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METHODS FOR USE OF CAGED MOLLUSCS FOR IN SITU BIOMONITORING OF MARINE SEWAGE DISCHARGES

Edited
by

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FOREWORD

Environmental measurements are required to determine the quality of ambient water, the character of effluents, and the effects of pollutants on aquatic life. The Environmental Monitoring and Support Laboratory-Cincinnati conducts research to develop, evaluate, standardize and promulgate methods to:

- Measure the presence and concentration of physical, chemical and radiological pollutants in water, wastewater, bottom sediments, and solid waste.
- Concentrate, recover, and identify enteric viruses, bacteria, and other microorganisms in water.
- Measure the effects of pollution on freshwater, estuarine, and marine organisms, including the phytoplankton, zooplankton, periphyton, macrophyton, macroinvertebrates, and fish.
- Automate the measurement of the physical, chemical, and biological quality of water.
- Conduct an Agencywide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

Section 301(h) of the Clean Water Act of 1977, as amended by the Municipal Wastewater Treatment Construction Grant Amendments of 1981, provides publicly owned treatment works (POTW's) currently discharging or proposing to discharge to coastal and saline estuarine waters an opportunity to apply for variances from secondary treatment requirements. This report provides recommended monitoring methods for use by POTW's seeking to determine compliance with the statuatory criteria listed under Section 301(h) and applicable Water Quality Standards.

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PREFACE

This manual was prepared with the assistance of the following workgroup members.

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ABSTRACT

This manual describes methods for use of caged molluscs in biomonitoring programs related to Section 301(h), Public Law 95-217. Molluscs collected at relatively contaminant-free locations are placed in cages and exposed for one month at a minimum of two stations: (1) in the plume, within the zone of initial dilution, and (2) at a nearby reference (control) station, outside of the area of immediate influence of the discharge. At the end of the exposure period, the organisms are retrieved, checked for mortality, analyzed for toxic substances, and examined for indications of sublethal biological effects, including scope for growth, and the distribution of toxic metals in the detoxification system.

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SECTION 1

INTRODUCTION

Section 301(h) of the Clean Water Act of 1977, as amended by the Municipal Wastewater Treatment Construction Grant Amendments of 1981, describes provisions under which publically owned treatment works (POTW's) may apply for variances from secondary treatment requirements for discharges to marine waters. A modified NPDES permit may be granted if the applicant can demonstrate that the less-than-secondary discharge would not impair the integrity of the marine receiving waters and biota. Following receipt of a modified permit, the POTW is required to maintain a monitoring program to demonstrate continuing compliance with applicable water quality standards and 301(h) requirements.

The major goals of 301(h) monitoring are to identify chemicals in the discharge that should be controlled and to determine whether discharges cause adverse biological effects in the receiving water. Monitoring for priority pollutants discharged by POTWs is an important facet of the program. Marine organisms do not bioaccumulate all chemicals equally. Some chemicals may be in low concentration or even below detection limits in wastewater, yet accumulate to high and/or toxic levels in marine organisms. Conversely, some materials in high concentration in the effluent may not be bioconcentrated. Accordingly, a field monitoring system is needed that identifies chemicals of biological significance.

Many approaches have been used in monitoring for adverse biological effects in receiving waters, including studies of natural planktonic and benthic communities. However, changes in the structure of natural communities are not as sensitive to pollution as changes in the health of individual organisms, which can be adversely affected at low but chronic levels of exposure to toxic chemicals.

Extensive use of filter-feeding bivalve molluscs during the past decade to determine the distribution and persistence of toxic substances in marine waters and to detect and measure adverse effects of pollutants on aquatic life has resulted in the development of methodology which is applicable to the 301(h) program (Bayne et al., 1978, 1981; Davies and Pirie, 1980; Goldberg, 1975; Goldberg et al., 1978; Phelps and Galloway, 1980; Phelps et al., 1981; Phillips, 1976, 1977a, 1977b; Stephenson, et al., 1979, 1980, 1981; Widdows et al., 1981). In recent years, attention has focused on only a few species, principally in the genus Mytilus.

This manual describes recommended methods for the exposure of molluscs to discharges from POTW's to determine the bioaccumulation of toxic substances, to detect acutely toxic conditions in the plume, and to measure the degree of stress (sublethal toxicity) to which the test organisms may have been subjected. In this protocol, caged molluscs are collected at a non-polluted site, exposed for one month at a minimum of two stations--in the zone of initial dilution and at a reference station --and analyzed for toxic substances in tissues and for sublethal biological effects.

Caution must be exercised in the use of caged molluscs in biomonitoring programs, such as the 301(h) program. Some organic contaminants which have a low octanol:water partition coefficient are not bioconcentrated by organisms (Gossett et al., 1982). Organisms in the plume may accumulate organic contaminants reflective of historical rather than current discharges (Young et al., 1976). A significant portion of contaminants (e.g. 39 percent for copper, Phillips et al., 1980) found in molluscs may be associated with sediments in the gut that may not be absorbed. Therefore, measurements of metals in undepurated organisms may not give a true measure of actual bioaccumulation of contaminants. Also, often in these organisms there are large (greater than 3-fold) variations in concentrations of organic contaminants related to both the stage in the reproductive cycle, which varies seasonally, and the amount of upwelling of contaminants from sediments (Brown et al., 1982d), which might make it difficult to see differences between stations. Seasonal changes also occur in histology (Reynolds et al., 1980), and in the rates of metabolism and detoxification of contaminants (Brown et al., 1982d). These factors must be taken into account when designing the field studies and interpreting the data.

SECTION 2

EXPOSURE SITE SELECTION

A minimum of two exposure sites must be used: (1) one in the plume, within the zone of initial dilution (ZID), and (2) a reference site, "upstream" from the zone of initial dilution and outside of the area affected by the discharge.

The plume exposure apparatus should be placed as close as possible to the outfall diffuser (i.e. at the center of the ZID). Additional exposure sites may be necessary or desirable to define contaminant gradients in the vicinity of the outfall, and in the case of receiving waters that are already stressed, to determine the contribution of other pollutant sources to bioaccumulation levels.

The control site must have hydrographic and water quality characteristics similar to those at the outfall. The test organisms are sensitive to salinity, and the use of more than one control site (i.e. such as upstream and downstream) may be required in estuarine environments where salinity gradients are present.

Exposure locations to be avoided include shipping lanes and dredging sites. Swift currents may preclude the use of some stations, but exposure gear has been successfully maintained in currents as high as 5 knots.

Station positions may be established by use of surface buoys, visual sighting (shore transects), use of fathometers to fix depth, acoustic transducers (pingers), Loran C navigation aid, satellite navigation aids, and portable navigation aids (e.g. Motorola Miniranger^{R}).

SECTION 3

EXPOSURE DEPTH

Test organisms are exposed at a minimum of one depth at each station. At the discharge, exposure cages are placed in the ZID, but at least one meter above the bottom to avoid the overriding influence of toxic substances released during the exposure period by the sediments, which might have been deposited by historical pollution not representative of the current discharge. At the control station, cages are placed at the same depth(s) as are used at the discharge. Under some circumstances, it may be desirable or necessary to expose the organisms at additional depths to determine concentration gradients or to detect the release of toxic substances from the sediment. In cases where the plume depth is expected to vary during the exposure period, it may also be appropriate to use multiple exposure depths to ensure plume exposures.

