitive to water level fluctuations; phytoplankton biomass is higher in low-water years. Decreases in the N/P ratio and improvement of light conditions in the mixed water give a competitive advantage to N_2-fixers at low WL. Small lake volume leads to low oxygen storage and increases the risk of winter fish kills in Lake Võrtsjärv in low-water years. Climate signals, that are usually strongest in winter and spring, are remembered for long periods because they establish the spring peak of water level and determine the water level throughout the rest of the year. Thus, climate forcing is the main driving force for the ecosystem of Lake Võrtsjärv and can mask the influence of eutrophication to some extent. In deeper Lake Peipsi, the role of WL on the ecosystem is less pronounced since it experiences only a maximum of 26% change in mean depth with a 57% corresponding change in lake volume. Climate forcing in Lake Peipsi is more direct than in Lake Võrtsjärv. Severe summer/autumn blue-green algae blooms occur in Lake Peipsi during warm, windless periods, causing summer fish kills (the most recent occurred in 2002). In Lake Võrtsjärv, fish-kills have been recorded only in winter (the most recent occurred in 1995/1996 and 2002/2003).

INTRODUCTION

Regular investigation of these two large, shallow, nonstratified lakes, Peipsi (3,555 km^2, mean depth 7.1 m) and Võrtsjärv (270 km^2, mean depth 2.8 m), began in the 1960s. To date, over 35-years of time series data on water chemistry and biology have been collected. Lake Peipsi is one of the most important lakes in Europe, with the fourth largest surface area after Lakes

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Ladoga, Onega, and Vänern. Located on the Estonian-Russian border, Lake Peipsi is a transboundary waterbody and the largest international lake in Europe. Lake Võrtsjärv is the largest lake belonging entirely to Estonia (Figure 1).

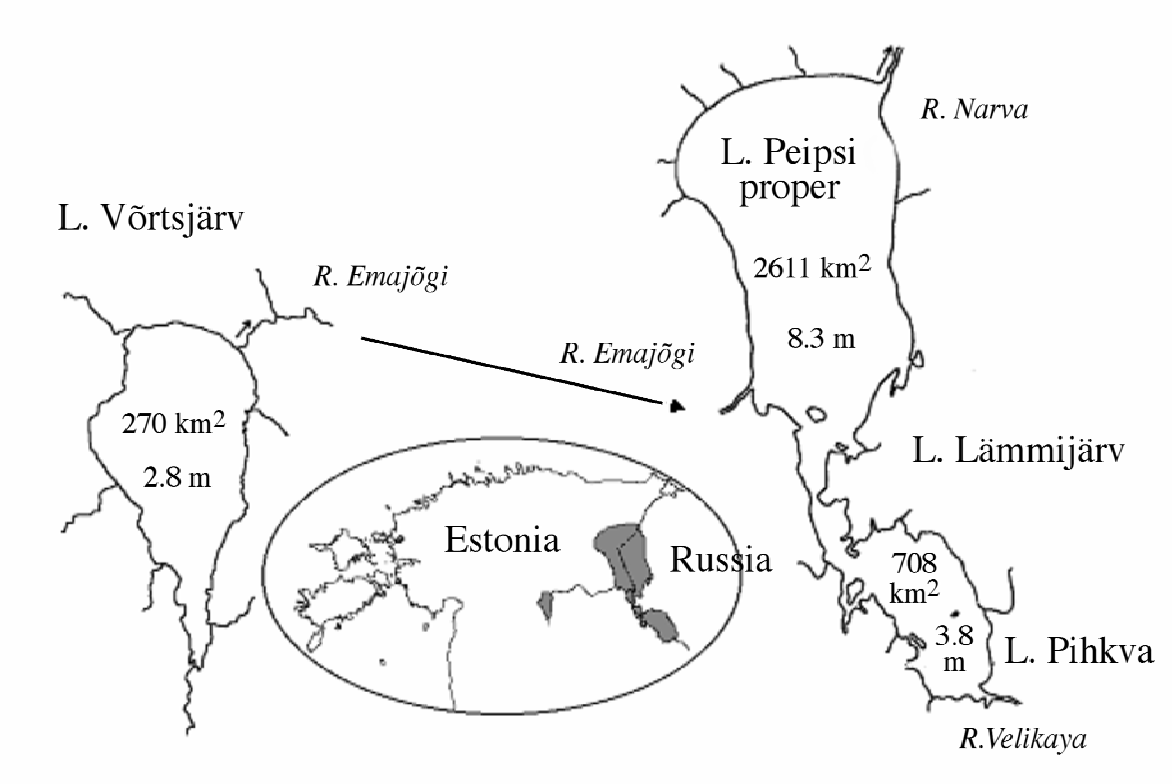

Figure 1. Location map of Lakes Peipsi and Võrtsjärv.

