Proceedings of the Integrated Research Planning Meeting for Gulf of Mexico Estuaries

November 2-3, 1993

U.S. Environmental Protection Agency
Environmental Research Laboratory
Gulf Breeze, Florida

November 1993

PROCEEDINGS OF THE INTEGRATED RESEARCH PLANNING MEETING FOR GULF OF MEXICO ESTUARIES

James E. Harvey[1], and Foster L. Mayer[2]

Avanti Corporation[1]
Environmental Research Laboratory
Gulf Breeze, FL 32561

U.S. Environmental Protection Agency[2]
Environmental Research Laboratory
Gulf Breeze, FL 32561

contract number 68-C3-0309

Work Assignment Manager
Michael A. Lewis

Project Officer
Raymond G. Wilhour

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
GULF BREEZE, FLORIDA 32561

DISCLAIMER

This document has not been peer and administratively reviewed within the EPA and is for Agency use/distribution only. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

CONTENTS

1. Introduction (Scope and Purpose) 1

2. Research Descriptive Summaries 4

3. Aquatic Ecological Evaluation Framework 6

4. Advisory Committee Recommendations 16

5. Literature Cited 20

Appendix A, Meeting Participants 21

Appendix B, Advisory Committee Members 23

Appendix C, EPA Request for Assistance (RFA) 25

Appendix D, Cooperative Agreement Research Summaries 27

FIGURES

Figure 1. Organizational Categories of Indicators 7

Figure 2. Candidate Selection Criteria for Indicators 9

Figure 3. Draft Site Selection Criteria Matrix 11

Figure 4. Watershed Approach to Sample Collection and Evaluation 12

Figure 5. EMAP Sampling Strategy 13

EXECUTIVE SUMMARY

A research planning meeting for the Aquatic Ecological Criteria Research Program was held November 2-3, 1993 to discuss and coordinate the development and field validation of an ecological diagnostic framework for Gulf of Mexico estuaries. Such a framework is essential for developing a systems-level diagnostic approach and developing relevant protocols to realistically and statistically assess the condition of a given ecosystem, drainage system or major watershed region. A brief review was given of this project's relationship to the Office of Research and Development's Aquatic Ecologically-Based Criteria and Characterization Issue Plan, and other EPA research initiatives: Gulf of Mexico Program, Environmental Monitoring and Assessment Plan, etc., and it's significance to EPA Regional and Headquarters Offices (Office of Water). Current and near-term research was presented in the framework of the Aquatic Ecological Criteria Research Program. Assessments of any missing areas of expertise in the overall project were addressed. Criteria to identify the site(s) to be studied were discussed. An advisory committee composed of representatives from academia, state and federal agencies, and industry provided suggestions relative to the selection of sites and protocol development, assessment and validation activities, and will eventually provide guidance through evaluating progress by reviewing reports and performing site visits. The meeting resulted in a peer-reviewed conceptual approach and directives for planning and implementation. The agreed-upon purpose of the next meeting will be to establish a research plan addressing such specifics as selecting a specific site, addressing temporal and seasonal variation, identifying an appropriate stress gradient, and delineation of responsibilities. As planning progresses, a peer review of this research plan will be conducted in the Spring of 1994. This report summarizes research discussions and presentations from the meeting, and also lists the Advisory Committee recommendations.

1. INTRODUCTION

Aquatic ecosystems are often subject to a complex and dynamic array of physical, chemical, and biological interactions. Ecologists do not have a complete understanding of how toxic chemicals and other stressors influence these interactions and how perturbations at one level of organization are expressed at other levels. There is a limited capability to compare or predict effects from one species to another and only simple qualitative approaches for dealing with population, community, or ecosystem comparisons. Assessments from single-species approaches for site-specific and media-specific (e.g., effluent, water column, sediment) situations must be extended to a fundamental, quantitative understanding of exposure-response relationships for larger ecological units.

Regulatory actions require the best, most consistent, most rational guidance from scientists regarding ecological characterization and the choice of underlying endpoints and indicators. The biogeographical distributions of discrete species and populations (e.g., streams, lakes, Great Lakes, Gulf of Mexico, North Atlantic, and Pacific coastal regions) and the influence of seasonal factors (e.g., temperature, salinity, and nutrients, will likely require that regulatory actions be based upon unique characteristics of each region. Researchers and managers must develop prudent, reliable, and cost-effective approaches to compare and predict the effects of various stressors at all levels of ecological organization in aquatic ecosystems of varying complexity and size within defined biogeographical regions.

In support of these needs, a research planning meeting was held November 2-3, 1993 to discuss and coordinate Gulf of Mexico estuarine research within the framework of the Aquatic Ecological Criteria Research Program. The meeting resulted in a conceptual plan. Such a plan is essential for developing a systems-level approach and developing protocols, since optimally, endpoints would need to be evaluated at the same site(s) at the same times and on the same organisms to realistically and statistically assess their utility. Assessments of any missing areas of expertise in the overall project were addressed. Criteria to identify the site(s) to be studied were discussed. A presentation was assembled by the meeting participants (Appendix A). This information was then presented to an advisory committee composed of representatives from academia, state and federal agencies, and industry. The Advisory Committee (Appendix B) provided suggestions relative to the selection of sites and protocol development, assessment and validation activities, and will eventually provide guidance through evaluating progress by reviewing reports and performing site visits. The purpose of the next meeting will be to establish a research plan addressing such specifics as selecting the site, addressing temporal and seasonal variation, identifying an appropriate stress gradient, and delineation of responsibilities.

As planning progresses, a peer review of the overall research plan will be conducted in the Spring of 1994.

Dr. Foster Mayer, U.S. EPA, called the meeting to order and articulated the agenda, introduced participants, outlined broad goals and provided background information on the purpose of this meeting. He called attention to the goals of the meeting and asked participants to assist in 1) obtaining consensus purpose and goals for this estuarine research project, 2) developing the research program matrix, 3) reviewing the sampling site approach, and 4) identifying and discussing deficiencies and watershed approach needs. Dr. Mayer also related portions of this programmatic mission to the similar goals of a 1991 workshop[1] on estuarine assessment and contaminant identification. The 1991 workshop goals reflected the importance of criteria for selection of appropriate endpoints and indicators that provide unambiguous information on the condition of an ecosystem. Because of the acknowledged importance of these goals to the EPA program and to this particular project, the goals of that workshop warrant restatement.

1.) To define, through consensus, a set of ecological assessment procedures to:

   A) Describe, at a screening level, the condition (physical, chemical and biological) of Gulf coast estuaries.

   B) Identify and characterize ecological problems caused by contaminants.

   C) Determine the causes of observed problems, focusing on pesticides and toxic contaminants.

2.) To establish selection criteria for demonstration sites in the Gulf of Mexico to field test the applicability and predictive capability of the procedures.

These goals helped to provide a basis for a subsequent Request for Assistance (RFA) from EPA that was issued in the Spring of 1993 for collaborative research. The RFA was developed to solicit proposals in support of development and validation of ecosystem diagnostic techniques for Gulf of Mexico estuaries and associated bayous. Appendix C lists the complete RFA solicitation. After competitive review, available resources allowed funding of three proposals. Part of the selection criteria for these proposals was their ability to complement current and proposed aquatic ecological research at Gulf Breeze Environmental Research Laboratory (GBERL).

"Development and Evaluation of Diagnostic Indicators of Ecological Condition for Gulf of Mexico Estuaries and Associated Bayous;" Dr. Kenneth Dickson, University of North Texas, Denton, TX (a consortium including Gulf Coast Research Laboratory, Ocean Springs, MS - University of Southern

Mississippi; University of Miami, FL; University of Texas at Dallas; and ABC Laboratories, Columbia, MO).

"Development and Validation of Bioassays for Assessing the Contamination of Marine Estuarine Environments;" Dr. Stephen Safe, Texas A&M Research Foundation, College Station, TX.

"Validation of the Mummichog Fundulus heteroclitus as a Histopathological Indicator of Pollution Along the Eastern United States;" Dr. Wolfgang Vogelbein, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA.

Research summaries were presented from each of these funded proposals to familiarize all the participants with the research and collective expertise at this meeting. EPA personnel also presented short research summaries of toxicological and histopathological analyses performed at the GBERL for the Pensacola Bay system.

As part of EPA's Office of Research and Development Aquatic Ecological Criteria Research Program researchers from GBERL and extramural cooperators will share appropriate approaches, methods, and samples to identify and develop appropriate and unambiguous endpoints and endpoint indicators that are sensitive, accurate and precise. These measurements contribute to the short-term program goal: characterize and assess a smaller aquatic ecosystem, estuarine drainage system or bayou, and where possible, identify diagnostic capabilities of endpoints and their suites of indicators. The long-term program goal is to utilize an integrated watershed approach to developing a characterization and assessment framework that will be applicable not only to estuaries and bayous, but to wetlands, marshes and terrestrial components of the ecosystem. This characterization and assessment framework, once developed and verified, would become a valuable monitoring and regulatory tool. Future goals could include expanding the usefulness of this framework to include an "early warning" indicator monitoring aspect as well as predictive abilities. The Aquatic Ecological Criteria Research Program provides fundamental knowledge regarding the ecology of near-coastal ecosystems to better enable formulations of risk assessment by EPA Regional personnel, EPA headquarters regulatory personnel, and interested state and local regulators and scientists in the Gulf of Mexico watershed region. Advisory committee members represent this broad regulatory and scientific diversity.

2. RESEARCH DESCRIPTIVE SUMMARIES

Complete descriptions of objectives and approaches for each of the three cooperative agreement research projects are contained in Appendix D.

The first proposal, "Development and Evaluation of Diagnostic Indicators of Ecological Condition for Gulf of Mexico Estuaries and Associated Bayous" helps to identify and evaluate ecological indicators, emphasizing those with aspects linked to diagnostic capabilities. The approach is to catalog and reduce sets of indicators across different spatial/temporal scales and different levels of biological organization to produce an evaluation framework. This evaluation framework will then be performance-tested and verified statistically. Application of the indicators to synthetic process-oriented simulation model output and field data comparison will contribute to a better understanding of the response of indicators to spatial-temporal changes in stressors. It is important to recognize that although this project has a modeling component, the purpose of the project is not to verify a model using field data. The field data will be used to assess sensitivity and reliability of indicator systems.

The second proposal, "Development and Validation of Bioassays for Assessing the Contamination of Marine Estuarine Environments" evaluates and validates the efficacy of a series of biomarkers to assess exposure of marine biota to organic contaminants, more specifically, polynuclear aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs). This project will determine if specific biomarkers indicative of organic chemical exposure have been induced and determine if exposure has caused specific physiological damage, such as modification of receptor binding and gene expression. An additional objective of this study is to assess the relative sensitivities of biomarkers such as EROD activity, P450IA mRNA levels, PAH metabolites in bile, and DNA adduct formation, and compare them to arrive at an approach that integrates the activity of contaminants in a mixture, providing a risk-based estimate at the molecular-genetic level. Information and experience gained from this study will be integrated into the overall development of the evaluation framework.

The third proposal, "Validation of the Mummichog *Fundulus heteroclitus* as a Histopathological Indicator of Pollution Along the Eastern United States" seeks to determine the efficacy of *Fundulus* sp. as a histopathological indicator of chemical contamination in aquatic environments. The scope of work for this project has been amended to include field evaluation of *Fundulus grandis* in selected Gulf of Mexico estuaries. As sediments are known to be efficient integrators of hydrophobic organic compounds, types and concentrations of sediment contaminants (PAHs, heterocyclic compounds, PCBs, chlorinated pesticides, trace metals, etc.) will be correlated with liver histopathologic responses.

These field studies will provide a method of pollution assessment that is able to link adverse health effects in a non-migratory fish to specific types, sources, and concentrations of environmental contaminants. This approach can be implemented locally and regionally and will help to identify specific localities where chemical contaminants are exerting adverse biological effects in an indigenous organism. Information and improved methods for use and development of histopathological indicators gained from this study will be integrated into the overall development of an aquatic evaluation framework.

3. AQUATIC ECOLOGICAL EVALUATION FRAMEWORK

The aquatic ecological evaluation framework will subsume many differing measurement endpoints, indicators, and coastline environments. This massive body of information eventually will be organized into a database from which one can combine and retrieve any number of biological, chemical, ecological, environmental, geographical, hydrological, and meteorological factors to assist in characterizing, comparing or assessing the condition of a given estuarine drainage system. Project-specific objectives include to:

- Analyze existing performance data of candidate diagnostic indicators gleaned from available literature and Federal and State ecological monitoring and assessment programs (such as EPA's EMAP).

- Develop methodology for testing and/or evaluating indicator performance using appropriate statistical analyses and simulation models.

- Assemble a set of habitat, exposure, stressor and effects indicators to characterize ecological conditions for specific spatial and temporal ranges in specific systems. Seek causal relationships or correlations between conditions and effects.

- Apply the candidate indicators in a known stress gradient in a selected estuary in the Gulf of Mexico.

These objectives will be accomplished through coordinated research by GBERL scientists and university collaborators in a stepwise fashion. **Phase 1** will consist of a comprehensive literature search undertaken to assemble and categorize documented ecological indicators of potential utility to assess estuarine drainage systems at differing spatial and temporal levels. Much of this information exists and has been captured in various workshops, symposia and journal articles and book chapters[2,3,4,5,6]. This includes molecular genetic indicators as well as physiological and gross anatomical indicators, geochemical indicators, etc. These indicators will then be grouped into endpoint categories: physical/chemical level, species-level, community level, ecosystem level, and landscape level. Rather than generate a comprehensive list of endpoints with subsets of indicators, these endpoint categories were chosen to represent organizational divisions within ecosystems (Figure 1). Endpoint categories include both assessment endpoints (impacts of interest) and measurement endpoints (parameters that can be physically measured). Endpoint selection is dependent on the particular ecosystem being examined and potential or suspected impacts or stresses[7]. The indicators for

SPECIES- AND POPULATION LEVEL ENDPOINTS - particular species of concern because of one or more of the following characteristics:

Direct Interest

- Economic, aesthetic, recreational, nuisance, or endangered species

Indirect Interest

- Interactions between species- e.g., predation, competition, parasitism
- Habitat role- e.g., physically dominant species such as mangrove tree species
- Ecological role
- Trophic relationships
- Functional relationships
- Critical species- e.g., keystone species that affect overall trophic structure or control important ecological processes

COMMUNITY-LEVEL ENDPOINTS

- Food-web structure
- Species diversity of ecosystems
- Biotic diversity of ecosystems

ECOSYSTEM-LEVEL ENDPOINTS

- Ecologically important processes- e.g., decomposition, nutrient recycling
- Economically important processes- e.g., wastewater treatment
- Water quality
- Habitat quality

LANDSCAPE-LEVEL ENDPOINTS

- Mosiac of ecosystem types- e.g., relative coverage of plant communities
- Corridors for migration- e.g., habitat for endangered species
- Spatial and temporal patterns of habitat- e.g., timing for and location of wetland areas necessary for bird nesting
- Feedbacks to regional and global-scale physical systems- e.g., albedo, evapotranspiration, or sources of biogenic gases

Figure 1. Organizational Categories of Indicators.

each endpoint will then be judged against specific selection criteria and incorporated into a site-coupled matrix diagram. Selection criteria for these endpoints and their respective suites of indicators will be determined by many factors and will largely be driven by practicality, regulatory needs, and scientific interpretability. Figure 2 displays some of the candidate selection criteria that were discussed at the meeting. Many site-coupled matrix diagrams will be constructed to provide the breadth of applicability for different site (estuarine drainage system) conditions. It is expected that indicators will differ among sites, but that some will appear throughout the matrices. These indicators will be designated as core indicators, and will allow comparisons, at a gross level, of differing sites. It is important to realize that core indicators should not be used as a basis for assessing the condition of a site, but merely as a first step in characterization. Further characterizing specific sites is important and will identify and delineate certain processes that drive the system under study, leading to a more complete understanding of its resiliency or capacity for perturbation. For example, salinity, dissolved organic carbon, dissolved oxygen, and turbidity may be much more important parameters in a shallow, poorly flushed bay than in a riverine system with flowing water. Measurements of these indicators may indicate trophic relationships that support the ecology of that system, and therefore the potential for adverse impacts if disrupted. Examining a perturbation that has registered across several ecosystem organizational (trophic) levels can provide a better estimation of the extent of an impact or potential impact.