To provide meaningful information, it is necessary for exposures to be conducted at a depth which will ensure maximum potential plume contact. It is the dischargers responsibility to demonstrate that exposures were actually conducted in the effluent plume. The spatial distribution of the plume may be determined by field water quality measurements (e.g. NH_3, turbidity), remote sensing (e.g. acoustic backscatter: Proni et al., 1976; Proni and Hansen, 1982), or by mathematical models (See Tetra Tech, Inc., 1982a and 1982b for examples and application). If models are used, site-specific water density data (i.e. temperature and salinity) for the exposure period should be used as input.

SECTION 4

EXPOSURE SYSTEMS

Gear that has been used successfully in the past and is recommended in the 301(h) program is described below. For additional information see Stephenson et al. (1979) and Phelps and Galloway (1980).

Because of the potential for loss of exposure gear due to natural events (e.g., storms, ice flows) or vandalism, it is recommended that two arrays be placed at each exposure site. The overall cost increase associated with an additional array is considerably less than that required for repeating the entire exposure if the test organisms cannot be recovered.

ANCHORS

Anchors that have been successfully used by various programs include:

(1) Train wheels (with axles removed), 340 kg.
(2) Degreased automobile engine blocks (use a commercial degreasing firm). Two blocks are used on each line, chained together.
(3) Cast concrete blocks, 25 - 160 kg.
(4) Fence anchors, auger type, 1 - 2 m length (for use in soft bottoms).

LINE

Sixteen millimeter (5/8 in) polypropylene line or 8mm (5/16 in) polypropylene encased steel cable (Rolyan Permaflex^{R}) is recommended for surface buoys. Smaller line (6mm; 1/4 in) may be used for subsurface buoys. The line should be kept bagged and off the deck of the surface vessel to prevent contamination.

BUOYS

Surface buoys are used primarily as station marker buoys, whereas subsurface buoys are used to support the mussel cages and/or bags to reduce losses due to ship damage and vandalism. Surface buoys placed in navigable waters must be Coast Guard approved (e.g. Rolyan^{R} 1352). The use of spar buoys is recommended.

Submerged buoys, such as a 30 cm diameter inflatable, phosphorescent orange, plastic float, plus a 20 cm diameter non-collapsable float, can be used to support the mollusc cages.

EXPOSURE CAGES/BAGS

Enclosures recommended for use with molluscs include polypropylene or nylon test tube baskets and bait bags.

(1) Test tube baskets - use non-contaminating material, such as polypropylene.

(2) Bags -
   (a) Nylon mesh bags - 8 cm x 1 m (3 X 36 in) nylon bait bags, 12 mm (1/2 in) mesh, 20 kg test*.
   (b) Polypropylene mesh bags - (Vexar^{R}), 15 cm X 225 cm, 12 mm (1/2 in) mesh.

GEAR CONFIGURATION

Examples of gear configuration are shown in Fig. 1. A commonly used configuration is where a USCG approved special purpose buoy (similar to Rolyan 1352^{R}) is attached by 8 mm (5/16 in) polypropylene encased steel cable (Rolyan Permaflex^{R}) to a 150 kg concrete anchor. Nylon lines (6 mm) are run about 6 m to satellite moorings of 25-50 kg each to which 6 mm polypropylene line is attached with 20 cm diameter hard plastic floats used to suspend mussel baskets 1 meter above the surface of the sediment. A float placed about 6 m up the mooring cable prevents entanglement with the subsurface floats, and baskets can be hung on the cable itself for profile work. Bags containing mussels can also be hung from a pipe framework as shown in Fig. 1e.

*Nylon Net Company, P.O. Box 592, Memphis, TN 38101

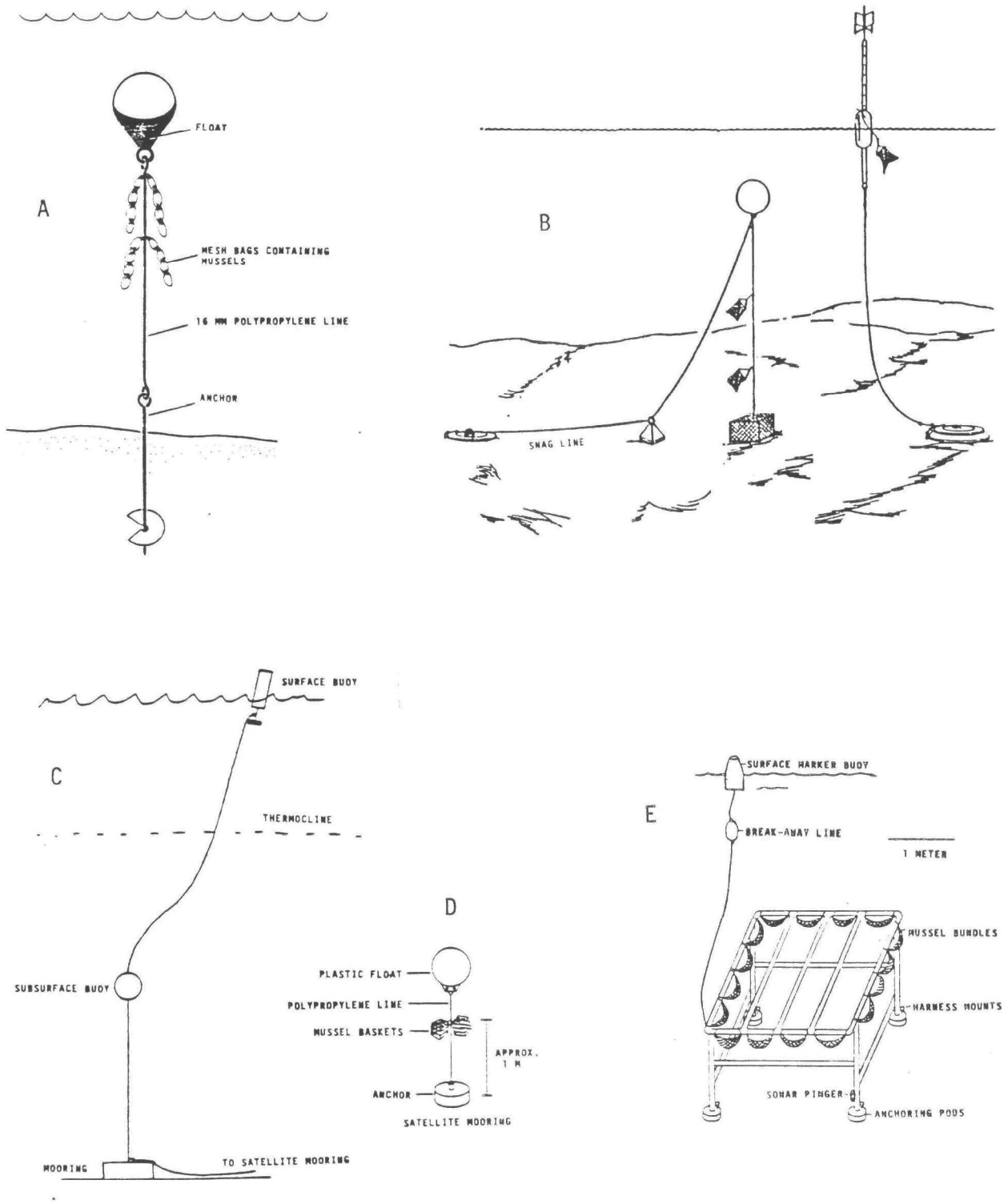

Fig. 1. Examples of exposure systems (not to scale): (a)from Stephensen et al., 1980; (b) From Young et al., 1976; (c) provided by D. Phelps, USEPA, Narragansett, RI; (d) from Phelps and Galloway, 1980; (e) provided by T. Fikslin, USEPA, Edison, NJ.