The watershed of Lake Võrtsjärv (3,104 km^2) lies within the basin of Lake Peipsi (47,800 km^2). Riverine transport is the most common pathway for nutrient input into both lakes. The four rivers Väike Emajõgi, Õhne, Tarvastu, and Tänassilma contribute 70–75% of the water inflow into Lake Võrtsjärv and 80–85% of the total pollutant load. In Lake Peipsi, the majority of phosphorous and nitrogen compounds (> 80%) are carried into the lake by the rivers Velikaya and Emajõgi (outflow from Lake Võrtsjärv), the first carrying biologically treated sewage from the Russian town of Pskov (201,400 inhabitants) and the latter transporting wastewater from the Estonian town of Tartu (101,200 inhabitants). A wastewater treatment plant has been in operation in Tartu since the end of 1998; prior to its installation, wastewater from Tartu was untreated. The River Emajõgi contributes approximately 70% of the total nitrogen (TN) and phosphorus (TP) loading into Lake Peipsi from Estonian territory. The Russian River Velikaya contributes about 65% of all nutrient loading into Lake Peipsi (Stålnacke, 2001), and about 85% of that comes from Russian territory.

Lake Peipsi consists of three parts: the northern and larges part, Lake Peipsi proper, 2,611 km^2, mean depth of 8.4 m and maximum depth of 12.9 m; the southern part, Lake Pihkva, with an area of 708 km^2 and mean depth of 3.8 m; and the narrow, river-shaped Lake Lämmijärv connecting Lake Peipsi with Lake Pihkva, with an area of 236 km^2 and mean depth of 2.6 m.

The entire catchment area (47,800 km^2) involves Estonian, Russian, and Latvian territories. Lakes Peipsi and Võrtsjärv are unstratified, eutrophic lakes. Lake Lämmijärv has some dystrophic features, while the trophic status of Lake Pihkva is the highest, reaching even hypertrophic levels (Table 1). The volume of water in Lake Peipsi is 25 km^3 at the long-term mean water level (30.00 m above sea level), and the mean residence time of water is about two years. The River Narva is fed by the outflow of Lake Peipsi, and runs into the Gulf of Finland.

Measurements of TP and TN in Lake Peipsi started in 1985, and in the Estonian rivers in 1984. The mineral forms of nutrients (ammonium, nitrites, nitrates, phosphates) have also been tested in the Russian rivers and the waters of Lake Peipsi. The data series on mineral N and P compounds started in 1968 in Lake Peipsi and in 1976 in the rivers. Regular biological data series date back to 1962.

Table 1. Indices reflecting trophic status of Lakes Peipsi and Võrtsjärv (modified from Haberman *et al*. 1998, Laugaste *et al*. 2001, Starast *et al*. 2001)

Parameter	Units	L. Peipsi mean	L. Pihkva	L.Lämmijärv	L. Peipsi s.s.	L. Võrtsjärv
TP	mg P m^{-3}	42	63	53	35	54
TN	mg N m^{-3}	768	1010	923	678	1600
Chlorophyll a	mg m^{-3}	18	26	25	14	24
Secchi depth	m	1.8	1.3	1.4	1.8	1

A long-term sinusoidal fluctuation of the water level (WL) with a period of about 30 years is characteristic of both lakes (Figure 2). Smooth and continuous decreases (1928-1940) and increases (1940-1957; 1965-1990) of WL resemble a long-term trend, and can be distinguished only in the context of a long time series. Apparent periodicity is probably associated with large-scale fluctuations in solar activity and atmospheric processes. Similar periodic changes have occurred in other large geographic areas, including the WL dynamics of Lake Saimaa, Ilmen, and Onega, with spectral density maximums of 28-32 years (Masanova and Filatova 1985), as well as for different hydrological elements of Lake Ladoga (20-30 years) (Malinina *et al*. 1985) and Lake Müggelsee (Behrendt and Stellmacher 1987).

In the present paper, we provide an overview of how runoff, nutrient concentrations, and riverine loadings have changed during the last four decades, and how these changes have influenced the ecosystem. The consequences of global climate change are discussed as well.