Thus far, we have discussed the use of measurement endpoints and indicators and their selection criteria. Certain of these indicators can be used to diagnose the condition of a particular ecosystem. These "diagnostic" indicators differ from other indicators in that they are usually associated with a particular stress; exhibit a causal relationship or correlation; are process oriented and possess a high signal to noise ratio, which means that few false positives would be expected. An example of a diagnostic indicator is the use of biomarkers to assess exposure of marine biota to polynuclear aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) as measured by molecular biological methods such as EROD activity, P450IA mRNA levels, PAH metabolites in bile, and DNA adduct formation.

Another class of indicators can be termed "early warning" indicators. Guided by experience, certain indicators may be identified that provide very limited information about the ecological system, but serve as a flag or trigger for additional measurements. We must stress development of these types of indicators are of low priority as compared to indicators which

- Relates to an assessment endpoint
- Availability of people to run assay
- Data on laboratory and field application available
  - Variability - Power
  - Sensitivity
- Temporal practability
- Specificity/selectivity/generality
- Ease of performance/cost
- Sample requirements
- Based on fundamental principles, mechanisms understood
- Large dynamic range
- Responsive - Reflects change in ecosystem condition
- System - Wide applicability
- Unambiguous
- Integrates effects
- Low natural variability
- Interpretability

Figure 2. Candidate Selection Criteria for Indicators.

provide site characterization information leading to an assessment of the condition of a given system. Early warning indicators will most likely have a rapid response time; be lower in the trophic level; be physiological or biochemical measurements; have a low signal to noise ratio, therefore a large number of false positives would be expected. As the development of the evaluation framework proceeds, it is expected that the screening process for indicators will suggest assignment of certain ones having high potential as early warning indicators.

Phase 2 of the project is to prioritize and select a field site. Initially, the evaluation framework will focus on one site; that site will be chosen using the criteria listed in Figure 3. The final evaluation framework will be composed of many ecologically diverse sites, representative of a wide range of habitats and aquatic environments. As more sites are characterized, they will be incorporated into appropriate site categories of the evaluation framework still to be decided. Initially, sites will be chosen based on such selection criteria as: diverse biological populations, acute exposure gradient for a stressor, availability of a reference area, well characterized stressors, known hydrology, close proximity to a research facility, etc. A site selection matrix (Figure 3) shows considerations that must be addressed to choose an appropriate site to initiate this project. Based on the aforementioned selection criteria and the proximity of GBERL to the Pensacola Bay drainage basin the choices have been narrowed to areas within Escambia Bay. To attain the sampling density needed for such a project and to obtain meaningful data at the appropriate spatial and temporal scales, small bays and bayous are under consideration for the first year's effort, and may be expanded in the following years. It was decided by meeting participants that a small bay or bayou would be chosen and a sampling approach selected at the next project coordination meeting. The general concept is to sample along a stress gradient, incorporating land-based watershed samples (streams, industrial effluents, etc.) and extend sampling into the receiving bay, reiterating the watershed approach of the evaluation framework (Figure 4).

Figure 5 displays a draft sampling scheme as developed and employed by GBERL EMAP. Note physical/chemical measurements coupled with biological measurements designed to characterize an area leading to an environmental assessment. Much work has gone into developing this sampling regimen, however it should be noted that the EMAP sampling scheme was developed for a specific spatial and temporal scale. Thus, it can serve as a starting point to modify according to the smaller spatial scale of this project. A more detailed draft of a sampling scheme appears in the proposal

SELECTION CRITERIA	POTENTIAL SITES			
	BACK BAY BILOXI	MOBILE BAY	ESCAMBIA	PENSACOLA
Diverse Community	Yes	Yes	Yes	Yes
Acute Exposure/Gradient	Yes/?	Yes	Yes	Yes
Large Watershed	Yes	Yes	Yes	Yes
"Reference" Area	Yes	Yes	Yes	Yes
Historic Information	Limited	Yes/Limited	Yes	Yes
Known Stressors/Sources	Yes	Yes	Yes	Yes
Localized Stressors	Yes	Yes	Yes	Yes
"Known" Hydrology	Limited	Yes/Limited	Yes/Limited	Yes/Limited
Research Facility	Yes	Yes	Yes	Yes

Figure 3. Draft Site Selection Criteria Matrix.

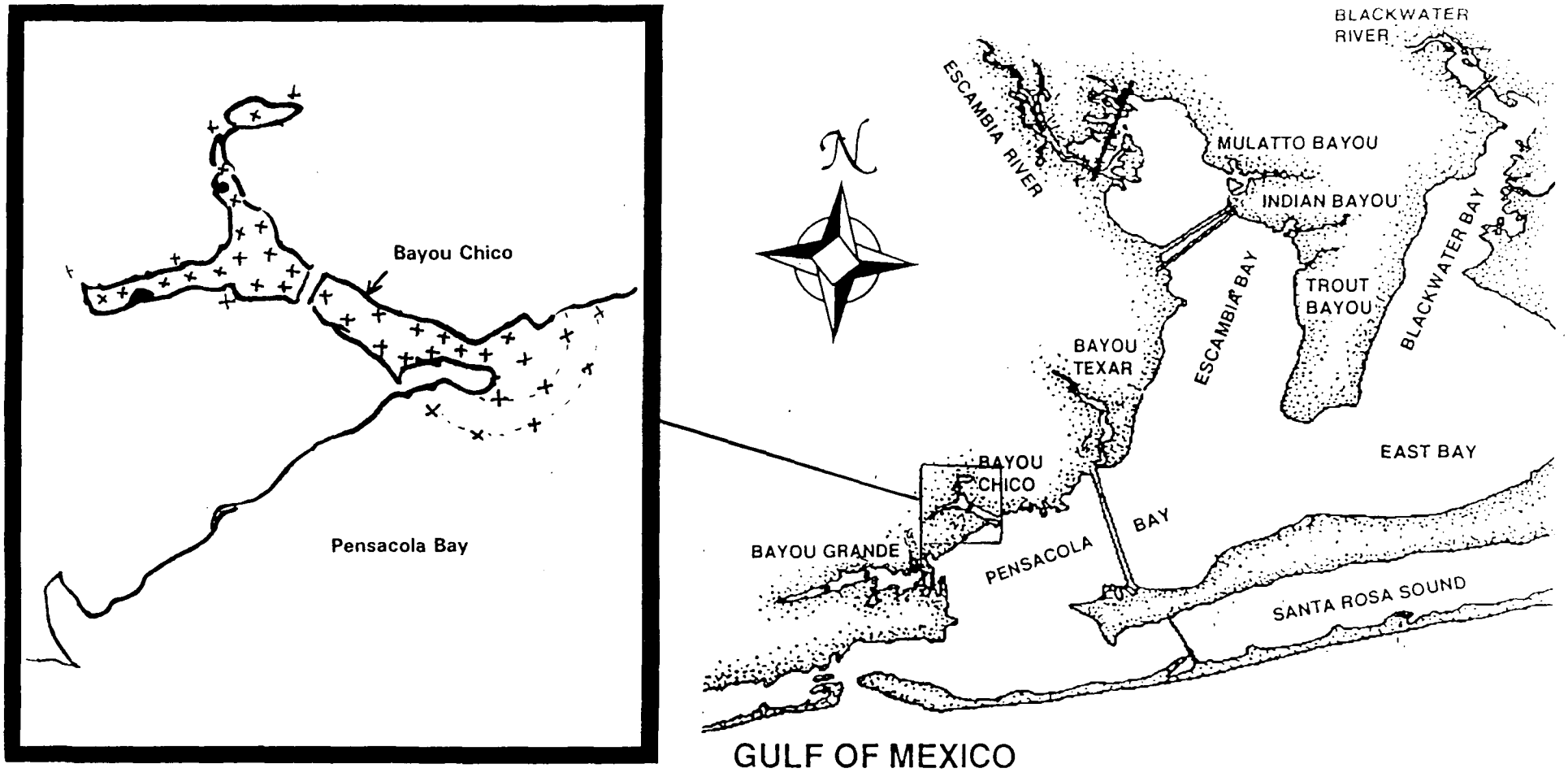


Figure 4. Watershed Approach to Sample Collection and Evaluation

This figure illustrates the approach strategic sampling of a site incorporating land-based samples, then extending into the receiving small bay or estuary along a gradient, and ending as the gradient dissapears into the larger receiving body of water. The sites shown have not been selected are merely illustrate the concept of the watershed approach.

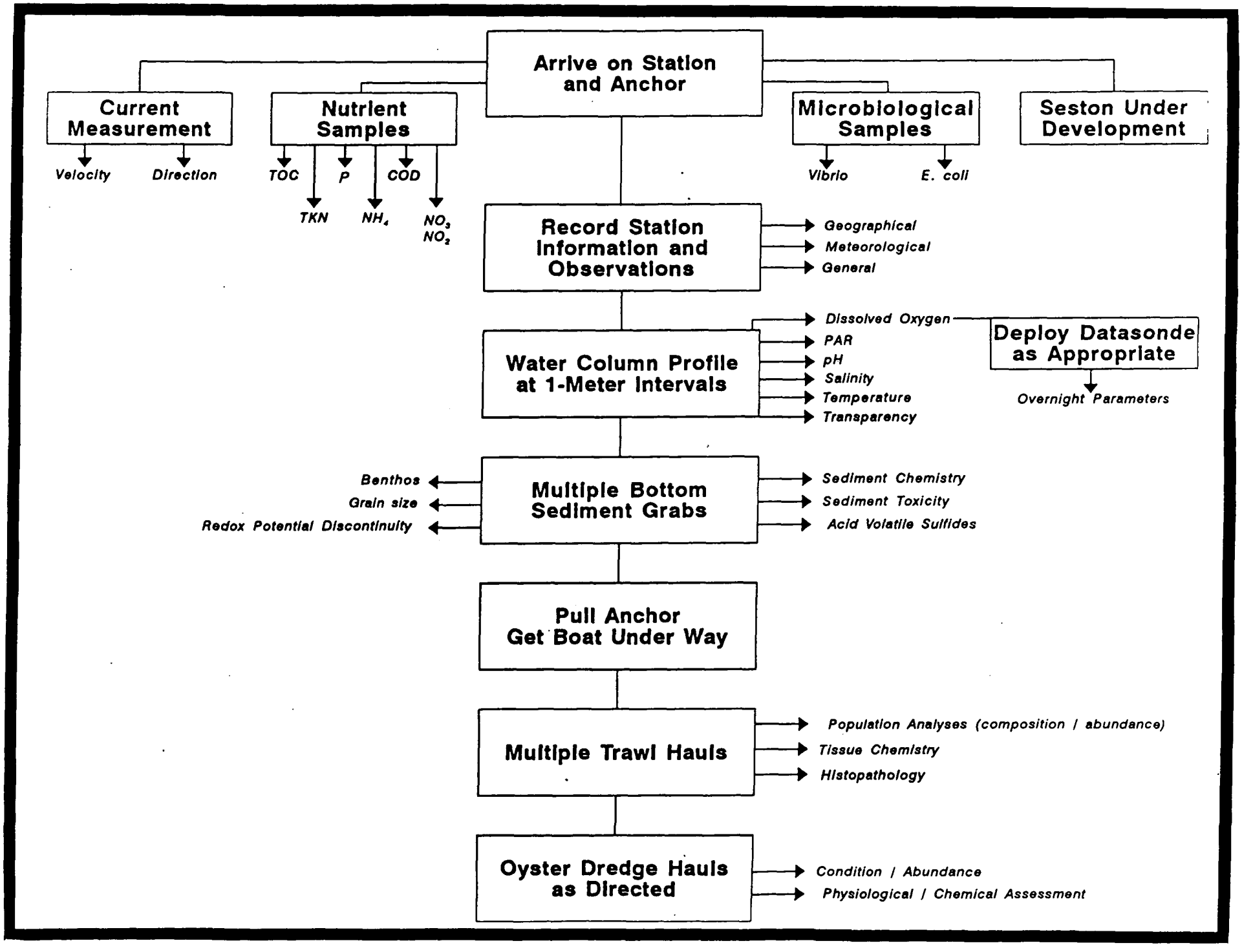


Figure 5. EMAP Sampling Strategy

"Development and Evaluation of Diagnostic Indicators of Ecological Condition for Gulf of Mexico Estuaries and Associated Bayous;" Dr. Kenneth Dickson, University of North Texas, Denton, TX (a consortium including Gulf Coast Research Laboratory, Ocean Springs, MS - University of Southern Mississippi; University of Miami, FL; University of Texas at Dallas; and ABC Laboratories, Columbia, MO).

Phase 3 of the project is to develop the field study design component which will be dependent on the final selection of indicators (phase 1) and the selection of the estuarine site (phase 2). The field study component will be designed to focus the best (most appropriate) sampling approaches and methods to bear on characterizing a particular ecosystem. The EMAP Field Operations Manual for the Louisianian Province[8] will be consulted to provide guidance for selection of approaches and methods to be tested on a smaller scale. The field study program does not seek to duplicate the EMAP sampling strategy; modifications to methods and changes to sampling strategies will be implemented to reflect appropriate scale differences between our selected site and other sites used originally to develop those methods. EMAP field sampling activities usually occur from July to October to reflect the "worst case" scenario of low dissolved oxygen. It is proposed in this project that field activities be scheduled outside the EMAP sampling window so that EMAP equipment may be used. A secondary benefit to this approach is that data obtained using the same equipment will provide more confidence in the measurements, and be more comparable to the historical EMAP data. A similar strategy was successfully employed in a demonstration project in the Back Bay of Biloxi, MS[9].

An important part of this project is to test and validate indicators by comparing their performance to simulation models of estuarine processes. This same approach has been suggested both as a part of EMAP[3] and at the 1992 Biloxi workshop[1]. The simulation models will test the sensitivity of indicators with respect to gradients of selected environmental conditions. This modeling effort is not a surrogate to the statistical analyses of the field-collected data, but will use the data to "truth" the validity and sensitivity of an indicator to a particular system. Variables to be included in the models will be related to the hydrodynamics, chemistry and biology of the system. For modeling at the landscape scale, grid cells (ca 1-10 ha) will be superimposed, encompassing the site. The exact horizontal and vertical resolution will be defined by the preliminary descriptions of the surrounding site. The hydrodynamics will include patterns of horizontal and vertical circulation, and its relationships to temperature, density and salinity. Freshwater inflow and tidal action may be incorporated depending on the location of the selected site. Salinity gradient measurements will be used to calibrate the dispersion parameters. Water quality relationships will include BOD/DO and a sub-set of stressors selected from the full set measured in the field. The selected site will be more intensively examined, emphasizing

chemical and biological processes at the sub-grid level. Ecosystem, community, population, organism and biochemical interactions will be examined at this smaller scale. Nutrient and energy flows in trophic chains, various biotic interactions, and bioavailability and bioaccumulation will be addressed at this level.