SECTION 5

TEST ORGANISMS

RECOMMENDED SPECIES:

Mussels: *Mytilus californianus* - West coast
Mytilus edulis - West coast, East coast, and Gulf of Mexico

Oysters: *Crassostrea virginica* - East coast, and Gulf of Mexico
gigas - West coast

SIZE:

Mussels: 5-7 cm
Oysters: 7-10 cm

SOURCE AND CONDITION

Test organisms should be collected from an area that is relatively free of contaminants. If no previous data are available on the level of contaminants in tissues, and the physiological condition of the organisms in the proposed collection area(s), representative samples should be collected and tested. The proposed source of organisms selected for use in the 301(h) program should be reviewed by the permitting authority before organisms are collected for transplanting to the exposure sites. It should be noted that a state permit may be required for collecting test organisms.

In the source area, test organisms should be collected from approximately the same depth, preferrably below mean-tidal level. Test organisms can be collected by dredge or removed from rocky substrates with stainless steel pry bars. Organisms should not be collected from steel or man-made wooden structures. Collectors should wear clean polyethylene gloves at all times. Care should be taken to avoid contamination of the organisms during collection and transport. Organisms should be of approximately the same size, to minimize the natural variation in chemical and biological parameters.

A random subsample of 20-25 organisms should be removed from the collection to determine mean length and weight, the condition factor, the incidence of parasites and disease, the stage in the gametogenic cycle, and body burden of toxic substances.

Upon collection, the organisms should be triple bagged in 4-mil cleaned polyethylene bags and placed in ice chests. The polyethylene bags and ice chests should be cleaned with detergent (Micro^{R}) and triple rinsed with distilled water prior to use.

The molluscs should be transplanted to the exposure sites within 48 hours after collection.

SECTION 6

SAMPLE EXPOSURE AND RETRIEVAL

EXPOSURE PROCEDURES

At the exposure site, the test organisms are placed in cages or mesh bags. The bags are constricted every 6-8 inches with nylon cable ties to ensure uniform exposure of the organisms to the surrounding water. Four bags/cages, each containing 25 individuals (total of 100 individuals), are exposed at each depth (Fig. 1).

The test organisms should be protected from surface contamination by enclosing them in cleaned 4-mil polyethylene bags until they are hauled overboard and lowered below the surface. The protective bag is then removed underwater.

EXPOSURE PERIOD

Organisms are exposed for one month. The choice of dates during which exposure should take place may vary with location. If pronounced seasonal changes occur, more than one exposure period is recommended. If only one exposure period is used, it should be the period of maximum exposure, i.e., when the sexual organs are well developed, the water temperature is such that the animals are metabolically active, and there is the least dilution of the discharge.

The period of maximum stratification and least dilution usually occurs in late summer. However, contaminant concentrations in tissues during this period may be the lowest of any time during the year because of spawning. For this reason, it may also be advisable to expose organisms during the winter. Exposures during periods of rapidly changing density gradients should be avoided because of uncertainties in maintaining plume exposures at a given depth.

Exposure periods greater than one month may be necessary at discharges where certain toxic substances with relatively slow uptake rates (e.g. Hg and Ag) are of concern.

SAMPLE RETRIEVAL

The potential for successful retrieval of exposure arrays is enhanced by the use of electronic navigation aids during deployment and retrieval. The use of such aids is especially important if subsurface buoys are

used. It is also recommended that an acoustic transducer (pinger) be attached to each array to aid in location during retrieval. Transducers are inexpensive (less than $200 each) and operate for 6 months. The use of acoustic releases is not generally recommended because they are expensive. However, they may be the best alternative for some applications.

For arrays with subsurface buoys, retrieval can be accomplished by a combination of electronic positioning and acoustic location, followed by diver retrieval of the exposure apparatus. The practical limit for diver retrieval is about 36 m. In situations where the subsurface buoy must be placed below diving depth, or in situations where diver retrieval is not feasible for other reasons, the exposure array may be retrieved by snagging a bottom line attached to the anchor (Fig. 1), or use of an acoustical release device.

FIELD OBSERVATIONS

a. Fouling

If fouling is severe, the flow of water to the molluscs may have been sufficiently reduced to interfere with feeding. The degree of fouling is observed and reported as the estimated percentage of mesh openings occluded by fouling organisms (Stephenson et al., 1980).

b. Mortality

Conditions in the ZID may be acutely toxic. Therefore, the percentage of test organisms surviving to the end of the exposure period should be determined for each exposure site/depth.

SAMPLE PRESERVATION AND TRANSPORT

Contamination from substances in the surface film can be avoided by placing the molluscs in polyetheylene bags before surfacing. When retrieved, the organims may be held briefly in cleaned ice chests until further processing. Excess water should be drained from the organisms on ship or after removing to shore.

a. Metal Analyses

Samples collected for trace metal analysis are placed in cleaned Ziploc^{R} bags, immediately frozen on dry ice and transported to the laboratory in the frozen state. In the laboratory, samples are stored at -20C until analyzed.

b. Priority Organic Pollutants and Metabolite Analyses

Samples collected for organic analysis are double-wrapped in precleaned, hexane-rinsed aluminum foil. The aluminum wrapped samples are then placed in Ziploc^{R} polyethylene bags, immediately frozen on

dry ice and transported to the laboratory in the frozen state. In the laboratory the samples are maintained at - 20°C until analyzed.

c. Cytosol Analysis

Samples collected for cytosol analysis are placed in cleaned Ziploc^{R} bags, and immediately frozen on dry ice. They are stored at -80°C upon return to the laboratory (experiments have shown that metallothionein is stable at this temperature, but not at -20°C; Oshida, 1982).

d. Biological Analyses

Samples collected for biological analyses returned to the laboratory in cleaned polyethylene ice chests.

SECTION 7

CHEMICAL ANALYSES

WATER AND WASTEWATER ANALYSES

Methods for water and wastewater analyses are described in USEPA, 1979, 1982.