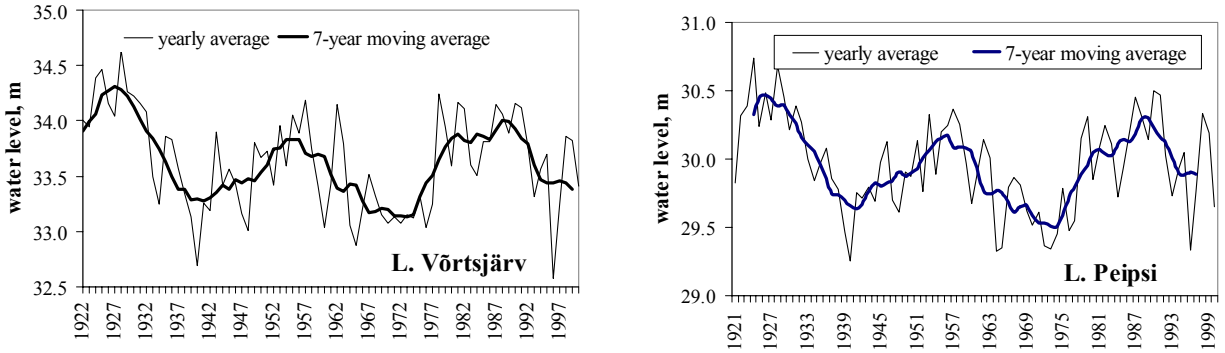

Figure 2. Long-term dynamics of the water level of Lakes Võrtsjärv and Peipsi.

CHANGES IN NUTRIENT LOADING

Riverine discharge of nutrients into Lakes Peipsi and Võrtsjärv increased during the 1980s, but a sharp decrease occurred in the early 1990s (Nõges *et al.* 2003, Järvet 2001). This was mainly evidenced in TN loadings (Figure 3). The reduction resulted from the collapse of soviet-type agriculture that was characterised by heavy fertilization of fields and often accompanied by substantial nutrient leakage into water bodies. Significant reductions in fertilizer use, typical for the transitional economy during recent years, diminished nutrient losses from the catchment area. Only 5–10% of N, P, and K mineral fertilizers and 30% of manure were applied to agricultural lands at the end of the 1990s compared to the levels being applied at the end of the 1980s (Järvet *et al*. 2002). Since TN loading decreased faster than TP loading, the TN/TP loading ratio also decreased (Figure 4).

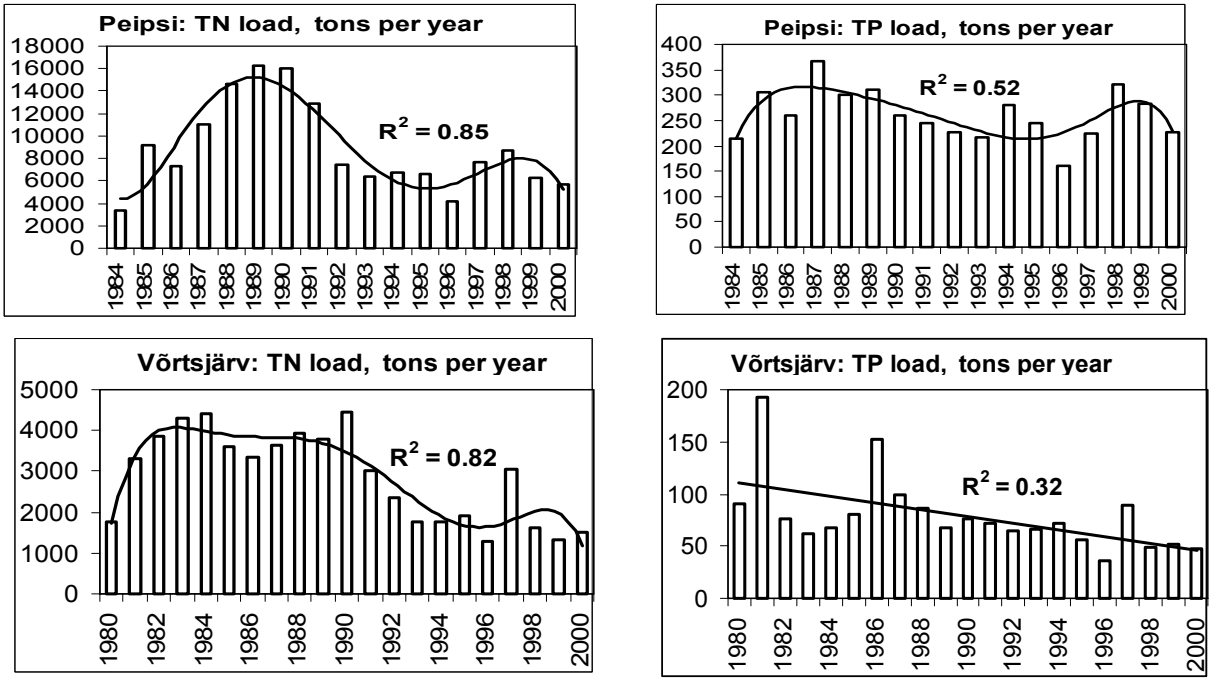

Figure 3. Annual runoff of total nitrogen (TN) and phosphorus (TP) into Lake Peipsi from Estonia from 1984–2000 (Nõges *et al.* 2003), and into Lake Võrtsjärv from 1980–2000 (Järvet 2001).