Laboratory data pertaining to the responses to toxicants at the organism level will be used to generate population-level model responses. Simulations of the gradual increase of pollutant loading to the model estuary and the response time of the indicators as applied to the model output can be used as an estimation of the minimum detectable trend in response indicators, and may help categorize certain indicators as being "early warnings" of impending effects.

4. ADVISORY COMMITTEE RECOMMENDATIONS

1. Selection of Indicators

The approach proposed for reviewing biological indicators in ecological studies and selecting sets of indicators for specific purposes is a sound approach. The program should proceed with the plan to evaluate existing ecological endpoints (assessment, measurement) and their suites of indicators (early warning, diagnostic) and classify their proven or potential utility for various uses in selected habitats or community types (wetlands, seagrasses, benthic communities, phytoplankton, etc.). A matrix diagram developed from a master list of biological and ecological indicators reviewed during the initial efforts for this program would be very useful for quick review by research teams and monitoring programs, and to provide a summary of potential applications for these indicators.

The panel realizes that it is crucial to the ultimate success of the program that the applications of some portion or subset of the biological indicators be based on criteria that focus the assessment conclusions along guidelines used for regulatory decisions or water quality classifications. The panel recognizes that a great deal of subtle understanding and inference can be built into a select suite of ecological indicators, but many bottom-line decisions and priorities must be set within regulatory frameworks where clear distinctions are necessary. Some information useful for ecological research communication may be lost through data reduction or manipulation of indices developed to address regulatory decisions, but a goal of this program should be development of indicators that communicate ecological conditions consistent with classifications defined by regulatory criteria. Thus, a means to relate selected ecological indicators to regulatory criteria such as "fishable or swimmable waters", "unacceptable adverse effect", "maintenance of balanced flora and fauna", will be needed if the end products are to be widely utilized by environmental scientists.

The panel recognizes that the proposed development of multiple indicators, i.e., a suite of indices based on various trophic levels or habitats, could lead to conflicting or inconclusive assessments for specific sites. This could lead to confusion as to the directions necessary to improve or restore habitats. Although much of the focus of the program may be on anthropogenic chemical stressors, many of the ecological indices will integrate other stresses such as low dissolved oxygen, habitat losses, eutrophication, etc. These factors must be considered during the site characterization process and evaluation of an ecological indicators.

Early warning indicators do not have to be causally related to specific stressors to be of value to the proposed program. They

can be used as early and inexpensive indicators that alert an investigator to potential problems, or conditions that require more detailed assessment. Their development and application should be followed by additional criteria that are used to pinpoint the nature of a problem and define ecological condition. Development of early warning indicators should be a secondary goal, after the development of suites of risk characterization and assessment criteria for designated environments (wetlands, shallow bays, open water, etc.).

2. Indicator Applications for Watershed Assessments or Ecological Risk Assessments.

The potential to apply ecological indicators to enhance ecological assessments of watersheds is an exciting development which is pertinent to new EPA programs, and should be evaluated in its entirety. The panel recognizes that the development and interpretation of ecological information relevant to site characterization at the watershed level will be challenging, but watershed approaches may become the basis for focusing and prioritizing limited resources for remediation or restoration programs. Ecological indicators that can help direct and focus these efforts are needed.

The watershed approach under consideration is based on large watersheds conceived at large physical scales (thousands to millions of hectares) reflecting conditions that developed over lengthy time scales (decades or more of various and changing land uses). However, site characterizations and remedial actions must be implemented at smaller spatial and temporal scales due to resource limitations. Ecological criteria or indicators appropriate to a number of scales of study and implementation must be developed. The panel appreciates the challenge of addressing problems within bays, estuaries, and watersheds, and encourages the investigators to address this issue. Much of the initial scoping may come from EMAP and other national, regional or local monitoring programs, but a thorough search and evaluation of existing ecological indicators is necessary to determine their utility for this new characterization framework.

Because the ultimate application of these ecological indicators may be risk-based resource management decisions, it is important to recognize the need for appropriate baseline or reference conditions for indicator development and application. The research team should have clearly defined criteria for selecting or defining reference conditions, as it is unlikely that the research team can identify representative pristine sites for comparison of all indicators. Historical information, ecological analogues, models, mathematical analogues, and professional judgement may all be necessary in development of reference conditions or criteria.

The panel encourages development of ecological indicators that clearly discriminate between reference/baseline conditions and impacted or changed ecological condition. We encourage the research team to avoid pitfalls such as:

- ecological indicators that provide only tones of gray distinctions, without bright line differentiation between impacted and acceptable conditions;

- the tendency for indicators to give conflicting results, especially as the number of inputs and assessment endpoints increase;

- the preference of many researchers to include too many endpoints or criteria, without focusing on critical measurements or driving factors.

We suggest that the team start with a broad scope in reviewing the kinds of ecological indicators that might be used for site characterizations, but then begin to focus on those indicators that provide reliable information for critical or priority characteristics. A quantitative approach, with statistical and modelling support will be necessary for scientifically defensible products. Nevertheless, the need for definitive conclusions and clear directions for restoration or remedial actions will require that professional judgement enter into final interpretations and applications of site assessment approaches.

3. Practicality/Utility of the Final Products

The committee believes that practicality of the tools and measurements will be an important aspect of the development of ecological indicators. The selection and weighting of indicators should be representative of the scale of study relevant to the site (bay, estuary, river basis), and should be relevant to the resources common to state or local resource agencies. Highly sophisticated or costly research approaches will have merit within a total program, but advocation of ecological indices that require tools or data out of the realm of day-to-day operations of state or local agencies will result in limited real world applications.

The panel views this program as an ecological assessment initiative, with <u>primary emphasis</u> on assessment criteria and indicators or ecological condition. The program should have a <u>secondary emphasis</u> on development of diagnostic and early warning indicators, as these tools will be relevant only when we agree on the appropriate criteria by which a normal or healthy condition is characterized.

Due to the location and expertise available at the Gulf Breeze ERL, we hope that there will be close collaboration between the cooperative agreement researchers and GBERL scientists. Selection

of a study site for indicator evaluation that is near Gulf Breeze is strongly recommended, in order to maximize potential participation by EPA scientists. The cooperative research program should maximize the extent to which it can utilize information from ongoing EPA research and monitoring programs.

4. Overall Direction

Senior management in regulatory or resource agencies often require that information presented for decision making be free of confusing scientific detail or caveats. The application of these ecological indicators for site characterization must, at some level, be useful under these circumstances. The final indices must incorporate various levels of data reduction and manipulation and still reflect scientific credibility for ecological significance, sensitivity, and reproducibility. Regulators often request a single number for each important criterion, or some type of bright-line distinction between impacted and unimpacted systems. Therefore, indicators which are easily described, and which both regulatory personnel and the lay public can relate to, are favored. The research team should strive to develop a suite of ecological indicators that can integrate accepted scientific approaches, stand up to rigorous statistical and scientific review, and be defensible in terms of regulatory criteria.

5. LITERATURE CITED

1. Mayer, F., T. Duke, and W. Walker. (eds.) 1992. Estuarine assessment and contaminant problem identification. Summary of a workshop held in Biloxi, Mississippi, April 23-25,1991. NOAA Technical Memorandum NMFS-SEFSC-330, 63 p.

2. McKenzie D.H., D.E. Hyatt, V.J. McDonald (eds.) 1992. Ecological Indicators. Vol. 1 and 2. Elsevier Applied Science.

3. Olsen, A.R. (ed.) 1992. The indicator development strategy for the Environmental Monitoring and Assessment Program. U.S. Environmental Protection Agency, Environmental Research Laboratory, Corvallis, OR.

4. Huggett, R.J., R.A. Kimerle, P.M. Mehrle, Jr., and H.L. Bergman. 1993. Biomarkers: Biochemical, physiological and histological markers of anthropogenic stress. Lewis Publishers, 121 S. Main Street, Chelsea, MI. 347 p.

5. Shugart, L.R., J.F. McCarthy and R. Hallbrook. 1992. Biological markers of environmental and ecological contamination: An overview. Risk Analysis 12(3):353-360.

6. Peakhall, D. 1992. Animal Biomarkers as pollution indicators. Chapman & Hall, London, U.K. 290p.

7. U.S. Environmental Protection Agency: Risk Assessment Forum. 1991. Summary report on issues in ecological risk assessment. EPA/625/3-91/018. Washington, D.C.

8. McCauley, J.M. 1993. Near Coastal Louisianian 1993 Sampling. Field Operations Manual, 4th Revision. ERL GB Number SR119. U.S. Environmental Protection Agency, Office of Research and Development, Environmental Research Laboratory, Gulf Breeze, FL. 98 p.

9. Walker, W. 1993. Evaluating ecosystem health: A demonstration project in the back bay of Biloxi, Mississippi. Gulf Coast Research Laboratory Report, Ocean Springs, MS.

Appendix A

Meeting Participants

Dr. Miguel Acevedo
University of North Texas
Institute of Applied Sciences
Box 13078, NT Station
Denton, TX 76203-3078

Mr. David Burke
University of Southern Mississippi
Gulf Coast Research Laboratory
P.O. Box 7000
703 East Beach Drive
Ocean Springs, MS 39564-7000

Dr. Kenneth L. Dickson
University of North Texas
Institute of Applied Sciences
Box 13078, NT Station
Denton, TX 76203-3078

Dr. William Fisher
U.S. Environmental Protection Agency
Environmental Research Lab
1 Sabine Island Drive
Gulf Breeze, FL 32561-5299

Dr. John Fournie
U.S. Environmental Protection Agency
Environmental Research Lab
1 Sabine Island Drive
Gulf Breeze, FL 32561-5299

Dr. Mark Harwell
RSMAS
Miami, FL
University of Miami

Mr. Mahlon C. Kennicutt, II
College of Veterinary Medicine
Texas A&M University Research Foundation
College Station, TX 77843

Dr. Michael Lewis
U.S. Environmental Protection Agency
Environmental Research Lab
1 Sabine Island Drive
Gulf Breeze, FL 32561-5299

Dr. Foster Mayer
U.S. Environmental Protection Agency
Environmental Research Lab
1 Sabine Island Drive
Gulf Breeze, FL 32561-5299

Dr. Stephen H. Safe
College of Veterinary Medicine
Texas A&M University Research Foundation
College Station, TX 77843

Dr. J. Kevin Summers
U.S. Environmental Protection Agency
Environmental Research Lab
1 Sabine Island Drive
Gulf Breeze, FL 32561-5299

Dr. Michael E. Unger
College of William and Mary
Virginia Institute of Marine Science
Gloucester Point, VA 23062

Dr. Wolfgang Vogelbein
College of William and Mary
Virginia Institute of Marine Science
Gloucester Point, VA 23062

Dr. William W. Walker
University of S. Mississippi
Gulf Coast Research Laboratory
P.O. Box 7000
703 East Beach Drive
Ocean Springs, MS 39564-7000

Appendix B.

Advisory Committee Members

Dr. Don Axelrad
Florida Department of Environmental Protection
2600 Blair Stone Road, Room 560
Tallahassee, FL 32399-2400

Dr. Thomas A. Bailey
U.S. Environmental Protection Agency
PPTS/OPP/EFED (H7505C)
401 M Street, S.W.
Washington, DC 20460

Mr. Glen Butts
Florida Dept. of Environmental Protection
160 Governmental Center
Suite 202
Pensacola, FL 32501-5794

Dr. James Clark
Exxon Biomedical Sciences, Inc.
Mettlers Road, CN-2350
East Millstone, NJ 08875

Dr. Carroll Cordes
U.S. Fish and Wildlife Service
National Wetlands Res. Ctr.
700 Cajundome Blvd.
Lafayette, LA 70504

Mr. Philip Crocker
U.S. Environmental Protection Agency
Region VI
1445 Ross Ave., Suite 1200
Dallas, TX 75202-2733

Dr. Thomas Dillon
U.S. Army Corps of Engineers
Waterways Experiment Station
WESE-R, P.O. Box 631
3909 Halls Ferry Road
Vicksburg, MS 39180

Mr. Henry Folmar
Dept. of Environmental Quality
121 Fairmont Plaza
Pearl, MS 39208

Mr. Jim Harrison
U.S. Environmental Protection Agency
Region IV
345 Courtland Street (4WM-OPO)
Atlanta, GA 30365

Dr. Fred Kopfler
U.S. Environmental Protection Agency
Gulf of Mexico Program, Building 1103
Stennis Space Center, MS 39529

Dr. J. Kevin Summers
U.S. Environmental Protection Agency
Environmental Research Lab
1 Sabine Island Drive
Gulf Breeze, FL 32561-5299

Dr. Becky Powell
Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

Dr. Maurice Zeeman
U.S. Environmental Protection Agency
OPPTS, HERD, EEB (TS-796)
401 M Street, S.W.
Washington, D.C. 20460

Appendix C

EPA Request for Assistance (RFA)
Issued Spring 1993

The Aquatic Ecological Criteria Research Program - Gulf Breeze within the Office of Research and Development of the U.S. Environmental Protection Agency anticipates funding new RESEARCH COOPERATIVE AGREEMENTS in FY93 for non-profit and academic institutions. Research must relate to the development of ecological assessment methods for estuaries in the Gulf of Mexico region. Full proposals identifying innovative research ideas and approaches in the research topic listed above are being sought. Proposals should include research objectives, significance to ecological criteria development, and experimental approach. In addition, a time-frame for accomplishments, a budget (annual and total), names and qualifications of investigator(s), and a quality assurance plan should accompany the proposal.

RESEARCH TOPIC OF INTEREST IS: Development and Validation of Ecosystem Diagnostic Techniques for Gulf of Mexico estuaries and associated bayous.

The research will provide fundamental knowledge of the ecology of near coastal ecosystems to better enable formulations of ecological risk assessment. The research will provide tools to assist the Agency in determining the effectiveness of management decisions in protecting estuaries. The research will:

1. Develop procedures to measure the current health, predict future health and to assess recovery of estuaries. The diagnostic protocol will be field tested to assess its applicability and predictive capability.

2. Identify and prioritize causes of impact on the biotic communities, e.g., point and non-point toxics.

3. Determine the tolerance limits of major floral and fauna communities and establish effective biocriteria to protect the biota.

The research may include synthesis of historical data bases and assessment of exposure in the water column and sediment. Bioavailability may be assessed by analysis of contaminant residues in indigenous biota. Toxicity assessment may include sediment and water column toxicity tests, biological community assessments, biochemical, physiological and pathological evaluations, and productivity measurements.

The anticipated funding period is up to three years. Multiple projects (2 to 3) may be awarded. The funds available for the research program are between $500,000-600,000 annually. This level is subject to availability and resources may limit the number of research projects funded. Peer review of proposals will provide a competitive basis for selection. Proposals will be evaluated on the basis of innovation, approach, relevance to topics of interest, scientific staff and facilities capabilities, and cost. Questions of a technical nature should be directed to Dr. Michael Lewis at 904-934-9382. Application kits and assistance are available from Ms. Janice Kurtz, 904-934-9286. Four copies of the proposal should be sent to Ms. Kurtz (U. S. Environmental Protection Agency, Environmental Research Laboratory, 1 Sabine Island Drive, Gulf Breeze, Florida 32561-5299). The original proposal and 3 copies should be sent (postmarked no later than April 30, 1993) to:

Grants Administration Division
PM-216F
U.S. Environmental Protection Agency
499 S. Capitol Street, SW
Washington, D.C. 20460

Appendix D .

Cooperative Agreement Research Summaries

"Development and Evaluation of Diagnostic Indicators of Ecological Condition for Gulf of Mexico Estuaries and Associated Bayous;" Dr. Kenneth Dickson, University of North Texas, Denton, TX (a consortium including Gulf Coast Research Laboratory, Ocean Springs, MS - University of Southern Mississippi; University of Miami, FL; University of Texas at Dallas; and ABC Laboratories, Columbia, MO).