TISSUE ANALYSES

Whole organism (soft part) composite samples are used for analyses of toxic substances. Three replicate composite samples (15-20 organisms per sample) should be analyzed from each exposure site/depth. Tissue samples are analyzed for (1) the full list of 129 priority pollutants and six pesticides, (2) for metabolites of toxic organic substances, and (3) for the distribution of toxic metals and organics in the cytosol (metallothionein/enzyme/glutathione pool). A subset of the priority pollutants and pesticides may be analyzed if it can be demonstrated that only those substances occur in the effluent. Data are reported in ug/g or ng/g dry weight, with a wet weight conversion factor.

a. Sample Preparation

Immediately prior to analysis, frozen mussels are removed from the bags, one at a time, scrubbed in deionized water to remove debris (use polyethylene gloves), and thawed in polyethylene, borosilicate glass, or stainless steel trays. The adductor muscle is severed with a clean, stainless steel scalpel, the gonad is excised, and the remainer of the soft parts are placed in a preweighed acid-cleaned containers. The quantity of tissue required for analysis is approximately as follows: (1) Hg - 1 gram; (2) remainder of metals - 5 grams; (3) organic priority pollutants - 50 grams; (4) metabolites - 5 grams.

b. Priority Pollutant and Pesticide Analyses

Methods for the tissue analysis for priority pollutants and pesticides are described in USEPA, 1981. The percent lipid (USFDA, 1970) also should be determined for each sample because it is may help explain the variability in the concentration of organics. The moisture content of an aliquot of tissue (dry weight conversion factor) is determined by drying at 103C for 12 hr (Stephenson et al., 1980).

c. Metabolite Analysis

Recent studies indicate that metabolites represent the major form of xenobiotic organics in marine organisms (Brown et al., 1982b,c,d). In addition, it appears that chronic effects of organic compounds are caused by their metabolic products, while acute effects, which would occur under only the most extreme circumstances, are caused by parent organic compounds (Young et al., 1979; McKinney, 1981). However, most studies on the presence of organic contaminants in the environment do not report levels of metabolites. These omissions may occur because most metabolites cannot be extracted by normal procedures since they are bound to proteins, DNA, glutathione, glucuronic acid, and other substances in organisms (Reid and Krishna, 1973; Roubal et al., 1977; Varanasi and Gmur, 1980; Miller and Miller, 1982). Therefore, to determine their levels, they must first be released from substances to which they are bound by a heat-catalyzed base hydrolysis (Miller and Miller, 1966; Miller 1970; Gingell and Wallcave, 1974; Gold et al., 1981; Brown et al., 1982b). Results obtained by Brown et al. (1982b), indicate the recovery of metabolites from tissues may be increased by one to two orders of magnitude when this procedure is used. Since metabolites appear to be the predominant form of xenobiotic organics in organisms, usually representing over 90% of the total of parent compounds and their metabolites, it is important that these analyses be included in programs designed to measure the bioaccumulation of organic compounds. In fact, it may be that those compounds which are rapidly metabolized after biological uptake may not be detected by normal procedures.

The methods for extraction of metabolites are similar to EPA standard procedures (Federal Register, 1979; USEPA, 1981), but with the addition of a step in which the extract is heated to 90°C for 30 minutes after extraction of the base/neutral extractable fraction and before extraction of the acid extractable fraction. The procedure is as follows:

(1) Homogenize 5 g (wet weight) of tissue in 20 ml of deionized (DI) water in a blender. Rinse the blade twice with DI water.

(2) Dissolve 1.2 g NaOH in the sample homogenate.

(3) Extract the homogenate three times with 50 ml of hexane/acetone (1:1, V:V). Centrifuge if necessary to obtain complete separation of the layers.

(4) Take the hexane (top layer) as the base/neutral extractable fraction and analyze for parent organic compounds.

(5) Heat the remaining aqueous phase to 90C for 30 min to hydrolyze possible conjugates (Gingall and Wallcave, 1974; Gold et al., 1981).

(6) Allow the solution to cool, adjust to pH 1 with 6N HCl, and extract three times with 50 ml of methylene chloride with centrifugation if necessary.

(7) Take the methylene chloride (bottom layer) to dryness with a roto-vaporizer.

(8) Add 10 ml of methylating agent (5 mg 3-methyl-1-p-tholyl-triazene/1 ml diethyl ether) to the dried sample.

(9) Blow-dry the sample under a stream of nitrogen.

(10) Redissolve the sample in methanol.

(11) Analyze the final methylated extract for the presence of metabolites using GC/EC, GC/FID or GC/MS (Brown et al., 1982b).

The distribution of metabolites between a site of detoxification, the glutathione-containing (GSH) pool, and sites of toxic action, including the metallothionein-containing (MT) pool and the enzyme-containing (ENZ) pool, can be determined by analyzing the composited cytosolic pools using the above method, starting at (2) above (Brown et al., 1982b).

Both metals and organics share a common site of toxic action, the ENZ pool, while organic metabolites also appear to act adversly on the MT pool, reducing metal-binding and detoxification by this pool (Brown et al., 1982b; Jenkins et al., 1982b). When all three cytosolic pools are analyzed for both metals and organic metabolites, it is possible to determine which specific contaminants are present at sites of toxic action and therefore responsible for direct toxic effects. When this procedure is used in combination with general stress indices, such as scope for growth, it is possible to ascertain both the sum total of direct toxic effects and indirect effects related to the metabolic cost of detoxification.

d. Analysis for Toxic Substances in the Cytosol

The following simple procedures are used to determine the partitioning of trace metals between a site of detoxification, the metallothionein-containing (MT) pool and a site of toxic action, the enzyme-containing (ENZ) pool (Brown et al. 1982a).

(1) Tissues are thawed and individuals (when practical) or composites of 15-25 organisms are suspended in three volumes of chilled buffer (0.05 M Tris-HCl, pH 7.4).

(2) Suspensions are homogenized with an antoxidant (2-mercaptoethanol) for 15 sec at high speed in a Sorval Omnimix homogenizer at 4°C. The homogenate is centrifuged for 10 min at 10,000xg in a refrigerated centrifuge, and the resulting supernatant is recentrifuged for 60 min at 100,000 x g. The final supernatants

(cytosols) from each sample are combined and rehomogenized for 5 sec to ensure homogeneity. At this point, cytosols can be stored at -80°C until further processing.

(3) Frozen cytosols are thawed, vortexed and 7 mL applied to a 1.6 x 70 cm column packed with Sephadex G-75 gel. The sample is eluted with 0.05 Tris-HCl (pH 8.2) at a flow rate of 28 mL hr^{-1}, and 3 mL fractions are collected for metal analysis (Jenkins et al., 1982c). A standard solution of proteins of known molecular weights, such as albumin, should be used to characterize the Sephadex column.

(4) Fractions are analyzed for metals using flame atomic absorption spectrophotometry when possible (e.g. usually Zn and Cu), or by graphite furnace atomic absorption spectrophotometry when necessitated by low metal levels (e.g. usually Cd and Ag).

The first peak to elute, as located by the metal profiles, is the high molecular weight enzyme-containing (ENZ) pool; the second peak is the medium molecular weight metallothionein-containing (MT) pool; and the third peak is the low molecular weight glutathione-containing (GSH) pool (Fig. 2). To save time for metal analyses, the location of these pools can be determined by doing a Zn profile, and then combining fractions constituting each of these pools for the remainder of the metal analysis.

A more rapid procedure has been developed, utilizing HPLC. Whereas each Sephadex G-75 column run takes about 8 hours, HPLC runs take only 40 minutes. In the HPLC procedure, 0.1-0.5 mL samples are injected on a Toya Soda TSK SW 3000 column (5 mm x 600 mm) and eluted at one mL/min with 0.2 M Tris HCl (pH 7.4). One mL fractions are collected and analyzed for metals as described above (Jenkins et al., 1982b).