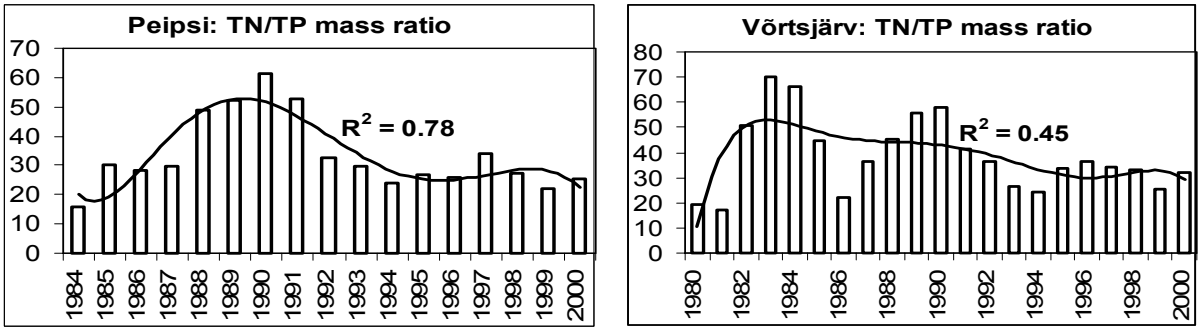

Figure 4. The ratio of total nitrogen to total phosphorus (TN/TP) in the annual external loading of Lake Peipsi (from Estonia) and of Lake Võrtsjärv (from Nõges *et al*. 2003).

RELATIONSHIP BETWEEN CHANGED NUTRIENT LOADING AND PHYTOPLANKTON

In Lake Võrtsjärv, average phytoplankton biomass between May and October from 1963-2001 was 20 gWW m^{-3}, varying between 1 to 100 gWW m^{-3}; in Lake Peipsi proper the respective values from 1962-2001 were 10 gWW m^{-3}, and 0.35 to 61 gWW m^{-3}. Many species of bloom-forming cyanobacteria use molecular nitrogen at low N/P ratios if N is the limiting nutrient. Observing the changes in cyanobacterial dominance from the beginning of the 1960s in Lakes Peipsi and Võrtsjärv, one can notice the increase during the late 1960s and early 1970s, the decline during the late 1970s and 1980s, and another increase during the late 1990s in both lakes (Figure 5, Nõges *et al.* 2003). Changes are much less pronounced in Lake Võrtsjärv than in Lake Peipsi. In Lake Võrtsjärv, the dominant cyanobacterial species *Limnothrix planktonica, L. redekei,* and *Planktolyngbya limnetica* are not able to fix N_2; the main N_2-fixing species, *Aphanizomenon skujae*, does not achieve dominant status. In Lake Peipsi, N_2-fixing species *Aphanizomenon flos-aquae* and *Gloeotrichia echinulata* prevail in summer phytoplankton. Since the beginning of the 1990s, the biomass of N_2-fixing species has increased in both lakes.

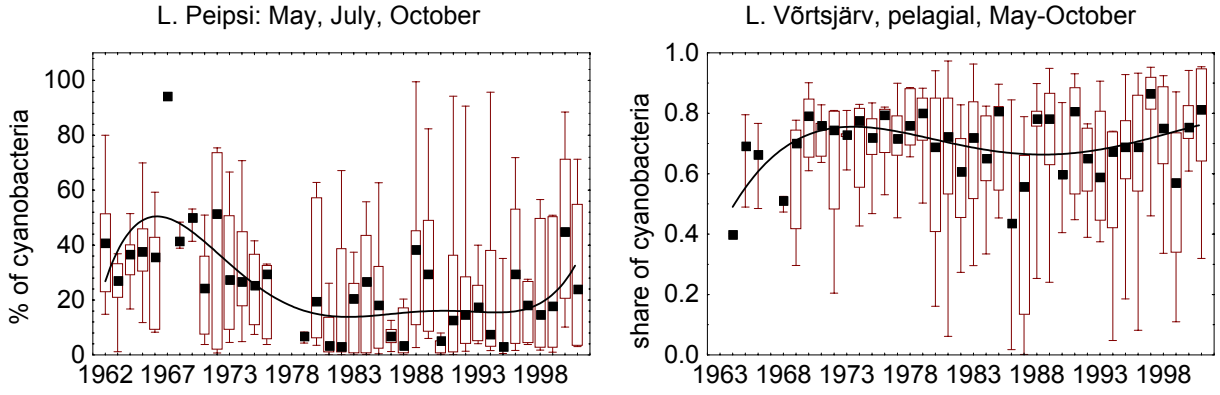

Figure 5. Changes in the dominance of cyanobacteria in phytoplankton biomass of Lake Peipsi and Lake Võrtsjärv since the 1960s (from Nõges *et al.* 2003).