"Development and Validation of Bioassays for Assessing the Contamination of Marine Estuarine Environments;" Dr. Stephen Safe, Texas A&M Research Foundation, College Station, TX.

"Validation of the Mummichog Fundulus heteroclitus as a Histopathological Indicator of Pollution Along the Eastern United States;" Dr. Wolfgang Vogelbein, Virginia Institute of Marine Science, Gloucester Point, VA - College of William and Mary.

DEVELOPMENT AND EVALUATION OF DIAGNOSTIC INDICATORS OF ECOLOGICAL CONDITIONS FOR GULF OF MEXICO ESTUARIES AND ASSOCIATED BAYOUS

A proposal for a Cooperative Research Program
Submitted to:

Aquatic Ecological Criteria Program
U.S. Environmental Protection Agency
Gulf Breeze Environmental Research Laboratory
Gulf Breeze, Florida

by

Kenneth L. Dickson
Miguel Acevedo
William Waller
James Kennedy

Institute of Applied Sciences
University of North Texas
Denton, Texas

in collaboration with

William Walker
David Burke

Gulf Coast Research Laboratory
University of Southern Mississippi
Ocean Springs, Mississippi

with consultants

Mark Harwell
RSMAS, University of Miami
Larry Ammann
University of Texas at Dallas, Richardson, Texas
Clarence Reed
ABC Laboratories, Columbus, Missouri

April 30, 1992

OBJECTIVES

General:

Development and evaluation of diagnostic indicators capable of estimating the ecological health conditions of Gulf of Mexico estuaries and associated bayous and of establishing associations between stressors and ecological responses.

Specific:

Analysis of existing field data, especially data sets collected using EMAP methodology, for one selected estuary in the Gulf of Mexico.

Collection of further field data using EMAP methodology supplemented with additional diagnostic indicators in the selected estuary in the Gulf of Mexico.

Development of a set of habitat, exposure, stressor and response indicators to detect ecological conditions as well as to diagnosis likely causes of these conditions.

Assure consistency of the set of indicators across different spatial scales and different levels of biological organization.

Organize existing data on tolerance limit of major estuarine biological organisms in the Gulf Coast populations and communities, in order to be able to relate biological effects to stressors.

Development of methodology for testing or evaluating indicator performance by means of statistical analysis, using output from process oriented simulation models and data from field measurements.

SIGNIFICANCE TO ECOLOGICAL CRITERIA DEVELOPMENT

This project will address the issue of ecological indicators, emphasizing those aspects related to diagnostic capabilities, i.e. ecological conditions as well as the stressors causing that condition. This work will contribute to the EMAP efforts by addressing the issues of: simplification of the set of indicators for devising cost effective strategy, maintaining consistency of the indicators across different spatial scales and different levels of biological organization, and of evaluating indicator performance by means of statistical analysis. Application of the indicators to synthetic process-oriented simulation model output

as well as field data will contribute to a better understanding of the response of indicators to spatial-temporal changes in the stressors.

SUMMARY OF EXPERIMENTAL APPROACH

The work plan to achieve the above mentioned objectives is divided into several phases;

1) select an initial group of indicators to include in a protocol for diagnosing ecosystem health,
2) select an estuarine system in the Gulf Coast meeting adequate criteria (existence of historical field data, EMAP-E data, and pollution gradients),
3) evaluate indicator (from set derived in phase 1) performance in diagnosing conditions of the selected estuarine ecosystem (from phase 2) based on historical data and output from process oriented models, by using statistical analysis.
4) perform data collection in the field for testing the proposed diagnostic protocol in the selected estuary,
5) apply the indicators to the field data (from phase 4) and use statistical analyses to assess the diagnostic capability of the indicators,
6) comparative analysis of indicator performance from tests using field data (from phase 5), historical data and model output (from phase 3).

Details on activities in these phases are given in the following sections.

SELECTION OF INDICATORS

Key to the success of this effort will be the selection of appropriate ecological indicators for gauging ecosystem health and inferring causal relationships. Specific problems addressed by the Near Coastal component of EMAP are low dissolved oxygen, eutrophication, chemical and biological contamination, habitat modification, and cumulative impacts. The EMAP sampling program will provide the basic framework upon which the diagnostic protocol will be based. A suite of chemical, biological, and toxicological measures rather than relying on a single type of response, will most accurately assess ecosystem health.

The main goal of this phase of the project will be to select relevant indicators for inclusion in the diagnostic protocol. The indicators should be exposure, stressors and habitat type as well as response type (Knapp et al, 1990) in order to infer cause and effect relations. This set should cover different scales of biological organization from biomarkers to ecosystems, as well as

different spatial scales, from sites to landscape. The list will likely include biological markers, and diversity, biotic, and similarity indices. While the ultimate indicators of ecological effects are adverse changes in exposed populations, such population responses (e.g. occurrence, abundance, reproduction) do not provide an indication of their cause. Inclusion of indicators detecting and documenting exposure and environmental stressors is fundamental (Shugart et al, 1992).

Cairns and McCormick (1992) compiled a list of desirable characteristics of ecological indicators. While each indicator cannot possess all of the desirable characteristics, reference to such a list may aid in the selection of ecologically relevant non redundant indicators. We will utilize these criteria in making our initial selection of effects and diagnostic indicators. In this phase we will not limit our evaluation to EMAP-E indicators, but will consider a broad array of candidate diagnostic indicators. We will examine EMAP-E results from studies conducted at Indicator Test Evaluation Stations, as well as candidate diagnostic indicators evaluated by other programs (i.e. Virginia Institute of Marine Science's - comparative biomarkers evaluation project on the Elizabeth River). Two recent reviews of the use of biomarkers as indicators of exposure and effects were recently published (Huggett et. al. 1993 and Peakall 1992). These will be carefully screened to identify additional candidate diagnostic methods.

ESTUARINE SYSTEM SELECTION

To be useful in validating the indicators, the estuarine system should be affected by several discrete and identifiable stressors, with gradients of impact and recovery. The presence of reference sites is critical. Availability of an historical data base will be an important factor in site selection. Sites for which EMAP data are available would have distinct advantages. Potential sites include Pensacola Bay and Mobile Bay. Personnel at the Gulf Coast Research Laboratory have experience conducting field studies in both of these systems and are familiar with the historical data bases for both systems. We will coordinate with the EPA Gulf Breeze Laboratory in selecting an appropriate estuary to conduct the field portion of the proposed project.

FIELD ACTIVITIES

The final location and number of sampling sites for this phase will be determined after selection of the study estuary and examination of available data (i.e. final experimental design will be based on examination of existing data). For scoping purposes and for budget estimation we have assumed that

approximately 50 sites (stations) will be studied. Proposed field sampling approaches are outlined in Appendix A, and follow guidelines of EMAP Field Operations Manual for the Lousianian Province (McCauley, 1991). The goal of the field sampling program is not just to duplicate EMAP-E efforts at the Province scale, but to focus efforts to best analyze a particular ecosystem, i.e. Tiers 3 and 4, thereby developing a diagnostic protocol. Some modifications to the proposed activities may be warranted after examination of historical data and final selection of the ecological indicators to be included in the analysis. Thus it should be considered as an example not as the final approach to the field study. The final field study design will depend on the final selection of indicators (Phase 1) and selection of the study estuary (Phase 2)

EMAP field sampling activities usually occur from July to October. This time frame has been selected by EMAP as an effort to sample during the period when lowest dissolved oxygen values are expected ("worst-case" scenario). We propose to schedule field activities during a time period **outside** the EMAP sampling window (perhaps early summer or late fall). By coordinating effort with the ongoing EMAP program, we may be able to utilize some of the EMAP equipment, thus substantially reducing the cost of the project. Using the same equipment as EMAP has the second advantage of rendering our data more comparable to the existing data. Similar strategy was employed by the research team at the Gulf Coast Research Laboratory in conducting a demonstration project in the Back Bay of Biloxi, Mississippi (GCRL, 1993).

EVALUATION OF INDICATORS BY USING HISTORICAL DATA

Analysis of data collected in previous EMAP-E efforts, such as the set of estuaries used in the Virginian Province Demonstration Project (EPA, 1992) and the Lousianian Province Demonstration Project (Summers, et al 1993), will be developed at an estuary level for the purposes of testing indicator performance.

Historical data from the selected estuary will also be used for evaluation of indicator performance. Data along gradients of pollution will be sought for this purpose.

EVALUATION OF INDICATORS BY MEANS OF SIMULATION MODELS

This part of the proposed research will contribute to the development of modeling methodologies for the analysis of indicator performance. We propose to test the indicators by applying them to the output data resulting from simulation runs of process models of estuarine systems. This approach has been suggested in phase four of the EMAP methodology (Knapp et al,

1990) as one of several potential methods to evaluate indicator performance, and it will play an important role in Step 3, Tier 3 of the estuarine assessment methodology developed in the Biloxi workshop (Mayer, et al, 1992).

The virtue of this technique hinges on the capability of setting up a large number of numerical experiments that can be statistically analyzed to evaluate the performance of the indicators. Using the simulation models, complete control of the environmental conditions of the simulated estuary can be achieved in order to test the sensitivity of the indicator with respect to gradients of these conditions. This modeling effort is not a surrogate to the statistical analysis of the field data to be developed during this project, but will complement and support the relationships to be uncovered by the statistics.

For this part of the project, the data previously collected in the field (e.g. from the Back Bay of Biloxy, GCRL, 1992) and to be collected during the course of the project will be used for model calibration and validation. Compilation of data from other sources will also be necessary to complete the required data base. The models will emphasize temporal and spatial variability so that the response of the indicators can be evaluated with respect to the time and space grids of the sampling design (Knapp et al, 1990).

The variables to be included in the models will be related to the hydrodynamics, the chemistry and the biology of the system. For the purpose of testing indicators across spatial scales, consistent models at different scales will be developed. Two different spatial scales will be selected: estuary or landscape and site specific. The estuary or landscape models will emphasize the transport of pollutants, and the evaluation of stressor and habitat indicators. The site specific models will primarily deal with the biological effects, and the evaluation of exposure and response indicators.

Landscape or estuary model:

At the landscape scale, the estuarine system will be modeled using grid cells of a relatively large horizontal size (ca 1 - 10 ha) and a few (three or four) vertical layers. The exact horizontal and vertical resolution will be defined on the basis of the extent of the selected estuary. The hydrodynamics will include the patterns of horizontal and vertical circulation, and its relationships to temperature, density and salinity. Freshwater inflow and tidal action (if significant for the selected estuary) will be incorporated. Salinity gradients measurements will be used to calibrate the dispersion parameters. Water quality relationships will include BOD/DO and a sub-set of

pollutants selected from the full set measured in the field. Transport of sediments and pollutants will be the emphasis of the models at this scale. Spatial distribution of habitats, flora and fauna at a very limited taxonomic resolution will constitute the biological variables at this scale. Remote sensing and GIS linkages of the models will provide support to this activity. Habitat and stressor indicators will be applied and tested at this scale

Site specific model:

Selected sites within the estuary will be subject to more intensive modeling. For each selected site, the driving physical variables such as temperature, salinity, etc. and pollutant concentrations will be derived from the estuary or landscape level model, but the chemical and biological processes at the sub-grid level will be emphasized. Ecosystem, community, population and organism level interactions will be included at this scale by using a limited (but finer than the one used at the landscape scale) taxonomic resolution. Nutrient and energy flows in trophic chains, various biotic interactions and population dynamics will be modeled. The kinetics of bio-availability will be linked to the biotic interactions, and an effort will be made to incorporate models of the kinetics of bio-accumulation.

Exposure and response indicators will be applied and tested at this scale. Cause and effect relationships helpful for designing indicators, can be inferred using the models. Laboratory data pertaining to the responses to toxicants at the organism-level will be used to generate population-level model responses. Simulations of gradual increase of pollutant loading to the model estuary and the response time of the indicators as applied to the model output can be used as an estimation of the minimum detectable trend in response indicators (Knapp et al, 1990).

STATISTICAL ANALYSIS

It is difficult to specify precisely the statistical methods to be used before a detailed examination of the data sets. This section describes, however, some principles that will be followed during this project.

Within the EMAP framework, the sampling design should evaluate the conditions of the selected estuary on the basis of sampling only a limited number of sites in the estuary. The tiered approach to be followed in this project requires the use of statistical methods taking into account this characteristic.The final experimental design will reflect measures to maximize randomness and uniformity in sampling for evaluation of indicators. Specific statistical evaluations to be utilized will

be selected during the project. Appropriate statistical analyses to describe and quantify observed differences will be performed. Choices relative to which to use in this study will be made cooperatively with USEPA personnel.

It is likely that the data will not satisfy the standard distributional assumptions required of classical statistical techniques, and hence require alternative approaches to the analysis, such as robust statistics (Hampel et al, 1986; Ammann, 1990). Another necessary feature of the statistics would be the flexibility to accommodate different experimental designs.

Multivariate statistical methods that incorporate the entire range of stressful conditions as well as recognized reference sites will also be used. Statistical analysis of biotic integrity indicators together with exposure indicators should provide a practical and objective means to identify ecological conditions and causative factors. Dependent variables could include various measures of community structure and function, such as taxonomic or trophic categories. Suites of environmental factors also could be integrated into such models as independent variables, including natural environmental parameters as well as measures of anthropogenic impact, such as contaminant levels.

If developed carefully and systematically, output from such refined statistical methods could be used to evaluate the consistency of the ecological indicators. The statistics also must discriminate among multicausal alternatives, including noncontaminant factors and natural environmental fluctuations.

TIME FRAME

The project will be conducted over a two year period. During the first year, emphasis will be placed upon indicator and site selection, historical data analysis and modeling. The first set of measurements in the field will be collected in the first year. During the second year, emphasis will be placed upon the evaluation of indicators by using field data and simulation output. The field work will continue during the second year for a second set of measurements.

QUALITY ASSURANCE

To maximize quality assurance in the protocol development, an Advisory Committee will be formulated to provide guidance, to review, to amend, and ultimately to approve the study protocol, and to assess progress during the course of the study. Guidance and requirements for quality control of measurements in each of the stations are addressed in appropriate Standard Operating

Procedures which presently exist for most activities specified in this proposal. Specific guidance will be provided by the EMAP QA Project Plan of the Lousianian Province (Heitmuller and Valente, 1992). Those activities not presently addressed will be included in developing remaining Standard Operating Procedures. A QA plan is included in Appendix B.

PROJECT ORGANIZATION AND RESEARCH TEAM

This project will be the collaborative effort of several institutions and researchers, in close cooperation with scientists of the Gulf Breeze Environmental Research Laboratory. All institutions are located in states with estuarine systems in the Gulf Coast: Florida, Mississippi and Texas. The research team has expertise in all aspects related to field and laboratory work in Gulf Coast estuaries, advanced capabilities of statistical analysis, simulation models, and indicator development and application. The proposed project team have prior experience working together. Drs. Dickson and Walker have both participated in a variety of Gulf of Mexico Program Toxics and Pesticide Committee activities. Drs. Harwell and Acevedo have collaborated extensively on ecological modeling projects. Drs Dickson, Waller, Kennedy and Ammann recently collaborated on an EPA cooperative agreement with the Duluth Environmental Research Laboratory examining the relationships between ambient toxicity and community response (Dickson et.al, 1992).

The Institute of Applied Sciences (IAS) of the University of North Texas will be the organization coordinating the project and contracting with EPA, Gulf Breeze. The IAS already has experience in successfully developing projects for EPA, and is currently examining estuarine data for ecological risk assessment. The following researchers from the IAS will be part of the research team: K. Dickson, who will be responsible for the overall project, T.W. Waller and J. Kennedy, who will be responsible for biological responses and eco-toxicological aspects related to the diagnostic capabilities of indicators, and M. F. Acevedo who will be responsible for the evaluation of performance of indicators using simulation modeling.