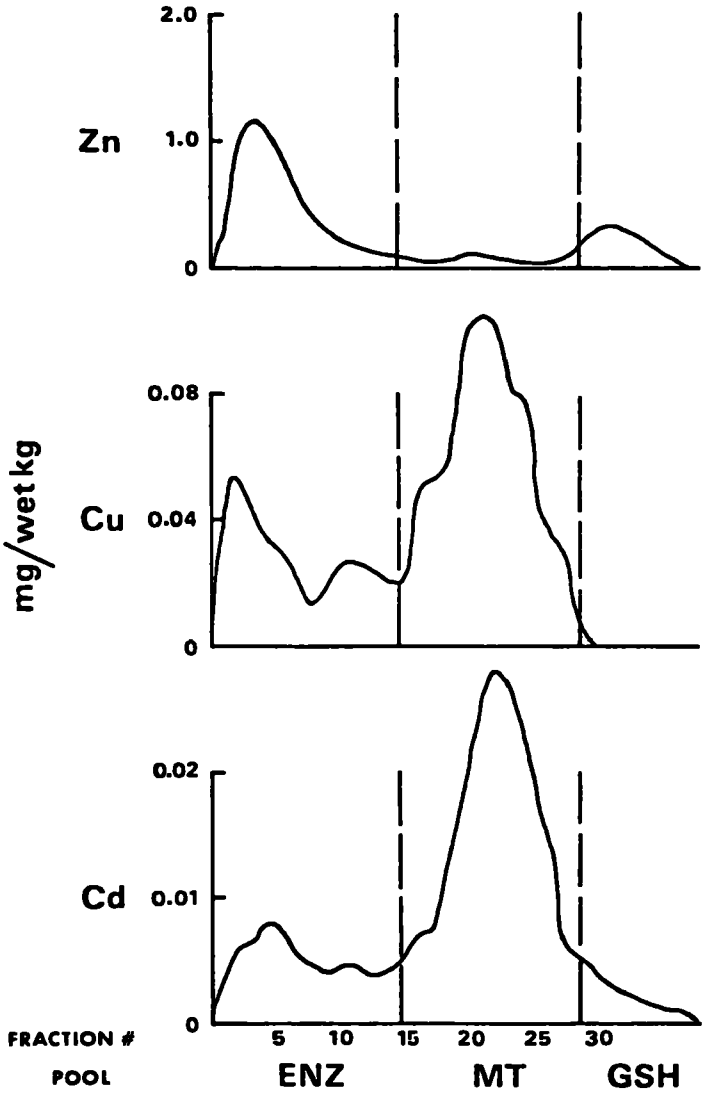

Fig. 2. Typical Sephadex G-75 elution profile for a control mussel (Mytilus californianus) liver showing the concentrations of Zn, Cu and Cd in individual fractions constituting each of the ENZ: high molecular weight enzyme-containing pool which contains Zn and Cu as essential components of metalloenzymes, but is a site of toxic action for excesses of metals; MT: medium molecular weight metallothionein-containing pool which serves a storage/detoxification function for essential (e.g. Zn and Cu) and non-essential (e.g. Cd) metals; and GSH: low molecular weight glutathione-containing pool which serves as a site of detoxification for organic metabolites (from Brown et al., 1982d).

SECTION 8

BIOLOGICAL ANALYSES

The recommended biological observations and analyses, arranged in approximate order of complexity and level of effort required, are as follows:

- Fouling
- Mortality
- Incremental Growth
- Condition Factor
- Gonadal Index
- Histopathological Effects
- Scope for Growth
- Oxygen:Nitrogen Ratio

FOULING

Fouling is determined in the field at the time of sample retrieval. The degree of fouling is reported as the estimated percentage of mesh openings occluded by fouling organisms (Stephenson et al., 1980).

MORTALITY

The percentage of test organisms surviving to the end of the exposure period is reported for each exposure site/depth.

INCREMENTAL GROWTH

The mean length of the shells (to the nearest 0.1 mm) is determined (Riisgard and Poulsen, 1981) before and after exposure at the reference site and in the plume to determine the change in length of the shells during the exposure period.

CONDITION FACTOR

The condition factor is the wet weight of the soft body expressed as a percent of the total organism weight (Bayne and Thompson, 1970; Boalch et al., 1981).

GONADAL INDEX

The gonads are removed and weighed, and the (gonad weight)/(soft body weight) ratio is calculated (Ouellette, 1978; Giese and Pearse, 1974). In M. edulis, the gonad develops within the mantle so that physical separation of the two tissues is difficult. Therefore, the entire gonadal/mantle complex is taken as gonadal tissue (Lobel and Wright, 1982).

HISTOPATHOLOGICAL EFFECTS

Histopathological analyses will provide useful information regarding the condition of the organisms and the site of toxic action, which could not be determined by other means. Methods for tissue preparation are found in Yevich and Barszcz (1981)

SCOPE FOR GROWTH

"Scope for Growth" (SFG) is a measure of the net amount of energy available to an organism for growth and reproduction, and is determined by subtracting the energy used for basic physiological processes from the food energy assimilated. This index has been found to be statistically correlated with the concentration of toxic substances in tissues and is considered a sensitive method to detect sublethal, adverse biological effects (Bayne et al., 1981; Phelps et al., 1981; Widdows et al., 1981; Martin et al., 1982a).

The size of the test organisms and the stage in the gametogenic cycle are important sources of natural variation in SFG measurements. At least 10 organisms of similar size should be used to minimize the experimental error.

SFG is determined by measuring four parameters - clearance (food uptake) rate, food energy absorption efficiency, respiration rate and ammonia excretion. The values for these parameters are converted into energy units (Joules), and substituted into the following equation:

$$SFG = (C \times A) - (R + E)$$

where:
- SFG = Net energy available for growth and reproduction
- C = Food energy consumed
- A = Food energy absorption efficiency (%)
- R = Energy lost through respiration
- E = Energy lost through excretion

The methods used for measuring these parameters for Mytilus edulis are described below. For the sake of consistency, physiological measurements are completed in the following sequence for each group of organisms tested:

Day 1: AM - Collection
PM - Clearance rate

Day 2: AM - Absorption efficiency
- Respiration rate
PM - Ammonia excretion rate

a. Clearance Rate

Clearance rate is the volume of water cleared of particles (diameter greater than 3um) per unit time. Seawater, filtered to 1 um, flows to a mixing chamber where algae (such as Tetralsemis suecica, Martin et al., 1982a) is added continuously to obtain a concentration of approximately 8-15x10^3 cells mL^{-1}. This water then flows at a rate of 50-75 mL min^{-1} through separate supply lines to ten 1-liter chambers, each containing one mussel (Fig. 3) . Each chamber is gently aerated to ensure uniform mixing and prevent settling of the algae. The algal uptake measurements are not initiated until after the animals have opened their values and ventilated for approximately 60 minutes. The inflowing and outflowing algal concentrations are determined at one hour intervals for three hours. To complete the algal counts in the timely manner, the use of an electronic counting instrument, such as a Coulter Counter (Model TA11, 100 um aperture tube, using channels 4-13), is recommended.

The clearance rate (CR) is calculated for each mussel at each hourly interval using the following equation:

$$CR\ (L\ h^{-1}) = \frac{C_1 - C_2}{C_2} \times F$$

where: C_1 and C_2 = incoming and outflowing algal concentrations, respectively.
F = flow rate through each chamber in Lh^{-1}.