The increasing dominance of cyanobacteria and the occurrence of water blooms in both lakes can be caused by reduced nitrogen loading and decreased TN/TP ratio. In Lake Peipsi, a TN/TP mass ratio less than 30 seems to be critical for the development of predominant cyanobacterial species, both N_2-fixing (*Gloeotrichia echinulata, Anabaena, Aphanizomenon*) and non-fixing (*Microcystis*) as seen in Figure 6.

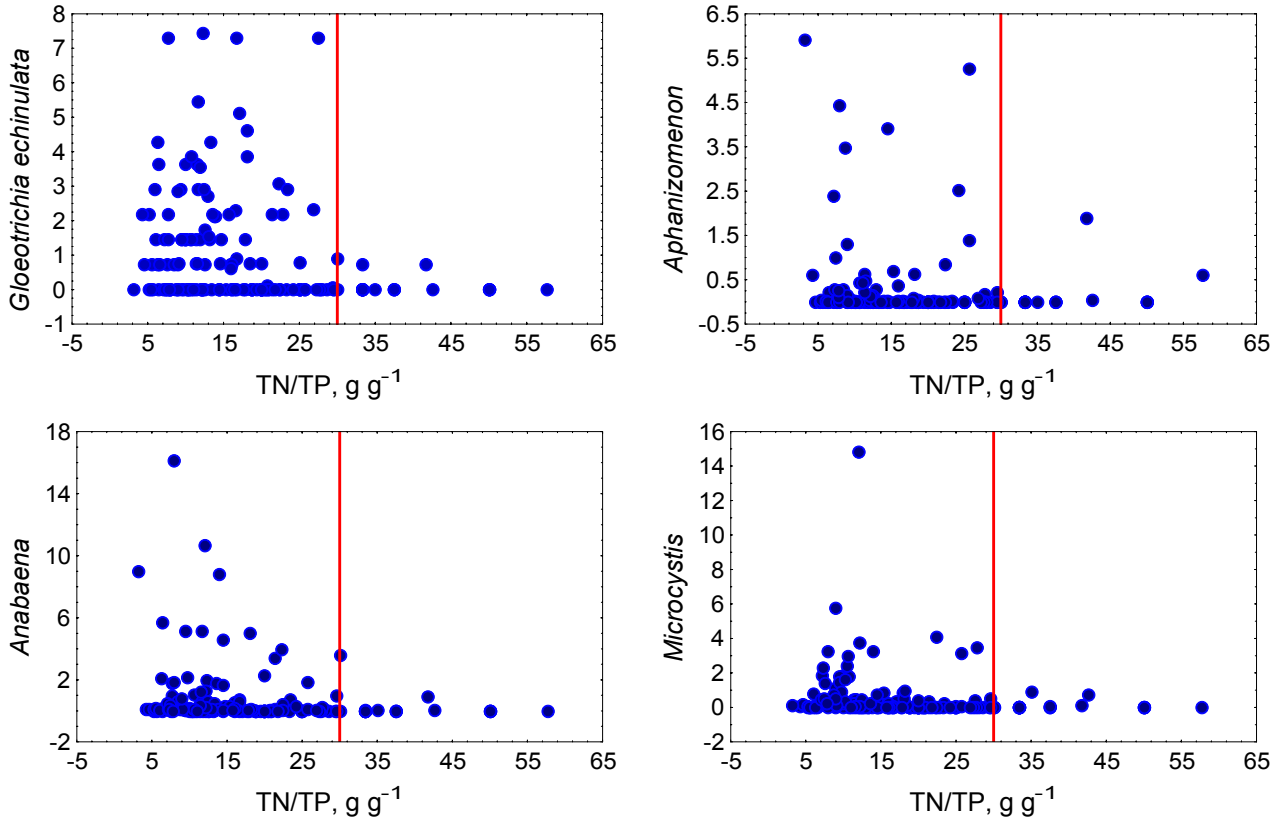

Figure 6. Biomass of dominating cyanobacteria gWW m^{-3} at different TN/TP ratios in Lake Peipsi in June-September (data of R. Laugaste).

CONSEQUENCES OF THE CHANGES OF GLOBAL CLIMATE ON WATER LEVEL AND THE STATE OF THE ECOSYSTEM

The North Atlantic Oscillation (NAO), defined by the variability of air pressure differences between the north (Iceland) and south (Azores), dictates climate variability over a large area of the Atlantic, North America, and Europe, especially during winter (Hurrell *et al*. 2001). Variation in heat and moisture transport between the Atlantic and surrounding continents affect water balance components of lakes, such as precipitation, riverine inflow, and evaporation, resulting in changes in water level. In both Lakes Peipsi and Võrtsjärv, mild winters associated with high NAO index bring about higher water levels (Figure 7A and B). In very shallow Lake Võrtsjärv where the annual amplitude of WL is 1.4 m and the absolute range is 3.2 m, fluctuations of WL have a strong influence on the ecosystem. In low-water years, increased water column illumination and increased release of phosphorus from resuspended bottom

sediments result in substantially higher phytoplankton biomass than in high-water years (Figure 7C). In deeper Lake Peipsi, where seasonal and absolute WL variation ranges represent approximately 1/5 and 1/2 of the mean depth, respectively, the direct influence of the WL is not so obvious (Figure 7D).