The Gulf Coast Research Laboratory (GCRL) of the University of Southern Mississippi will be in charge of the field work. This research unit has the personnel and facilities for this activity: including vessels and analytical laboratories. GCRL has been actively sampling estuarine systems in the Gulf Coast, and has recently performed a demonstration project at the Back Bay (GCRL, 1993). The following scientists will participate in the research team: William W. Walker, who will be responsible for the field work aspects of the project, William D. Burke, William E. Hawkins, Lisa S. Ortego, responsible for collection, processing

and quality of field data, and Chet Rakocinsky, who will provide expertise on the statistical analysis.

Additionally the following scientists will participate as consultants: Clarence Reed (ABC Labs), who will work on the application of the indicators to the field data, M. Harwell (RSMAS, University of Miami) who will provide advice on indicator development and evaluation and L. Ammann (University of Texas at Dallas) who will provide expertise in the statistical analysis.

Development and Validation of Bioassays for Assessing the Contamination of Marine Estuarine Environments

Dr. Stephen Safe
Texas A&M Research Foundation,
College Station, TX.

TABLE OF CONTENTS

		Page
1.0	Project Description	1
	1.1 Introduction	1
	1.2 Objectives	2
2.0	Results and/or Benefits	5
3.0	Approach, Background and Rationale	6
	3.1 Approach	6
	3.2 Galveston Bay	7
	3.3 Target Organism Selected	9
	3.4 Chemical Characterization	11
	3.5 Diagnostic *in vitro* Bioassays for Marine Ecological Assessment	12
	3.5.1 Background	12
	3.5.2 CYP1A(1) Induction Assay	14
	3.5.3 Antiestrogenicity Bioassay	16
	3.6 Diagnostic *in vivo* Bioassays for Marine Ecological Risk Assessment	17
	3.6.1 CYP1A1 Induction Assay	17
	3.7 Biliary PAH Metabolites in Fish	18
	3.8 PAH-DNA Adduct Formation	18
	3.9 Epidermal Growth Factor Receptor (EGF-R) Binding and Gene Expression	19
	3.10 Timeframe of Accomplishments	20
	3.11 Program Responsibilities	20
	3.12 Sampling and Methods	21
4.0	General Project Information	21
5.0	Quality Assurance	22
	5.1 Organic Extractions and Analyses	22
	5.2 Biochemical and Molecular Analyses	24
	5.3 Bile	24
6.0	References	24
7.0	Resumes	38

Stephen A. Safe 39
Susanne J. McDonald 41
Terry L. Wade 42
Mahlon C. Kennicutt II 43

Appendix A - Experimental Methods for Biochemical and Molecular Analyses A-1
Appendix B - Standard Operating Procedures (SOPs) for Organic Extractions, Instrument Conditions, and Chemical Analyses B-1

1.0 PROJECT DESCRIPTION

1.1 Introduction

The increasing use of synthetic and naturally occurring chemicals by society has resulted in widespread chronic contamination of many parts of the global marine environment. Diverse structural classes of organic and inorganic chemicals such as the pesticide DDT, its metabolites and the polychlorinated biphenyls (PCBs) have been detected in water, sediments, invertebrates, fish and wildlife species (1-28). Many of the more persistent halogenated pollutants; i.e., DDT, PCBs, polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) are bioconcentrated as they ascend to higher trophic levels (9,10,12,21).

Polynuclear aromatic hydrocarbon (PAHs) mixtures are another major class of organic contaminants in the marine environment. PAHs are typically derived from spills of crude oil and refined petroleum, runoff, and combustion sources (29-40). The PAH are susceptible to biodegradation and their presence in water, sediment, and invertebrate and fish tissues is usually due to relatively recent petroleum spills and/or chronic exposure to non-point sources.

Numerous studies have demonstrated that organic contaminants; such as PCBs and PAHs; are probable etiologic agents in many diseases which can result in adverse health effects in aquatic fish and wildlife populations (41-45). Increased incidences of cancer have been observed in fish populations which are exposed to PAHs (46,50). For example, in a non-migratory teleost fish (mummichog, *Fundulus heteroclitus*) which inhabited an area contaminated with PAH, there was a high incidence of hepatocellular carcinomas (50). Tanabe and coworkers (41-44) have analyzed for and assessed the potential adverse effects of halogenated aromatic hydrocarbons (HAHs) on biota. Their studies conclude that marine species may be highly vulnerable to the toxicity of these compounds. Recent studies by Tillitt and coworkers (45) have correlated the hatching success of double-breasted cormorant eggs with the relative concentrations of the "dioxin-like" PCBs in the eggs. A "dioxin-specific" bioassay of the egg extracts (induction of ethoxyresorufin O-deethylase, EROD activity in rat hepatoma H4IIE cells) was used to assay for PCB's. Historically, hydrocarbon contaminant studies measured their ambient concentrations in water, sediments and tissues

using highly sensitive analytical methods to quantify these chemicals (1-40). However, these mixtures are invariably complex and it is difficult to identify and quantitate individual compounds. Even if the concentration of individual components of these mixtures are obtained the extrapolation of this data in terms of potential adverse environmental effects is not possible. The biological significance of contaminants is difficult to assess due to the vagaries associated with a wide diversity of exposure routes, differences in biological availability, and individual and species differences in reactions to toxicant exposure (Figure 1). Biomarkers and bioassays for environmental contamination are potentially powerful alternatives for regulatory agencies that are attempting to evaluate chemical hazards in the enviroment. Biomarkers are sensitive and can be highly specific indicators that demonstrate that toxicants have the potential to elicit a response at the cellular level.

1.2 Objectives

The primary objective of the proposed study is to evaluate and field validate the efficacy of a series of biomarkers which will assess exposure of marine biota to organic contaminant in Galveston Bay, Texas. This study is designed to provide a measure of the ambient tissue and sediment concentrations of PAHs and HAHs using a combination of analytical (chemical) and bioassay procedures (Figure 2). The *in vitro* bioassay procedures which will be utilized for sediment and tissue extracts are highly sensitive to HAHs and PAHs and will serve to quantitate the bioactive components that may occur in these marine extracts. Several *in vivo* markers of exposure to contaminants will also be determined for Galveston Bay fish.

This project will (1) describe the organic contaminants to which fish from Galveston Bay are potentially exposed, (2) determine whether specific biomarkers of organic exposure have been induced, and (3) determine if exposure has caused specific physiological damage as indicated by a specific biomarker, such as modificiation of EGF-receptor binding and gene expression. Another objective of this study is to compare the relative sensitivities of biomarkers (EROD activity, P450IA mRNA levels, PAH metabolites in bile, and DNA adduct formation) currently used in marine

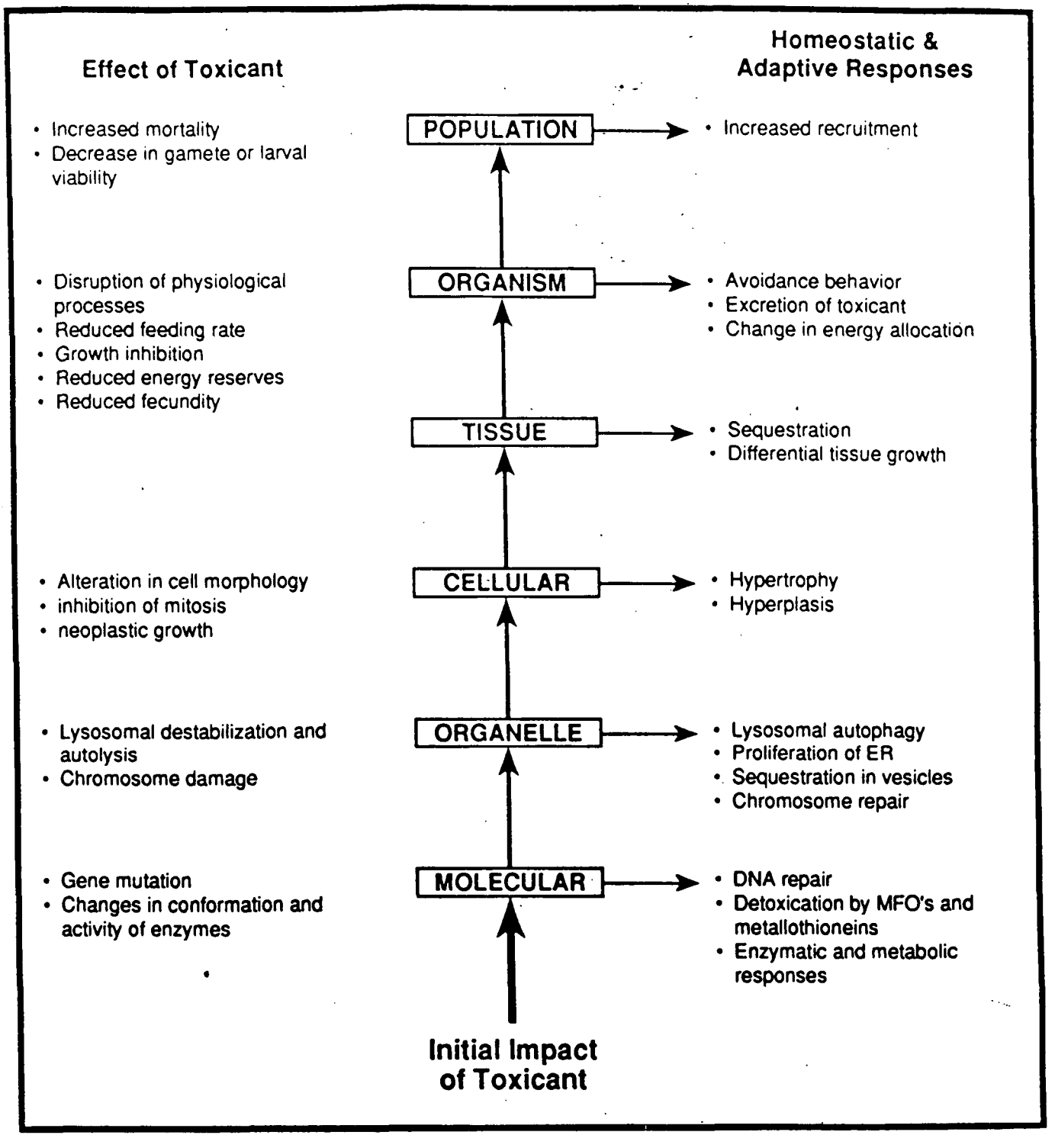


Figure 1. The impact of a pollutant may be dissipated through successive hierarchical organizational levels as a result of compensatory mechanisms. this adaptive process, along with variability intrinsic at all levels, may render such effects undetectable at the higher levels of organization (from Bayne *et al.*, 1985).

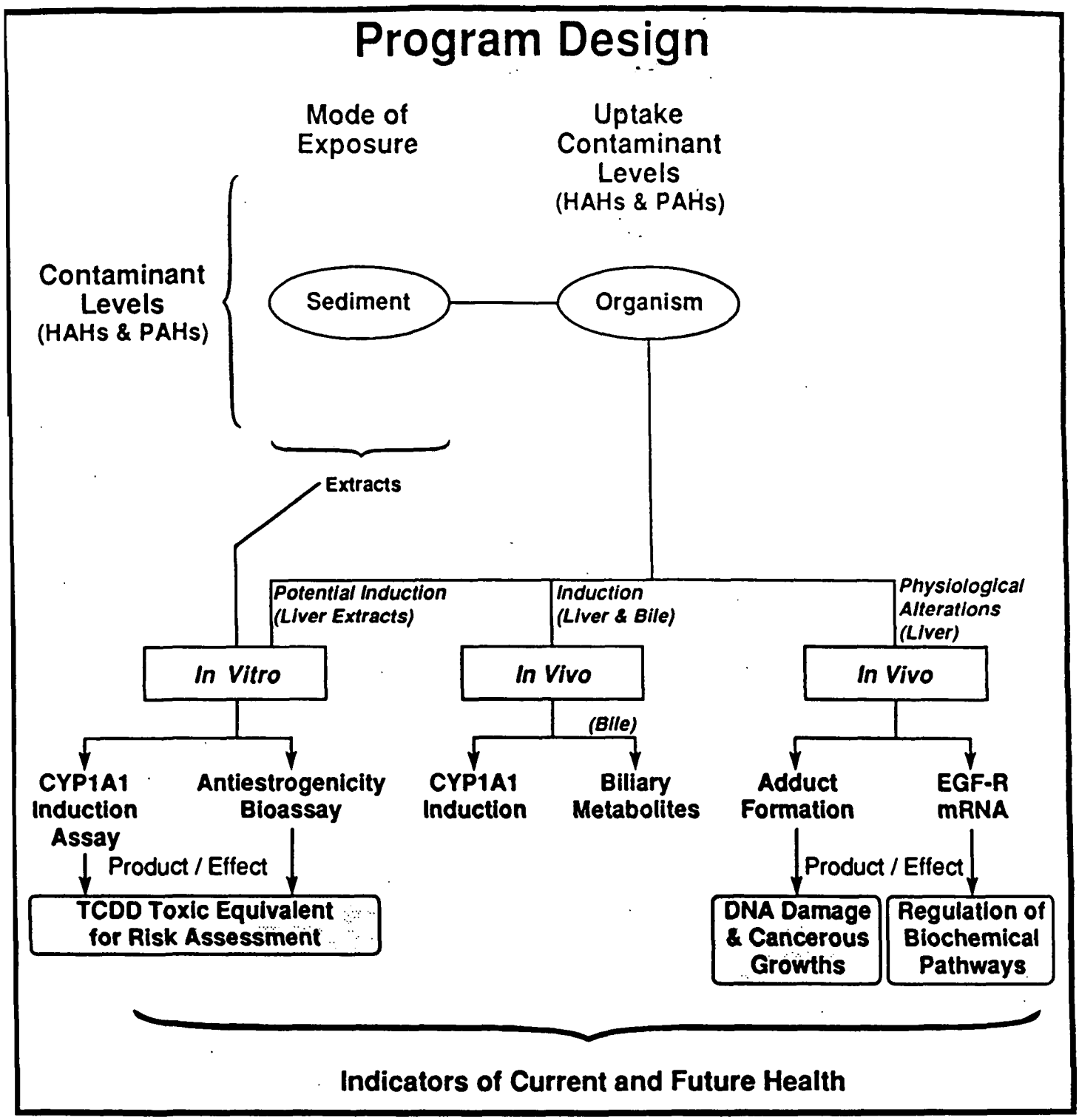


Figure 2. Summary of assays analyses proposed for this study.

studies to evaluate *in situ* environmental contamination and compare them to proposed *in vitro* and *in vivo* assays not currently used in marine studies.