The mean of the three hourly rates is then calculated and used as the representative clearance rate for each mussel.

b. Food Energy Absorption Efficiency

Mussels are maintained in clearance chambers overnight to allow for a sufficient amount of feces to be deposited by each individual. The following morning a sample of feces is collected from the bottom of the chamber with a pipette, deposited on a washed, ashed and weighed glass fiber filter using a Millipore system, and washed with 2.4% ammonium formate to remove any salts from the fecal pellets and the filter. The filter is dried overnight at 70°C and weighed to provide the dry weight of the material. The filter is then ashed in a muffle furnace at 500°C for at least four hours and weighed to provide an ash weight. The food material is also collected on a washed, ashed and weighed glass filter,

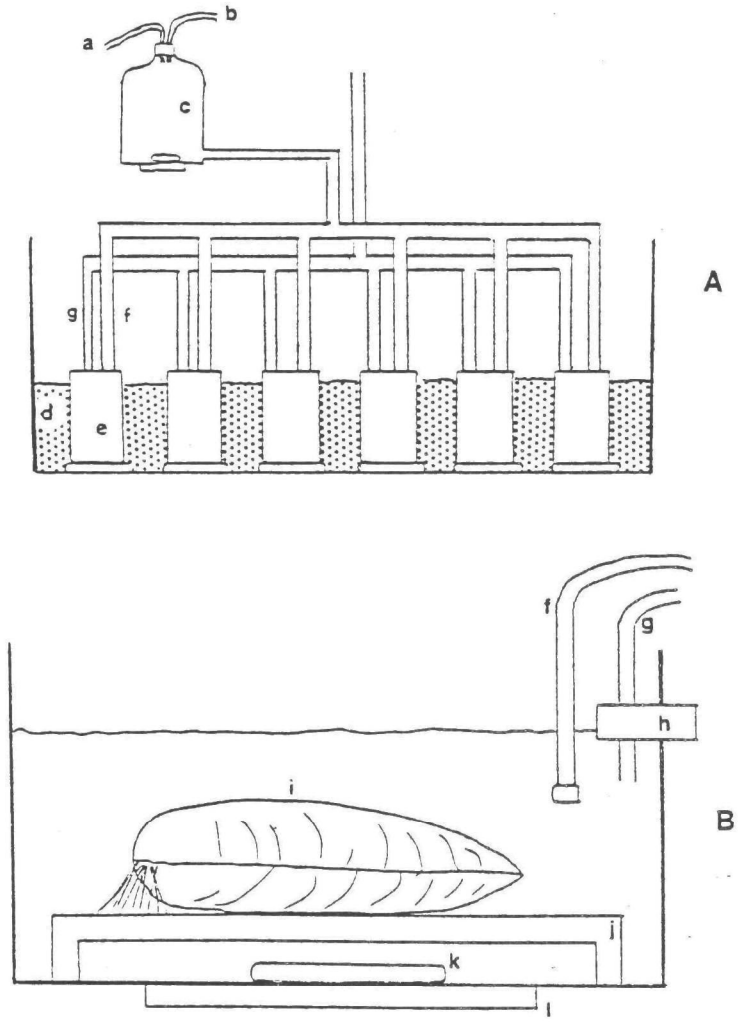

Fig. 3. Apparatus for measuring clearance rates and assimilation efficiency. A. Clearance rate apparatus. B. Enlarged side view of feeding chamber: (a) infloving algae; (b) infloving filtered seawater; (c) mixing chamber; (d) running water bath; (e) feeding chamber; (f) algae; (g) air; (h) overflow; (i) mussel; (j) pedestal; (k) stirring bar; (l) magnetic stirrer (from Martin et al., 1982a).

and treated in the same manner as the feces. The absorption efficiency (AE) is calculated as follows (Conover, 1966):

$$AE = \frac{(F-E)}{(1-E)(F)} \times 100$$

where: F = ratio, (ash-free dry wgt)/(dry wgt), for food.
E = ratio, (ash-free dry wgt)/(dry wgt), for feces.

c. Respiration Rate

Respiration rates are measured by isolating individual mussels in closed respirometer vessels equipped with a dissolved oxygen electrode (Fig. 4). After it is placed in the chamber, the organism is allowed to acclimate for a period of approximately 30 minutes, during which time seawater containing algae is pumped through the apparatus as described above. This ensures the measurement of routine (feeding) metabolism. At the end of the acclimation period, the chamber valves are closed, isolating the mussel. The decline in PO_2 is recorded using a Radiometer blood gas analyzer strip chart recorder. Water in the container is stirred continuously to give an accurate reading at the oxygen probe. The oxygen tension should not be allowed to decline below about 115 mm Hg, at which concentration mussels become oxygen conformers.

The respiration rate (RR), expressed in mL O_2 per animal per hour, is determined as follows (use period of steady decline only):

Convert mm Hg into mL O_2 L^{-1}:

$$DO\ T_0\ (mL\ O_2\ L^{-1}) = \frac{mm\ Hg}{160}$$

$$DO\ T_1\ (mL\ O_2\ L^{-1}) = \frac{mm\ Hg}{160}$$

$$RR = (T_0 - T_1) \times \frac{VV\ (L) - VA\ (L)}{1000} \times DOS$$

Where: DO T_0 = initial dissolved oxygen concentration, mL L^{-1}
DO T_1 = final dissolved oxygen concentration, mL L^{-1}
RR = respiration rate in mL O_2 $animal^{-1} hour^{-1}$
VV = volume of respiration vessel, mL's
VA = volume of animal, mL's
DOS = Saturation value for dissolved oxygen at the specified temperature and salinity, obtained from a table.

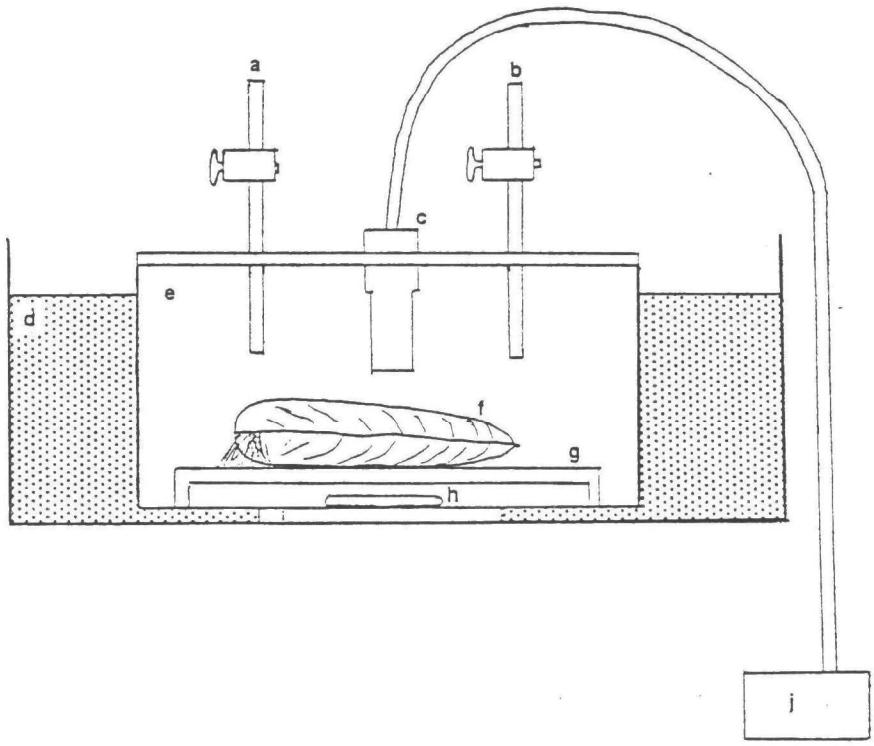

Fig. 4. Exposure chamber for measuring respiration rates: (a) inflow of water; (b) outflow of water; (c) O_2 probe; (d) water bath; (e) experimental chamber; (f) mussel; (g) pedestal; (h) stirring bar; (i) magnetic stirrer; (j) radiometer (from Martin et al., 1982a).