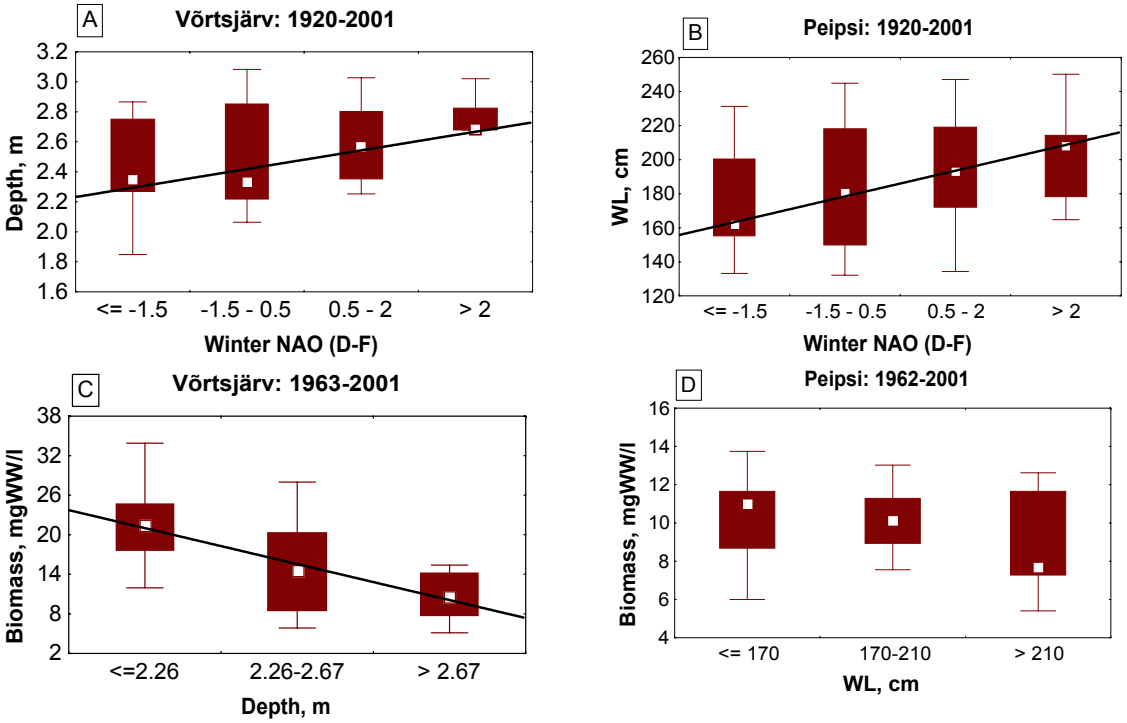

Figure 7. Relationship between winter NAO, yearly average water level (WL) or lake depth and phytoplankton biomass in Lakes Võrtsjärv and Peipsi (median, minimum, maximum, quartiles).

For the ecosystem of Lake Võrtsjärv, warmer and wetter climate could bring about higher water levels. The deeper the mixed water column, the lower the average light intensity, resulting in reduced phytoplankton biomass (Nõges and Nõges 1998). In deeper water, both resuspension and denitrification rates are lower. The former reduces phosphorus release from the bottom sediments and causes lower water phosphorus concentrations, while the latter increases nitrogen concentration (Nõges and Nõges 1999). Consequently, in a warmer world the N/P ratio in Lake Võrtsjärv would be higher and N_2-fixing cyanobacteria would have less chance to develop (Figure 8).

A warmer and wetter climate would also affect watersheds by reducing soil freezing times and increasing the amount of precipitation, causing higher nutrient leaching from the soil and accelerating the eutrophication of water bodies. We have noticed that during the 1990s the spring peaks of riverine nutrient discharges have shifted to an earlier period than was observed prior to and during the 1980s (from the beginning of April to mid March). Despite lower intensity of land use, wintertime nutrient losses are almost as high as during former years because of increased water discharge and greater amount of overland flow in winter.