It is proposed to collect samples from four sites within Galveston Bay. The contaminant field will be described by measuring the concentrations of HAHs and PAHs in sediments and fish livers. The biological effects of the organic contaminants on fish in Galveston Bay will be evaluated by using *in vitro* bioassays and *in vivo* markers of exposure and effect. The *in vivo* biomarkers of exposure that will be measured in fish include CYP1A (P450IA) induction as determined by hepatic EROD activity, P4501A mRNA levels and the concentration of PAH metabolites in bile. Other biochemical changes which will be measured in fish include levels of hepatic PAH-DNA adduct formation using the [^{32}P] postlabeling assay (an indicator of PAH-induced damage) and the levels of epidermal growth factor (EGF-R) receptor binding and gene expression. Recent studies have confirmed 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decrease EGF-R binding in both rodents and fish and therefore the assay indicates HAH and PAH induced changes. The relative toxicity of contaminants in tissue and sediment extracts will also be determined using *in vitro* bioassays which are sensitive to PAHs and HAHs. Previous studies in this laboratory have demonstrated that low levels of HAHs and PAHs induced CYP1A1-dependent activity in rat hepatoma H4IIE cells and elicit antiestrogenic activities in human MCF-7 breast cancer cells. The data generated from the *in vitro* bioassays can be utilized to determine TCDD or benzo[a]pyrene (BaP, for PAHs) toxic equivalents of a mixture, which can be calculated by comparing the amount of the mixture required to cause a specific induction response to the amount of TCDD or BaP (the toxic reference standards) required to cause the same response. This approach will, therefore, integrate the activity of all the contaminants in a mixture and provide an estimate of hazard and risk of contaminant levels in these extracts.

2.0 RESULTS AND/OR BENEFITS

The use of biomarkers to assess environmental contamination has become increasingly important to determine the exposure of marine biota to toxic chemicals and evaluate ecosystem health, both current and future. However, biomarkers must be carefully evaluated before their

implementation as routine field measurements. Consequently, the major benefits of this study are to develop and field test *in vitro* bioassays, using fish and sediment extracts, which have been previously shown to be highly sensitive bioindicators for HAHs and PAHs and to determine the levels of organic contaminants in Galveston Bay sediment and fish extracts using the *in vitro* bioassays. Additionally, chemical measurements which are traditionally used to evaluate organic contamination in the marine environment will also be compared to the results obtained from the bioassays.

This approach will generate data that not only determines the level of contaminants, but also determines if these contaminants have/can induce CYP1A(I) levels/activity in both *in vitro* and *in vivo* assays, and the extent of the associated physiological changes or damage. The products of this proposed study are assays/analyses which (1) measure the current health of Galveston Bay, (2) generate calculations of TCDD or BaP toxic equivalents, which translate into a risk assessment that can be used for standard settings of marine contaminant levels, and (3) measure specific physiological alterations in biota which have implications for future health and/or recovery of an ecosystem.

3.0 APPROACH, BACKGROUND AND RATIONALE

31. Approach

A series of biomarkers senstitive to PAHs and HAHs will be evaluated to determine which are the most applicable, sensitive, and offer the greatest potential for determining the health as well as future implications for biota in Galveston Bay. The selection of Galveston Bay is based on several factors. Galveston Bay is a highly industrialized estuary that supports a multi-million dollar commercial and recreational fishery. Although, Galveston Bay is still a relatively productive estuary it receives a wide range of environmental contaminants, including PAHs from the petrochemical industry, petroleum refining activities, agricultural runoff, and municipal waste water that could have serious impacts on the fisheries and health of the Bay.

It is proposed to collect fish and sediments from Galveston Bay at four sites twice a year. HAH and PAH concentraions will be measured for

sediment and fish liver samples to define the environmental level of contamination. The sites which will be selected are those that have been or are being currently sampled in other ongoing studies which are investigating contaminant levels in Galveston Bay (i.e., NOAA's National Status and Trends Mussel Watch Program, EPA's Environmental Monitoring and Assessment Program-Near Coastal, EPA's National Estuary Program, etc.). In addition, the selected sites must contain a range of contaminant levels (from relatively uncontaminanted to highly contaminated based on historical data) and have sufficient numbers of target fish species. Selecting sites with a historical and/or continuing data base will provide a temporal trend of contaminant levels at these sites and also provide additional supporting data for contaminant measurements made for this study. Sampling twice a year will provide information regarding short-term variablity.

Three species of fish will be evaluated for this study. It is anticipated only two of these species will be selected for this study. The species selected will depend upon their availability at the sites within the Bay. Although, we hope to collect at least one species at all sites, that may not be possible throughout the duration of study. Therefore, the selection of two target species should ensure continuity throughout the entire program.

The biomarkers selected for this study are specific indicators of PAH and HAH exposure. *In vitro* bioassays using rodent and human cell lines will be utilized on sediment and fish liver extracts. Traditional and innovative *in vivo* assays will be determined on fish liver and fish bile will be analyzed for PAH metabolite levels. The proposed assays/analyses range from semi-quantitative screening methods to new quantitative methods for assessing hydrocarbon contaminants in the marine environment. This study will integrate and statistically evaluate the data generated from all of these biomarkers. The products of this study will therefore provide results that show which techniques can be best utilized for evaluating the effects of PAH and HAH contamination in a marine environment.

3.2 Galveston Bay

Galveston Bay, with surface area of 1600 km^2, is one of the largest embayments on the U.S. coast. The Bay is very shallow, averaging only about 2 m in depth, and is isolated from the Gulf of Mexico by the Bolivar Peninsula and Galveston Island. Tides, which average about 40 cm in height,

exchange ocean and bay water primarily through the channel between these two land barriers. Winds, rather than tidal cycles, are often the dominant factor determining bay circulation and water exchange. Specific current patterns in the Bay and the residence time of water in the Bay are not well known.

For many years, Galveston Bay has been the recipient of various pollutant inputs because of an agressively growing urban and industrial region. Houston, Deer Park, Bayton, Texas City, and Galveston, surrounding Galveston Bay to the north and west, are some of the most heavily industrialized areas in the United States. Hundreds of industrial plants, including petrochemical complexes and refineries, bordering the Galveston Bay estuarine system contribute significant amounts of pollutants into the Bay. Early studies demonstrated that Galveston Bay has suffered ecological damage related to xenobiotic inputs. Hohn (116) and Chamber and Sparks (117) reported significant decreases in diatom species diversity and number of invertebrates and fish in the upper Houston Ship Channel. A change in species diversity from sciaenids to anchovy was also related to the influx of pollutants into the Bay (118). The conclusions of these studies were that the waters of Galveston Bay contained sublethal concentrations of pollutants which resulted in significant changes in the estuarine community structure. However, these contentions have never been fully assessed.

Recent studies performed by our laboratory provide an extensive high quality database on levels of hydrocarbon contaminants that indicates Galveston Bay provides an excellent setting for the proposed study (119). The concentrations of PAH and pesticides/PCB have been measured in oysters and sediments the U.S. Gulf Coast as part of the National Oceanic and Atmospheric Administration's (NOAA) National Status and Trends "Mussel Watch" Program. This program was designed to monitor the current status and long-term trends of selected environmental organic and trace metal contaminants, e.g., chlorinated pesticides, PCBs and PAHs along the Atlantic, Pacific and Gulf coasts of the U.S.A. Several overviews of the concentrations and distributions of PAHs and chlorinated hydrocarbons have already been reported (120-124).

Higher PAH and pesticide/PCB concentrations were measured in oysters from the upper portion of the Galveston Bay, e.g., Ship Channel and Yacht Club, and near the city of Galveston, e.g., Confederate Reef and Offatts

Bayou. Oyster samples from areas further away from urban centers, e.g., Hanna Reef, had average concentrations one to two orders of magnitude lower. In general, these concentrations are in good agreement with those previously encountered during temporal studies in Galveston Bay (125; Sericano, unpublished data).

Most contaminant concentrations determined for the NS&T Program are normaly distributed on a logrithmic scale (126). From frequenty distribution plots the median concentration for the Gulf Coast can be determined. The concentrations that are plus one sigma or minus one sigma from the mean can then be termed high or low as compared to the mean (unpublished data). This type of distribution for total DDTs is provided in Figure 3. The Galveston Bay NS&T sites are indicated and it is apparent that they cover the range of the distribution. Therefore a sufficient contaminant gradient exists within Galveston Bay for the purpose of this study. In addition, Galveston Bay is representative of all U.S. coastal NS&T sites, which will make the results of this study applicable to this expanded geographic area.

3.3 Target Organism Selected

Fish have been proposed as the target organism for this study because numberous labatory and field studies have shown that levels of the cytochrome P450IA and associated enzyme activities are increased following exposure to HAHs and PAHs. The fish cytochrome P450 system is similar to the mammalian system, although levels/activities are typically lower (60-76). Fish and other laboratory animals express the Ah receptor (51-58) and exposure to various HAHs and PAHs result in the induction of CYP1A(1) gene expression in these species (Table 1). Therefore the induction of the CYP1A1-dependent EROD and AHH activities has been historically used as biomarkers of fish exposure to aromatic hydrocarbons (64). In recent years multiple cytochrome P450 proteins have been identified in several species of fish. One form purified from fish livers has been identified as a P450 induced by HAHs and PAHs and is apparently a functional analogue of rat P450IAI (127-129). Although PAHs are Ah receptor agonists and induce CYP1A, these compounds are also metabolized by the induced P450 to form metabolites which may be highly toxic or carcinogenic (47-49). Some of these highly reactive PAH metabolic intermediates are known to alkylate

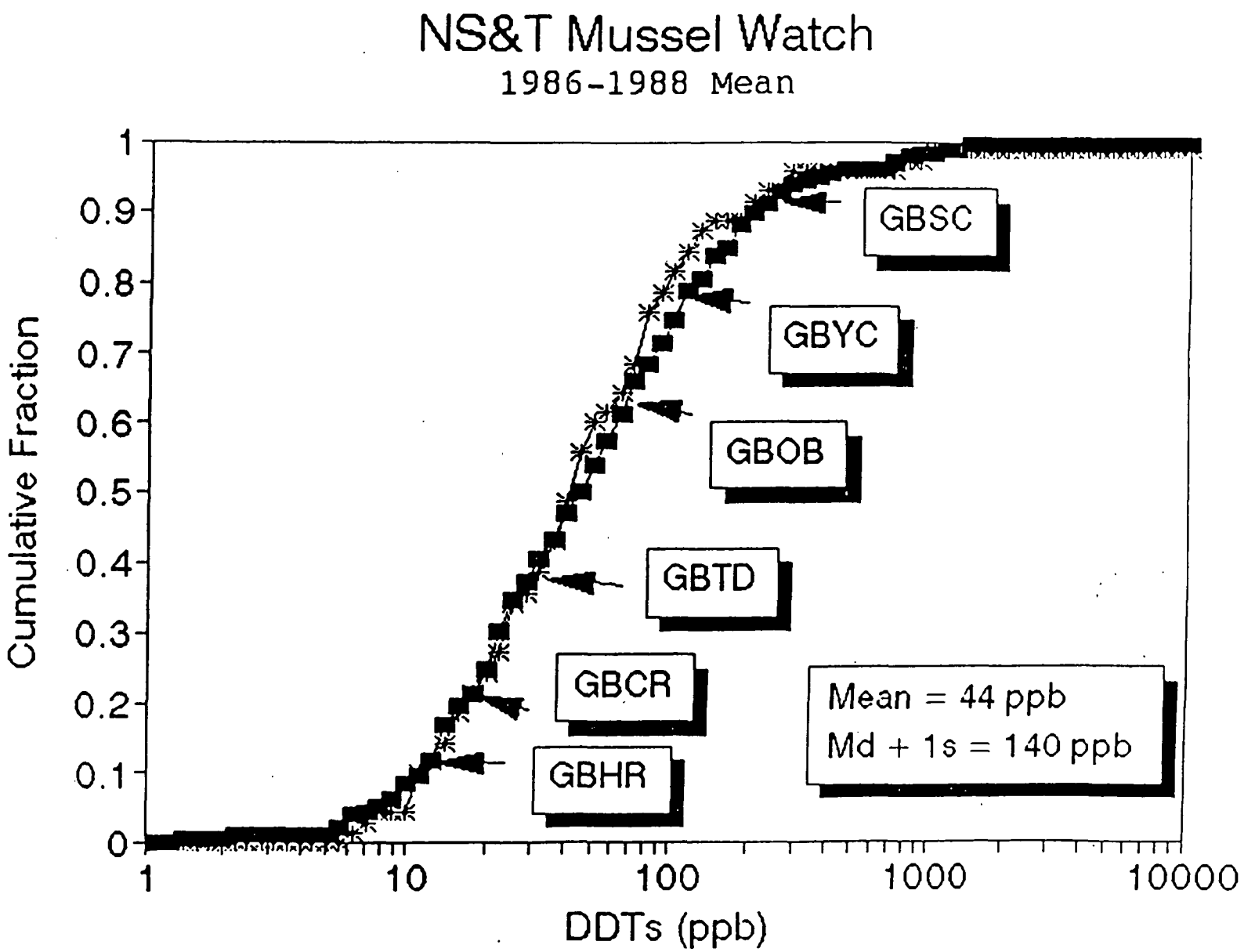


Figure 3. Cumulative frequency distribution of total DDT in bivalves from Galveston Bay and other U.S. coastal sites. (*-Summary of US Coastal NS&T sites; ■- Galveston Bay

cellular macromolecules such as protein, RNA and DNA in fish and these interactions have been associated with hepatotoxicity and the development of hepatic cancer (47-49). For this reason, this proposal will incorporate bioassays which will also assess the metabolism of PAHs and their interaction with cellular DNA in fish.

Table 1. Selected aromatic and chlorinated hydrocarbons tested as inducers of microsomal monooxygenase activity in fish.

Active inducers	Inactive as inducers
Benzo[a]pyrene	DDT
Dibenzanthracene	2,2',4,4'-Tetrachlorobiphenyl
Methylcholanthrene	2,2,4,4',5,5'-Hexachlorobiphenyl
3,3',4,4'-Tetrachlorobiphenyl	
2,3,7,8-Tetrachlorodibenzodioxin	
b-Naphthoflavone	

(from 64)

Three species of fish will be examined to determine which is (are) most appropriate for this study. The species proposed are *Micropogonias undulatus* (Atlantic croaker), *Arius felis* (sea catfish), and *Paralichthys lethostigma* (southern flounder) because they are commonly found in Galveston Bay during the warmer months and are associated with or feed on the bottom. Additionally, the croaker and flounder are important catches in commercial and sport fisheries. The Atlantic croaker has been sampled in Galveston Bay since 1984 for contaminant studies for the National Benthic Surveillance Project of NOAA's National Status and Trends Program. Additionally, the sea catfish has been sampled by local regulatory agencies as part of their monitoring studies. It is proposed to use two fish species in this study. However, which species or combination of species used in this study will depend upon availability at the proposed sites. To avoid spawning activities the best sampling times will most likely be during April-May and September-October.

3.4 Chemical Characteriziation

Three objectives will be addressed by the chemical characterization portion of this study. First contaminant concentrations within fish tissues will be determined to document the level of uptake. In particular fish livers will be analyzed. Secondly the level of contaminants in the environment will be documented by measuring analytes in the sediments. Third, extracts of sediments and fish will be used to estimate potential toxicological

contaminants contained in them. These data will provide a framework from which to describe potential exposure, expressed in body burden of chemicals, and potential inducible biological effects. In conjuction with the *in vivo* toxicology measurements a more complete understanding of exposure will be possible.

As a first step in a contaminant study design, ambient concentrations of specific analytes are measured. While the ambient concentrations cannot easily be directly extrapolated to a biological effect it does at least define what contaminants are present and at what levels. The expected or predicted impact on the associated flora and fauna is difficult to assess. The presence of contaminants does not infer effect or impact and various mitigating factors such as bioavailability and metabolism factors may in fact ultimately control exposure. However contaminant concentrations are essential to provide a freamework within which the observed ecological, physiological, and biochemical varitions can be interpreted.

The analytes of interest will include PAHs and HAHs, including AHH active congeners. The methods will be identical to those of EPA EMAP-NC and NOAA Status and Trends Program. Standard operating proceedures are provided in Appendix B. The quality assurance for chemical contaminant analyses will be equivalent to the EPA EMAP/NC Program.