d. Ammonia (Nitrogen) Excretion Rate

Mussels are placed in individual beakers containing 300 mL of 0.45um filtered seawater. The beakers are placed in a water bath at ambient temperature, and the mussels are left undisturbed for a period of three hours. One control beaker, lacking a mussel, is used to determine background ammonia levels.

At the end of the three-hour period, the seawater in each beaker is gently mixed, sampled and analyzed in duplicate for ammonia, using the salicylate-hypochlorite method of Bower and Holm-Hanson (1980).

The procedure is as follows:

(1) A 50 mL syringe is rinsed by filling it with water from an exposure beaker, and discarding the contents.
(2) The syringe is filled with a second 50 mL sample from the beaker, and a filter holder containing a glass fiber filter is attached to the end of the syringe

(3) A 10 mL sample is flushed through the filter and discarded.

(4) The remaining 40 mL are filtered into a pre-washed, HCl-rinsed, plastic container, covered and frozen at -20°C until the sample is processed. It is more convenient to delay the NH_4 analyses until a large number of samples are available.

The NH_4-N excretion rates (NER) are calculated as follows:

$$NER\ (ug\ NH_4\text{-}N\ h^{-1}) = (TC - CC)(VSW) \times 14 \times h^{-1}$$

Where: 1 uM NH_4 - N = 14 ug N
TC = Concentration of NH_4 (uM/L) in test beakers containing mussels
CC = Concentration of NH_4 (uM/L) in control beaker
VSW = Volume (L) of seawater in exposure vessels

If 300 mL are used in each beaker, and the exposure period is three hours, (TC - CC) x 1.4 = ug NH_4-N h^{-1}

e. Algal Cultures

Algae used in SFG experiments are from unialgal cultures (e.g. Tetralsemis suecica, Martin et al., 1982a) maintained in a healthy, log-growth phase by adding small pure cultures (400 mL) and commercially available nutrients to five gallon carboys of autoclaved seawater (collected on incoming tide). The energy content of the algae is determined using the wet oxidation method of Maciolek (1962).

f. Calculation of Scope for Growth Index (SFG)

Scope for growth is calculated as follows:

$$P = (C \times A) - (R + E),$$

where: P = Energy (Joules h^{-1}) available for growth and reproduction
C = Food energy (Joules h^{-1}) consumed
A = Food energy absorption efficiency (%)
R = Energy (Joules h^{-1}) lost through respiration
E = Energy (Joules H^{-1}) lost through excretion

(1) C = Joules of food energy consumed per hour

Where: (a) Clearance rate (Lh^{-1}) x food concentration (cells L^{-1}) = total No. cells consumed per hour.
(b) (No. cells h^{-1})/(No. cells mg^{-1} ash-free wgt) = mg algae h^{-1}
(c) mg algae h^{-1} x Joules mg^{-1} algae = Joules h^{-1}
The energy content (Joules mg^{-1}) of the algae is determined according to the method of Maciolek, 1962.

(2) A = Food energy absorption efficiency (%)

(3) R = mL O_2 consumed h^{-1} x 20.08 Joules per mL O_2

(4) E = mg NH_4-N h^{-1} x 24.81 Joules per mg NH_4-N

Additional information required to complete the calculations includes determining mussel volume and dry weight, to standardize calculations for a 1 gram animal. Calculation of standardized rates is only recommended when using animals that vary widely in length. If the lengths of the animals fall within a narrow range, weight-specific rates should be calculated for clearance, respiration, and ammonia excretion before calculating the SFG.

OXYGEN:NITROGEN (O:N) RATIO

The O:N ratio, which is the ratio of oxygen consumed to nitrogen excreted, is another useful physiological index of stress. This value can be calculated from the above data as follows (Bayne, 1975; Widdows, 1978b):

a. Convert ml O_2h^{-1} to mg O_2h^{-1} by multiplying by 1.428.

b. $$O{:}N = \frac{\text{mg } O_2h^{-1}}{16} : \frac{\text{mg } NH_4\text{-N } h^{-1}}{14}$$

SECTION 9

QUALITY ASSURANCE

A quality assurance plan must be prepared as an integral part of the 301(h) monitoring plan. Factors in the field that will affect the quality and utility of the data include the condition, uniformity in size, and stage in the gametogenic cycle of the organisms, the care taken in avoiding contamination and injury of the organisms during collection and transport of the test organisms, the attention given to the depth and positioning of the exposure gear, and the water quality conditions, such as salinity, at the exposure sites.

Laboratory quality assurance practices include the regular calibration of instrumentation, the use of duplicate analyses (i.e. every tenth analysis) and reference materials, and participation in interlaboratory studies such as round robins and performance evaluations. Detailed laboratory quality assurance guidelines are described in USEPA (1979, 1982). Reference materials for water, wastewater and tissue analyses are available from the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio.

SECTION 10

DATA ANALYSIS, INTERPRETATION AND REPORTING

The overall objective of the caged mollusc biomonitoring program is to provide the permittee and the regulatory agency with data useful in identifying discharges that cause adverse effects on marine life or otherwise pose a threat to the marine environment. Differences observed in the chemical and biological parameters at reference and plume stations must be analyzed for statistical significance to account for natural variations in chemical and biological data, which are often large. The use of composite samples is generally not recommended because it obscures the variation in the individual organims, and prevents an adequate determination of the precision of the analyses. However, compositing is sometimes necessary to obtain sufficient material for analysis, or to reduce an otherwise overwhelming analytical burden. The selection of test organisms of similar size and stage of gametogenesis will tend to reduce the variation in the biological data (Bayne et al., 1981), and enable the investigators to detect smaller differences in population responses between stations than otherwise possible.

Upon the completion of the statistical analyses (t-test, ANOVA, etc.), parameters which fail to show a significant difference between the reference and exposed stations are reported, together with an appropriate discusssion if the results were unexpected or otherwise unusual. Parameters which are significantly different are further evaluated to determine the magnitude of the difference, whether any FDA, EPA, or state criteria for standards have been exceeded, and what reduction in the concentrations must be achieved to reach acceptable biological conditions.