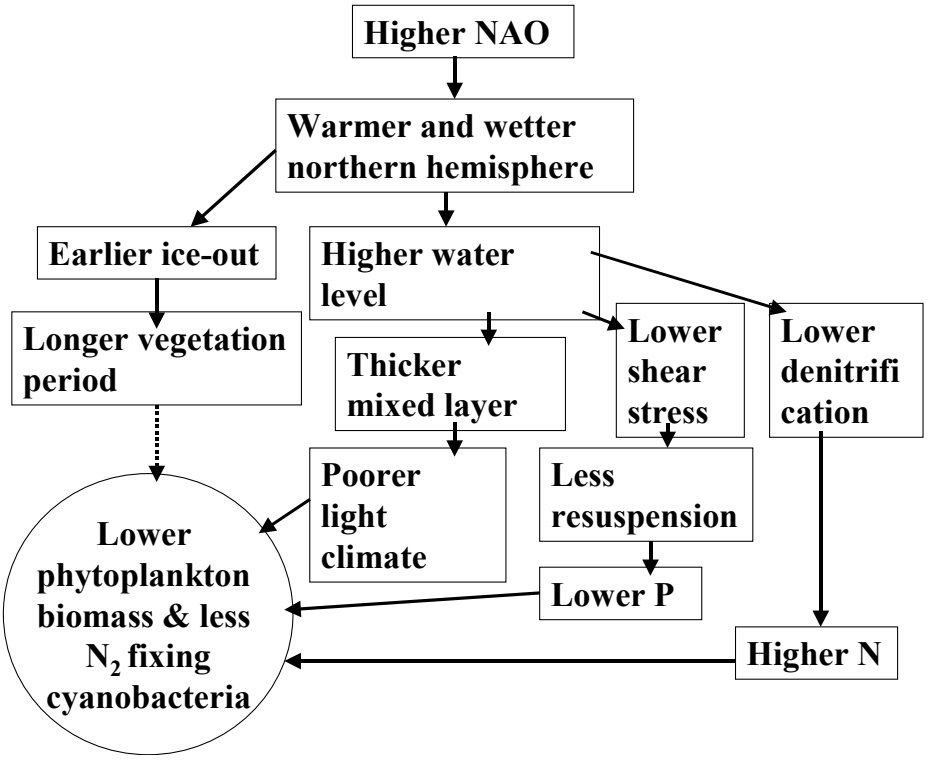

Figure 8. The assumed consequences of global warming on the phytoplankton of Lake Võrtsjärv.

ORIGIN OF FISH KILLS

Several winter fish-kills have been documented in Lake Võrtsjärv during the last century (in 1939, 1948, 1967, 1969, 1978, 1987, 1996). Fish-kills occurred mostly in wintertime, and dead fish subsequently were recovered in the spring. One reason for these fish-kills is the depletion of oxygen in low-water years during late winter (Figure 9) when the amount of oxygen trapped under the ice is decreased due to smaller lake volume. Such an oxygen depletion was documented in March 1996 (Nõges and Nõges 1999) and resulted in a massive kill of eel.
In Lake Peipsi, high water temperature and algal blooms resulted in massive summer fish kills during 1959, 1972, and 2002. During the algal blooms, phytoplankton biomass is built up faster than it can be consumed by zooplankton. Intensive photosynthesis produces much oxygen during the day; if the water gets oversaturated, some of this oxygen left the atmosphere. At night, when algal masses consumed but did not produce oxygen, a deficiency occurred. Such large scale diurnal fluctuations of oxygen concentration harm fish and make them more susceptible to other stressors. High water temperature associated with algal blooms makes the situation even more dangerous to fish. Other stressors accompanying algal blooms are high water pH, caused by intensive photosynthesis, and elevated concentrations of ammonium released during the decomposition of organic matter. At high pH (>9), most ammonium is converted to toxic ammonia (NH_3) that also can kill fish. Moreover, cyanobacterial toxins can also significantly influence fish populations (Figure 9).