3.5 Diagnostic *in vitro* Bioassays for Marine Ecological Assessment

3.5.1 Background

The most toxic member of the HAH class of environmental contaminants is TCDD which is formed as an industrial by-product and has been identified in aquatic ecosystems (11, 14-28). Several studies have reported that 2,3,7,8-TCDD and related toxic halogenated aromatics elicit a number of common toxic responses which include body weight loss, thymic atrophy, impairment of immune responses, hepatotoxicity and porphyria, chloracne and related dermal lesions, tissue-specific hypo- and hyperplastic responses, carcinogenesis, teratogenicity and reproductive toxicity (51-56).

One of the hallmarks of exposure to HAHs and PAHs is their induction of both phase I and phase II drug-metabolizing enzymes in laboratory animals and mammalian cell cultures (34,35, 48-56, 59-76). 2,3,7,8-TCDD and related toxic halogenated aromatics induce the cytochrome P4501A1

(CYP1A1) and cytochrome P4501A2 (CYP1A2) hemoproteins and their associated microsomal monooxygenases, which include aryl hydrocarbon hydroxylase (AHH) and EROD activities. These latter enzyme activities have been extensively used in fish as biomarkers of exposure to the TCDD-like HAHs and PAHs. In addition, several PCB congeners induce other forms of cytochrome P450 (in the rat) and these include cytochromes P4502B1 (cytochrome P450b), P4502B2 (cytochrome P450e), P4502A1 (cytochrome P450a) and P4503A1 (cytochrome P450p) and their associated monooxygenase enzyme activities. However, these enzyme are not inducible in fish and cannot be utilized as biomarkers of exposure to these compounds. Toxic aromatic and halogenated hydrocarbons also induce the phase II drug-metabolizing enzymes, glucuronosyl transferases, and glutathione S-transferases. HAHs also induce many other biochemical responses and these include the induction of diverse enzymes, the modulation of hormone receptor binding activities, the alteration of thyroid hormone and vitamin A levels, and enzymes which metabolize steroid hormones. Many of these induced responses are potentially useful as biomarkers of fish exposure to PAH and HAH; however, only minimal characterization of HAH-induced hormone/hormone receptor responses has been reported in fish and marine biota.

The mechanism of action of HAHs and PAHs have been extensively investigated (51-57, 59) and the results of genetic and molecular biology studies are consistent with the role of the aryl hydrocarbon (Ah) receptor as the initial cellular target for the HAHs (Figure 4). The Ah receptor has been identified in diverse species/organs (57) including seven species of teleost and elasmobranch fish (scup, winter flounder, killifish, rainbow trout, brown trout and dogfish) (58). The molecular biology of Ah receptor-mediated effects have primarily been derived from studies on the induction of CYP1A1 gene expression by TCDD and 3-methylcholanthrene (a PAH) (51). Initial binding of the toxin to the cytosolic Ah receptor is followed by an activation or transformation step and the subsequent accumulation of occupied nuclear receptor complexes. These nuclear complexes then interact with specific DNA sequences or dioxin regulatory elements (DREs) which are located in the 5'-upstream region from the CYP1A1 gene. These interactions lead to the enhancement of CYP1A1 gene expression. It is assumed that many of the toxic effects elicited by halogenated and aromatic aryl hydrocarbons are

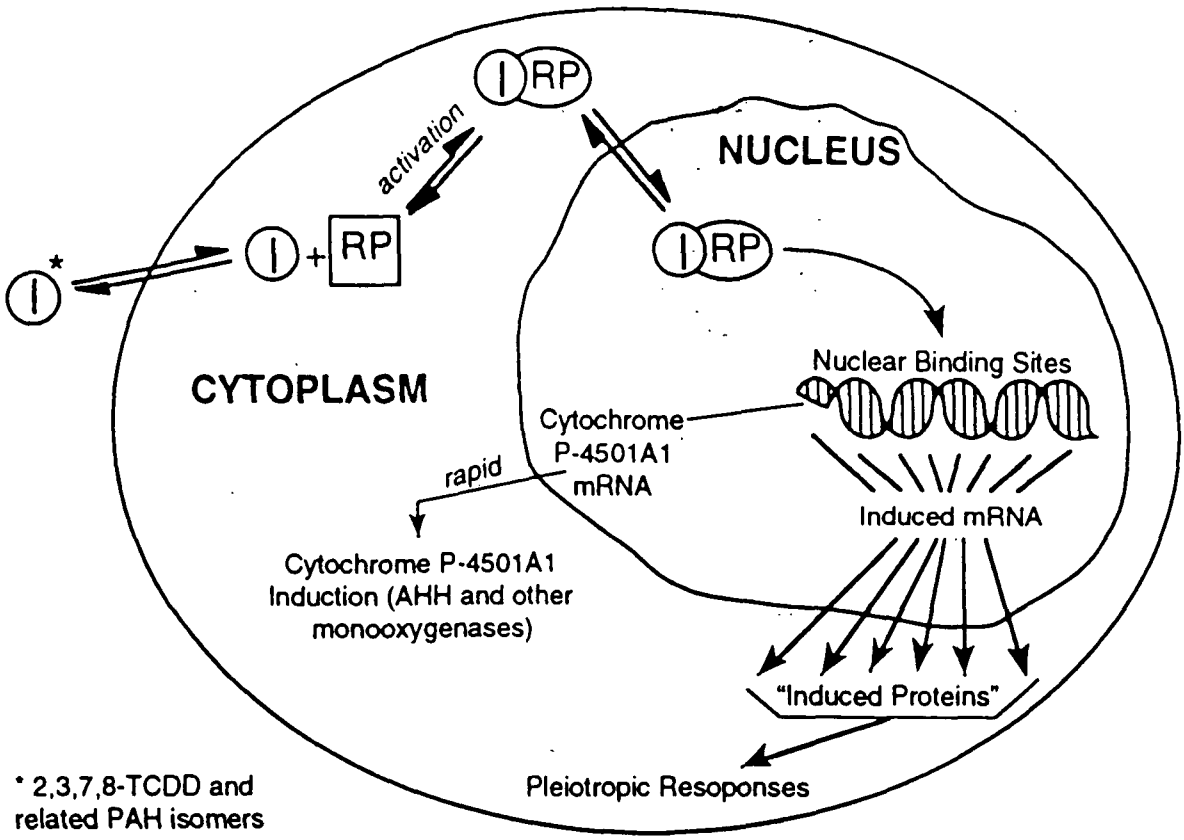


Figure 4. Proposed model for the mechanism of action of 2,3,7,8-TCDD and related toxic halogenated aromatic hydrocarbons. The initial formation of a cytosolic receptor complex is followed by an activation step, the formation of nuclear receptor complexes, and their interaction with specific nuclear-binding sites. This interaction contributes to an increase or induction of a 2,3,7,8-TCDD-inducible gene such as CYP1A1.

also the result of altered receptor-mediated gene expression, however, the molecular mechanisms of these responses are currently unknown. One of the hallmarks of the toxicity of HAHs are their remarkable structure-activity relationships which is determined, in part, by their parallel structure-dependent affinities for the Ah receptor. This induced activity has been adapted for determining the *in vivo* exposure of aquatic and marine biota to HAHs and PAHs and will be extensively utilized in this proposal.

3.5.2 CYP1A(1)Induction Assay

Current analytical techniques to measure PAHs and HAHs are costly and require sophisticated equipment and trained personnel. Therefore, the

development of bioassays for these compounds would be advantageous since the bioassay-derived toxicity is an integrated assessment of the potential biological effects of a complex mixture. Several groups have developed mechanism-based bioassays for HAHs and PAHs (77-81). Bradlaw and coworkers (82,83) first reported that HAHs readily induced AHH activity in rat hepatoma H4IIE cells in culture demonstrated the utility of this assay system for quantifying toxic halogenated aromatics in diverse matrices, including fish tissue extracts, PCB/PCDF-contaminated rice oil, diverse food extracts (including gelatin samples containing pentachlorophenol) and trace levels of higher chlorinated PCDDs. Research in our laboratory has focused on validating the utility of the P450 induction bioassay for predicting the toxicity of individual PAH and HAH congeners and HAH mixtures derived from diverse sources including fish tissue extracts (59, 85-94) (59,77,84-92). From these results, the TCDD or toxic equivalents of a mixture can be calculated by comparing the amount of the mixture required to cause a specific induction response to the amount of TCDD (the toxic reference standard) required to cause the same response (*e.g.* EC_{50} - half-maximal induction). Based on the dilutions of the extract, the toxic 2,3,7,8-tetrachlorobenzo-p-dioxin (TCDD or dioxin) equivalents (TEQs) or benzo[a]pyrene (BaP) for this extract can be calculated (Figure 5). This bioassay has been validated by comparing the "bioassay-derived" TEQs for a mixture to the *in vivo* toxicity of the same mixture and there was a good correlation between the results of both assays (92). Moreover, recent laboratory studies have demonstrated that *in vitro* the AHH or EROD induction bioassay can be used for extracts of aquatic samples (45, 95-97) (45,93-95) and the results have been utilized to implicate HAHs as etiologic agents in wildlife toxicity (45).

This project proposes to utilize the rat hepatoma H4IIE cells as a bioassay for HAHs and PAHs and the responses which will be measured includes a CYP1A1-dependent enzyme activity (EROD). This bioassay is one of the most sensitive and widely utilized procedures for determining TEQs and will be used as a quantitative bioassay of extracts from field samples.

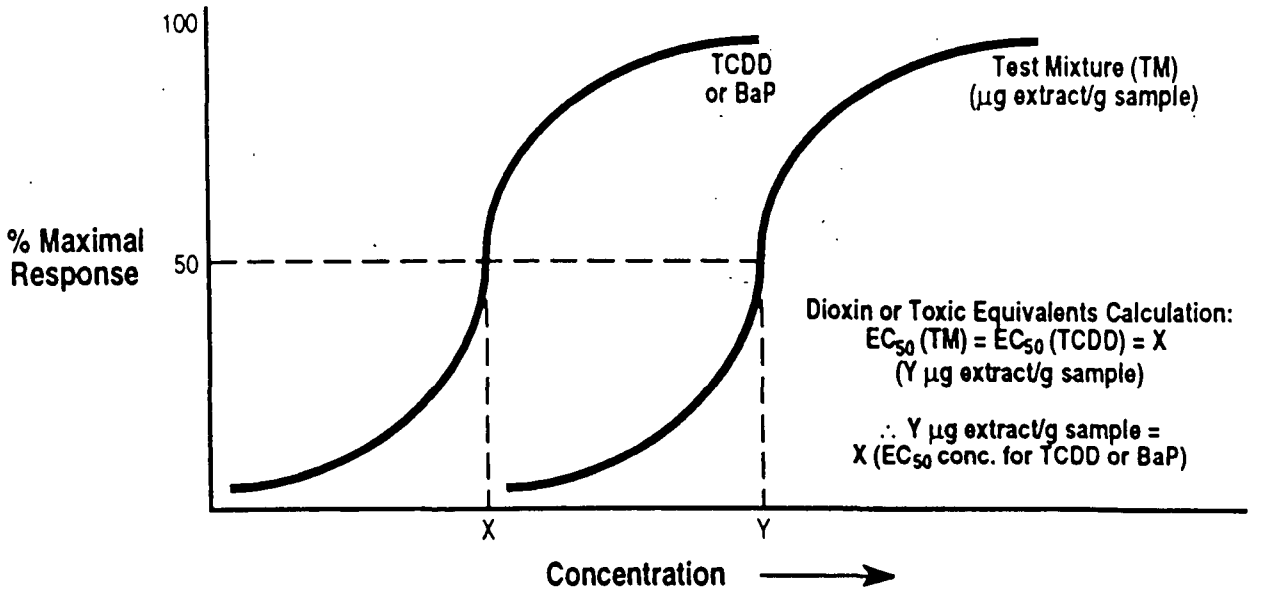


Figure 5. Calculation of TEQs from *in vitro* bioassays.

3.5.3 Antiestrogenicity Bioassay

Recent studies have demonstrated that HAHs and PAHs exhibit antiestrogenic activity in the female rat and in human breast cancer cell lines (98,99). In MCF-7 human breast cancer cells, TCDD and related HAHs inhibit several estrogen-induced responses including cell proliferation and the secretion of the 52-, 34- and 160-kDa proteins (98-102) and the results are consistent with an Ah receptor-mediated response. These same compounds also induce CYP1A1 gene expression; however, this induction response in MCF-7 cells is less sensitive than that observed in rat hepatoma H4IIE cells. Recent studies in this laboratory have shown that an electrophoresis assay coupled with double-staining of the resulting gels with an ISS ProBlue and silver staining solution can be used for determining the estrogen-induced secretion of the 52-kDa protein, and the antiestrogenic effects of TCDD and related HAHs of 2,3,7,8-TCDD (101). The results of these studies (see Table 2) show that for several classes of HAHs their antiestrogenic potencies were similar to those observed for other Ah receptor-mediated responses (102), including the induction of CYP1A1. The sensitivity of the antiestrogenicity assay was comparable to the CYP1A1 induction bioassay. The results of recent studies showed that for PAHs (103), the antiestrogenicity assay was more sensitive to these compounds

than CYP1A1 induction. For this reason, the antiestrogenicity bioassay will also be evaluated for sensitivity and specifically for contaminants extracted from marine samples.

Table 2. EC_{50} values for selected PCB, PCDD, and PCDF congeners as inhibitors of 17β-estradiol-induced secretion of the 52-kDa protein from MCF-7 cells[a].

Congener	Approximate EC_{50} value (nM)	Relative Potency Antiestrogenicity	Relative Potency Other Assays
2,3,7,8-TCDD	8.6	1.0	1.0
2,3,4,7,8-PeCDF	63	0.14	0.8-0.11
2,3,7,8-TCDF	31	0.28	0.43-0.006
1,2,3,7,8-PeCDD	69	0.12	0.64-0.007
1,2,3,7,9-PeCDF	680	0.02	---
1,3,6,8-TDDF	1900	0.004	---
3,3',4,4'-TCBP	170	0.05	0.009-0.0008
3,3',4,4',5-PCBP	30	0.29	0.3-0.0006
3,3',4,4',5,5'-HCBP	180	0.05	0.1-0.0012
2,3,3',4,4',5'-HCBP	>1000	<0.009	<0-001
2,3,3',4,4'-PCBP	>1000	<0.009	<0.001
2,3,4,4',5-PCBP	>1000	<0.009	<0.001

[a]The values for the relative potencies of these congeners compared to TCDD as Ah receptor agonists were recently reviewed (Safe, 1990)

3.6 Diagnostic *In Vivo* Bioassays for Marine Ecological Risk Assessment

3.6.1 CYP1A1 Induction Assay

The uptake and contamination of PAHs and HAHs by fish living in marine or aquatic environments has been extensively investigated utilizing the induction of hepatic CYP1A(1) in the exposed animals (50, 60-76). Numerous studies have correlated the magnitude of the induction response in fish with the levels of PAH/HAH contamination and, thus, the induction of CYP1A in fish is a quantitative *in vivo* bioassay which integrates the effects of all "TCDD-like" contaminants in these species. Both the *in vivo* and *in vitro* induction bioassays provide induction responses which give the total TEQs associated with a contaminated species or extract. This integrated value represents a quantitative assessment of levels or exposure to HAHs and PAHs. Thus, the induction of EROD activity and CYP1A mRNA levels will be utilized in this study as a measure of *in vivo* exposure of fish to pollutants derived from estuarine environments. Measuring both catalytic enzyme activity and mRNA levels can be advantageous. It has been documented that some inducers also inhibit the catalytic activity of P450 induced. Thus

measuring only catalytic activity may not represent the total induction response whereas induced mRNA levels will be a more consistent indicator of CYP1A induction.