CHEMICAL DATA

a. Water and Wastewater Quality

Water quality data should be reported to document conditions at the reference and plume stations, and should be evaluated in terms of the environmental requirements of the test organisms and confounding effects, if any, on the interpretation of the biological data.

b. Priority Pollutants in Tissues

Data on priority pollutants in tissues of organisms exposed at the reference and plume stations should be compared and evaluated in terms of

differences in biological responses at the stations. FDA action levels should also be considered if test species are being harvested for human consumption from the polluted zone.

c. Metabolites in Tissues

As mentioned above, it appears that chronic effects of organic compounds are caused by their metabolic products, while acute effects, which would occur under only the most extreme circumstances, are caused by parent organic compounds (Young et al., 1979). Body burdens of metabolites should be checked against data on toxicity of metabolities and parent compounds.

d. Distribution of Metals in the Cytosol

The metal levels in each pool of cellular proteins are added and expressed as an amount of metal per unit weight of tissue. The metal concentration in the MT pool can be compared to the loading capacity of this pool as determined by laboratory exposures. In this way, the degree of utilization (saturation) of the detoxification capacity of the organism can be determined (Brown et al., 1982a). Using this information, predictions can be made as to how much additional metal could be loaded into the biota before spillover of trace metals from the MT pool to the ENZ pool would occur, with resultant toxic effects. These toxic effects occur because excesses of essential metals or non-essential metals in the ENZ pool result in disruption of normal enzyme function.

It should be noted that a certain amount of Cu and Zn will always occur on the ENZ pool because these metals are essential components of metalloenzymes (Brown and Chatel, 1978; Jenkins et al., 1982c). However essential metals present in excess of that required in the metalloenzymes must be partitioned onto MT or they will have a toxic effect. Further discussions of the analytical methods and significance of the metallothionein data can be found in the following references: Brown et al., 1982c; Jenkins et al., 1982b,c,d,e; Kohler and Riisgard, 1982; Noel-Lambot et al., 1980; Piscator, 1964; Shiakh and Lucis, 1971; Simkiss and Taylor, 1981; Simkiss et al., 1982; Squibb et al., 1974; and Viarengo et al., 1980, 1981;

BIOLOGICAL DATA

a. Fouling

If severe fouling is observed at the end of the exposure period, the flow of water to the molluscs may have been sufficiently reduced to interfere with feeding. The degree of fouling, therefore, should be taken into consideration in evaluating data on growth and condition.

b. Mortality

The percentage of test organisms surviving to the end of the exposure period is reported for each exposure site/depth. Conditions in the ZID

may be acutely toxic. If less than 90 percent of the organisms exposed at the reference site survive, the test would be considered invalid and should be repeated. If survival at the reference site exceeds 90 percent, but survival at the plume site(s) is less than 90 percent, acute toxicity may be present. Under these circumstances, the bioaccumulation data would be invalid. To obtain adequate bioaccumulation results, it would be necessary to repeat the test using additional exposure sites.

c. Incremental Growth

This index is simply the increase in the mean shell length during the exposure period. Shell growth is dependent upon water temperature, available food, and other environmental factors, in addition to the presence of pollutants. Under normal conditions, a growth of several mm would be expected in 30 days. Riisgard and Poulson (1981), starting with organisms 2.26 mm in length, reported a increase in length of as much as 6.6 mm in M. edulis in 18 days. Additional observations on the growth of M. edulis were reported by Kautsky (1981). If the mean increase in shell length of organisms exposed in the plume is significantly ($P_{0.05}$) less than at the reference station(s), the likelihood of adverse environmental conditions is indicated.

d. Condition Factor

The condition factor is the wet weight of the soft body expressed as a percent of the total organism weight (Bayne and Thompson, 1970). Boalch et al., (1981) observed a mean condition factor of 6% in a composite of 20 M. edulis with a mean length of four cm. In their study, the condition factor was significantly correlated ($P_{0.05}$) with the $\log_{10}$ of the metal concentration for all metals except copper. They observed that the use of a composite sample reduced the variation from three orders of magnitude, for individual organisms, to approximately 50%. Stephenson et al. (1980), using a slightly different form of the condition factor, (soft body weight)/(length), observed that mussels with the highest condition factor were collected away from heavily industrialized areas.

A statistically significant ($P_{0.05}$) decline in the condition factor ratio during the exposure period, or a significantly lower CF at the plume site compared to the reference site(s), would indicate the likelihood of adverse environmental conditions at the exposure site.

e. Gonadal Index

The gonadal index varies with the stage in the gametogenic cycle of the organism. Within the gametogenic cycle, the proliferation of gonadal tissue and maturation of the gametes will be affected by the physiological condition of the organism, which in turn will be determined by the availability of food and other environmental conditions, including the concentration of toxic substances. Stephenson et al. (1980) reported gonadal indices ranging from 0.21 to 0.39. The low indices observed at some stations in their study were assumed to be related to high metal concentrations in tissues.

f. Histopathological Effects

Histopathological analyses provide information on the general condition of the organisms and the site of toxic action which could not be determined by other means (Barry and Yevich, 1975; Lowe and Moore, 1978; Lowe et al., 1981; Mix and Schafer, 1979; Mix et al., 1977; Mix et al., 1979a; Mix et al., 1979b; Reynolds et al., 1980: Thompson et al., 1978; Yevich and Barszcz, 1977). This information includes:

- Identity of Specific tissues affected by the toxic substances (site of toxic action)
- Whether the effects are reversible or irrersible
- The sex, stage in the reproductive cycle, and condition of the gonads
- Whether the poor condition of organisms that appear to be stressed was caused by parasites, pollutants, or nutrition

g. Scope for Growth

"Scope for Growth" (SFG), which is a measure of the net amount of energy available to an organism for growth and reproduction, has been found to be inversely related to the concentration of toxic substances in mollusc tissues and is considered a sensitive method to detect sublethal, adverse biological effects of toxic substances on molluscs (Phelps et al., 1981; Widdows et al., 1981; Martin et al., 1982a). A decline in SFG results in less rapid growth and a reduction in fecundity (Bayne et al., 1975, 1978; Bayne and Widdows, 1978; Bayne and Worrall, 1980). Gillfillan et al. (1976) observed an inverse correlation between SFG and the concentration of aromatic hydrocarbons in tissues, and a similar relationship was reported between SFG and the concentration of the water-accommodated fraction of North Sea crude oil by Widdows et al. (1982), and between SFG and tissue burdens of metals and organics by Phelps and Galloway (1980). An excellent discussion of factors related to the collection and analysis of SFG data can be found in Bayne et al. (1981).

g. Oxygen:Nitrogen Ratio

The Oxygen:Nitrogen (O:N) ratio provides information on the relative utilization of protein in energy metabolism compared to other carbon sources. A high rate of protein utilization, compared to carbohydrates and lipids, results in a low O:N ratios, which are generally indicative of a stressed condition (Widdows, 1978). According to Bayne (1973a,b), low O:N ratios (i.e. 20 or less) may result from low food concentrations (starvation), whereas at high food concentrations (1.5 mg/L or greater), O:N ratios will fall in the range of 40 to 50 in the absence of other adverse environmental conditions. They indicated that food levels available during most of the year support O:N ratios in the range of 25-30. Widdows et al. (1981), however, stated that O:N ratios of less than 30 were indicative of organisms that were very stressed. They observed O:N ratios of 50-75 in organisms that were well nourished and living under generally favorable environmental conditions. Although some variability in the O:N data is indicated, they may be useful in detecting stress.

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