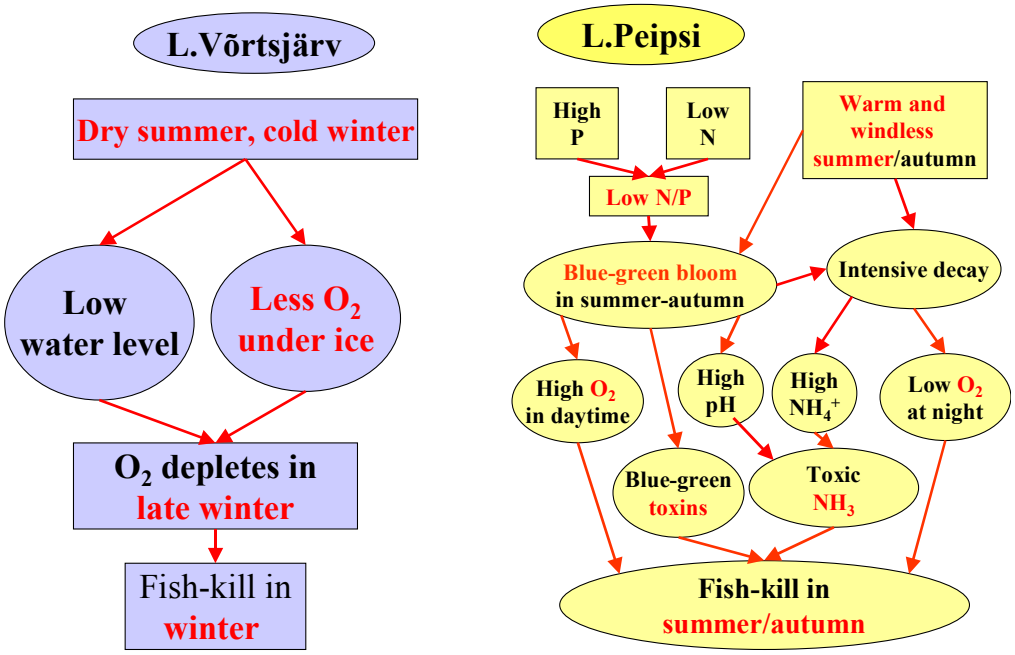

Figure 9. Origin of fish-kills in Lakes Võrtsjärv and Peipsi

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REFERENCES

Behrendt, H. and R. Stellmacher. 1987. Long-term Changes in Water Quality Parameters of a Shallow, Eutrophic Lake and Their Relation to Meteorologic and Hydrologic Elements. The Influence of Climate Change and Climatic Variability on the Hydrologic Regime and Water Resources. Proceedings of the Vancouver Symposium, August 1987. IAHS Publishers 168:535-544.

Haberman, J. 2001. Zooplankton. *In:* Lake Peipsi. Flora and Fauna, E. Pihuand and J. Haberman (eds.). Sulemees Publishers. Tartu, Estonia. Pp. 50-68.

Hurrell, J.W., Y. Kushnir, and M. Visbeck. 2001. The North Atlantic Oscillation. Science 291:603-605.

Järvet, A. 2001. Long-term changes of nutrient runoff in a Lake Võrtsjärv catchment area. Environmental Impact and Water Management in a Catchment Area Perspective. Tallinn Technical University. pp. 54–62.

Järvet, A., Ü. Mander, A. Kull, and V. Kuusemets. 2002. Nutrient runoff change in a rural catchment in South Estonia – Large Rivers, Vol. 13, No. 3–4. Archives of Hydrobiology Supplement 141/3–4:305–319.

Laugaste, R., T. Nõges, P. Nõges, V.V. Jastremskij, A. Milius, and I. Ott. 2001. Algae. *In*: Lake Peipsi. Flora and Fauna, E. Pihu and J. Haberman (eds.). Tartu, Estonia. pp. 31-49.

Malinina, T.I., I.V. Filatova, and N.N. Filatov. 1985. Long-term Changes in the Elements of Water Budget of Lake Ladoga. *In:* Problemy Issledovaniya Krupnyh Ozer. Leningrad. Pp. 79-81 (in Russian).

Masanova, M.D. and I.V. Filatova. 1985. Probability Structure of Interannual Water Level Changes in North-Western Lakes. *In:* Problemy Issledovaniya Krupnyh Ozer. Leningrad. Pp. 81-84 (in Russian).

Nõges, T., A. Järvet, R. Laugaste, E. Loigu, I. Tõnno, and P. Nõges. 2003. Current State and Historical Changes of Nutrient Loading and Ecological Status of Large Lakes Peipsi and Võrtsjärv. *In:* Proceedings of 4th International Lake Ladoga Symposium 2002, H. Simola, A.Yu. Terzhevik, M. Viljanen & I.K. Holopainen (eds.). University of Joensuu, Publications of Karelian Institute 138:199-203.

Nõges, P. and T. Nõges. 1998. The Effect of Fluctuating Water Level on the Ecosystem of Lake Võrtsjärv, Central Estonia. Proceedings of the Academy of Sciences, Estonia. Ecology Series 47:2:98-113.

Nõges, T. and P. Nõges. 1999. The effect of extreme water level decrease on hydrochemistry and phytoplankton in a shallow eutrophic lake. Hydrobiologia 408/409:277-283.

Stålnacke, P. (Editor). 2001. Nutrient Loads to Lake Peipsi. Environmental Monitoring of Lake Peipsi/ Chudskoe 1998-99. Subproject Report Phase 2, Jordforsk Report No. 4/01, 65 pp.

Starast, H., A. Milius, T. Möls, and A. Lindpere. 2001. Hydrochemistry. *In:* Lake Peipsi. Meteorology, Hydrology, Hydrochemistry, T. Nõges (ed.). Sulemees, Tallinn, Estonia. Pp. 97-131.