3.7 Biliary PAH Metabolites in Fish

Statham et al., (130) first proposed assessing hydrocarbon pollution by analyzing the bile of fish for hydrocarbon metabolites. A number of PAH metabolites formed in the livers of fish are excreted into bile. The gall bladder is the storage site of PAH metabolites and represents an important pathway for excretion (131). A nonradiometric technique employing high performance liquid chromatography (HPLC/fluorescene detection) was developed to estimate biliary PAH metabolites in fish (132,133).

Studies have shown that fish captured in areas with contaminated sediments have higher biliary concentrations of PAH metabolites than fish from less contaminated areas (134-136). Our studies on several species of fish from Galveston Bay, Texas showed that the concentrations of PAH metabolites in bile was variable between sites and was related to the degree of hydrocarbon contamination (unpublished data). Studies have also shown that fish exposed to spilled oil exhibit elevated concentrations of PAH metabolites in their bile (137-139). Additionally studies have correlated high biliary concentrations of benzo[a]pyrene equivalent metabolites in fish with idiopathic liver lesions (132,133), reduced ovarian maturation (140) and elevated AHH activity (141). Bile metabolites will be analyzed to demonstrate uptake and induced biochemical reaction to exposure.

This method has been useful as a semi-quantitative indicator of PAH exposure and complements the more quantitative P4501A1 bioassay and compound specific PAH-adduct assay which will be used in this study.

3.8 PAH-DNA Adduct Formation

Although PAHs can function as Ah receptor agonists and induce CYP1A(1) gene expression in fish, these compounds also elicit both toxic and carcinogenic responses via Ah receptor-independent pathways. The metabolism of benzo[a]pyrene and other PAHs to both non-toxic and highly reactive carcinogenic intermediates has been extensively investigated in diverse species including fish (34,35, 46-49, 102-104). The conversion of benzo[a]pyrene into the 7,8-epoxy and 7,8-dihydrodiol metabolites followed

by P450-dependent oxidation into the 7,8-diol-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene results in the formation of a highly reactive alkylating agent which forms covalent DNA adducts which are believed to be markers of PAH-initiated cancer in target tissue.

The metabolic activation of PAHs into carcinogenic metabolites can be monitored in exposed fish by measuring DNA adduct formation (102-104) using the [^{32}P]-postlabeling procedure described by Randerath, Gupta and coworkers (105,106). This bioassay quantitates all PAH adducts and thus serves to integrate overall exposure of the fish to those PAHs which are activated and form DNA adducts with the more carcinogenic PAHs. Thus, this assay will be evaluated in field collected fish as an *in vivo* biomarker of exposure to PAHs and can also serve as an indicator of DNA damage and risk assessor of future development of cancerous lesions.

3.9 Epidermal Growth Factor Receptor (EGF-R) Binding and Gene Expression

Recent studies in this laboratory demonstrated that relatively low doses of TCDD (≤0.016 μmol/kg) inhibited constitutive and estrogen-induced EGF-R binding in uterine and hepatic membranes and EGF-R mRNA levels in the uterus and liver of female rats (107). In laboratory studies, Newsted and Giesy also reported that TCDD caused a dose-response decrease in EGF-R binding in hepatic plasma membrane in rainbow trout and structure-activity studies suggested that this effect was Ah receptor mediated (108). Moreover, it was demonstrated that the TCDD-induced decrease in EGF-R binding was nearly two-orders of magnitude more sensitive than the CYP1A1 induction response in rainbow trout. This proposed study will, therefore, evaluate the EGF-R binding and EGF-R mRNA levels as *in vivo* bioassays for determining the impact of PAHs and HAHs on fish. The results will be compared with the P4501A1 induction data from the same fish samples and serve to validate the utility of the EGF-R binding and EGF-R mRNA levels in fish as bioindicators of estuarine pollution by HAHs and PAHs. Since the EGF-R is involved in the regulation of diverse biochemical pathways, the validation of this response in marine fish will provide a physiologically-relevant bioindicator of exposure to contaminants which may also play a role in contaminant-mediated adverse effects in these species.

3.10 Timeframe of Accomplishments

Task	Year I		Year II		Responsibility
	0-6 Months	6-12 Months	0-6 Months	6-12 Months	
Establish Study Sites	X				Safe/McDonald/ Wade/Kennicutt
Select Target Sites	X				Safe/McDonald/ Wade/Kennicutt
Standardize *in vitro* assays for sediments/fish	X				Safe
Standardize *in vivo* assays for fish livers	X				Safe/McDonald
Sample study sites	X	X	X	X	McDonald
Extract sediment and fish samples	X	X	X	X	Wade
Analyze sediment and fish livers for PAHs/HAHs	X	X	X	X	Wade/Kennicutt
Conduct *in vitro* assays	X	X	X	X	Safe
Analyze bile for PAH metabolites	X	X	X	X	McDonald
Conduct *in vivo* assays for fish livers	X	X	X	X	Safe/McDonald
Integrate and evaluate data				X	Safe/McDonald/ Wade/Kennicutt

3.11 Program Responsibilities

Dr. Stephen Safe will have overall management, supervisory and data synthesis responsibilities for the project. Dr. Safe will also be responsible for the development and implementation of *in vitro* bioassays and the following *in vivo* assays using fish livers, CYPIA mRNA levels, DNA adduct formation and EGF-R binding and gene expression. Dr. Safe will be assisted by two graduate students. Dr. Susanne McDonald will be reponsible for the field sampling, biliary metabolite analyses, *in vivo* EROD activities in hepatic fish samples as well as assist Dr. Safe. Dr. McDonald will be assited by a half-time technician. Dr. Terry Wade will be responsible for the extraction of all

samples for contaminant concentrations as well as *in vitro* bioassays and the analysis and interpretation of HAH analytical data. Dr. Wade will be assisted by a half-time technician. Dr. Mahlon C. Kennicutt II will be responsible for the analysis and interpretation of PAH analytical data.

All field and laboratory equipment and/or instrumentation are currently available at Texas A&M University for completion of this project.

3.12 Sampling and Methods

Sediment, water, and fish samples from Galveston Bay will be collected twice a year during April-May and September-October. Fish will be collected by trawling, gill nets, or hook and line. Fish will be processed immediately upon capture. Livers will be excised and subaliquoted. Liver aliquots to be used for *in vivo* assays will be frozen and stored in liquid nitrogen until analysis. Liver samples used to measure contaminant levels and in *in vitro* bioassays will be stored on ice in the field and upon arrival at the laboratory frozen and stored at -20°C until analysis. Bile will be collected using vacutainers, stored on ice in the field and upon arrival in the laboratory stored at -20°C until analysis.

Sediments will be collected using a boxcore. The top 2cm of the boxcore will be sampled for contaminant analysis. Samples will be stored on ice in the field and frozen at -20°C until analysis in the laboratory.

Details of analytical methods are in Appendices A and B.

4.0 GENERAL PROJECT INFORMATION

The amounts and composition of HAHs and PAHs wil be measured in sediments and the livers of two species of fish collected at four sites in Galveston Bay to assess the levels of these classes of contaminants. These samples will be extracted using EPA approved methods (see Appendix B). PAHs will be identified and quantified using gas chromatography/mass spectrometry. HAHs will be identified and quantified using gas chromatography/ECD detection. Duplicate extractions, excluding surrogates and internal standards, will be conducted on all sediment and fish liver samples for use in the *in vitro* bioassays.

Fish livers will be further analyzed to assess CYPIA induction. EROD activity is a measure of the catalytic rate of a CYPIA-associated

monooxygenase and determining the levels of CYPIA mRNA evaluates the induction of CYP1A gene transcription. Measuring the levels of DNA adducts is an *in situ* biomarker of environmental genotoxicity since the DNA damage can be expressed in mutations and disease. Additionally, levels of DNA adducts in ferrel fish is proported to be representative of cumulative exposure. The levels of EGF-R binding and gene expression are also *in situ* measurements of PAH/HAH contaminant exposure in which physiologically important growth factor-mediated signal transduction pathway is modulated. Based on the results of preliminary studies in rainbow trout (108) this assay should prove to be a highly sensitive indicator of (fish) estuarine exposure to toxic pollutants. As such this technique is a measure of both exposure and effect following exposure to genotoxic pollutants.

The levels of compounds fluorescing at naphthalene, phenanthrene and BaP excititation/emission wavelengths will be estimated using HPLC and fluorescence detection. The types of PAHs identified in bile can be useful in describing the type(s) of PAH to which fish are exposed. Anthropogenic-derived PAHs typically contain high concentrations of BaP, which can be a bi-product of combustion.

All samples will be collected in triplicate and all asssays/analyses will be performed in triplicate. Triplicate analyses will facilitate the use of statistical methods to evaluate the products of this project. This project will not use human subjects or research animals.

5.0 QUALITY ASSURANCE

5.1 Organic Extractions and Analyses

The quality assurance for this study is two-fold. First the availability of environmental test cases is especially helpful to attaining project goals. Only test cases with EPA approved quality assurance work plans or programs of comparable high quality will be chosen. QA routinely includes replication, system blanks, standard reference materials, matrix spikes, calibration checks, surrogates, and internal standards (see Appendix B-QAMP, QAPP, SOPs).

The PI's are highly experienced in trace contaminant analysis and are fully aware of the critical need for rigorous quality control to ensure program results and success. The principal investigators in this proposal

have had senior positions in a number of major environmental studies conducted for NOAA, the Minerals Management Service (MMS), U.S. Fish and Wildlife Service (USFWS), the Environmental Protection Agency (EPA), the Office of Naval Research (ONR), the National Science Foundation (NSF) and the Department of Energy (DOE). For example, GERG currently conducts the following high quality environmental programs that involve field sampling, design and analyses:

- **NOAA's National Status and Trends Mussel Watch Project for the Gulf of Mexico.** We have for the last 7 years conducted the Gulf of Mexico portion of this program. We have a long-standing record of intercalibration using the NIST/NOAA methods for a wide array of trace metal and trace organic analytes.

- **EPA's Environmental Monitoring and Assessment Program - Near Coastal (EMAP-NC) in the Gulf of Mexico and Virginian Province.** We are conducting the sediment analyses for the EMAP-NC program in the Gulf of Mexico (Louisianan Province) and tissue analyses for the Virginian Province (EPA's Narragansett Office).

- ***Exxon Valdez* Damage Assessment Studies (NOAA, USFWS and the State of Alaska).** We are conducting most of the analytical hydrocarbon analyses for the *Exxon Valdez* Trustee's as part of the oil spill damage assessment effort. We have also provided Exxon's contractors with hydrocarbon analyses as part of Exxon's Oil Spill Litigation studies.

- **U.S. FWS Contract Laboratory.** GERG is one of the U.S. Fish and Wildlife Service's two and four contract laboratories for trace organic and trace metal contaminant analyses, respectively.

- **EPA's National Estuary Programs (Galveston, Boston and Casco Bays).** GERG has conducted several of EPA's National Estuary Program analytical and sampling efforts in Galveston Bay, Boston Harbor and Casco Bay.

- **NSF's *Bahia Paraiso* Antarctic Oil Spill Project.** GERG was chosen by the National Science Foundation to provide the hydrocarbon component of the *Bahia Paraiso* oil spill rapid response in Antarctic in 1989.

- **IOC's International Mussel Watch.** GERG has been selected by the IOC to participate in International Mussel Watch as the only U.S. laboratory.

- **Cooperative Environmental Research Consortium for the Wider Caribbean.** GERG has been selected by EPA to participate in this Puerto Rico based Institute to work with government and industry in the wider Caribbean region on environmental issues and studies.

All contaminant analyses will be made by methods equivalent to those used in the above programs. QA will be equivalent to these programs.

5.2 Biochemical and Molecular Analyses

The proposed *in vivo* and *in vitro* bioassays are all currently ongoing in this laboratory. For the induction of EROD activity, CYP1A1 mRNA levels, EGF-R binding and gene expression and PAH adduct formation, the assays will be standardized using fish which have been treated witeither TCDD or BaP at a maximal or near maximal dose. The appropriate subcellular fractions are frozen and stored at -80°C and will be used in each assay as standards to ensure that assay procedures vary by less than 10%. The *in vitro* induction and antiestrogenicity assays will also be standardized for each run by determining dose response curves for TCDD and BaP.

5.3 Bile

Blanks, calibration standards, reference bile, and duplicates are analyzed with each run. Details of quality assurance are provided in the Standard Operating Procedure (SOP-9009) for the HPLC analysis of bile in Appendix B.

A PROPOSAL

Submitted to:

The United States Environmental Protection Agency

by

The College of William and Mary
School of Marine Science
Virginia Institute of Marine Science
Gloucester Point, VA 23062

VALIDATION OF THE MUMMICHOG, *FUNDULUS HETEROCLITUS* AS A HISTOPATHOLOGICAL INDICATOR OF POLLUTION ALONG THE EASTERN UNITED STATES

Project duration: 2 Years

Wolfgang K. Vogelbein, PI
Assistant Research Scientist
(804)642-7261

Craig L. Smith, Co-PI
Associate Research Scientist

Michael A. Unger, Co-PI
Assistant Research Scientist

David E. Zwerner, Co-PI
Marine Scientist

Robert J. Huggett, Head,
Dept. of Environmental
Science

Robert J. Byrne, Assoc.
Director of Research

Jane A. Lopez, Manager,
Sponsored Programs

INTRODUCTION

Laboratory and field studies indicate that exposure to toxic chemical agents can cause the development of characteristic pathologic disorders in aquatic organisms. In fishes, lesions can develop in almost every organ system, however, the liver appears to be the most common target organ. An important function of the vertebrate liver is the metabolic transformation of endogenous and exogenous chemicals, often resulting in the formation of reactive and potentially cytotoxic, mutagenic, or carcinogenic metabolites. The liver is therefore a prime target for the damaging effects of many xenobiotic chemical contaminants. For example, a spectrum of liver lesions including cancer develops in both laboratory rodents and fishes following exposure to potent hepatotoxic and hepatocarcinogenic agents (eg. Frith and Ward, 1980; Maronpot et al., 1986; Hawkins et al., 1985; Hendricks et al., 1985; Couch & Courtney, 1987). Similarly, fishes from waterways adjacent to urban areas exhibit a spectrum of pathological conditions, including liver neoplasms, associated with exposure to xenobiotic chemical contaminants (eg. Smith et al., 1979; Murchelano & Wolke, 1985, 1991; Malins et al., 1988; Vogelbein et al., 1990; Myers et al., 1993).

The chemical compounds most frequently implicated as causative agents of liver neoplasia in feral fishes are the polycyclic aromatic hydrocarbons (PAH). The U.S. EPA has identified 16 unsubstituted PAH as priority pollutants. Eight of these chemicals, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3,-cd]pyrene, dibenz[a,h]anthracene, and benzo[ghi]perylene, exhibit possible or probable carcinogenic activity in rodents (Menzie et al., 1992). Although significant concentrations of these eight compounds are often present in polluted aquatic environments, only benzo[a]pyrene has been examined for its carcinogenic activity in fishes (Hendricks et al., 1985; Hawkins et al., 1989). Further evidence of PAH carcinogenesis in fishes is provided by field studies (Malins et al., 1984, 1985a, 1985b; Baumann et al., 1989; Vogelbein et al., 1990). English sole, Parophrys vetulus from urban areas of Puget Sound exhibit a spectrum of liver lesions associated with 1) high PAH concentrations in surficial sediments and stomach contents of fish (Malins et al., 1984; Myers et al., 1987; 1991; 1993), 2) induction of

cytochrome P450 mediated xenobiotic metabolizing enzymes in liver (Collier et al., 1992), and 3) elevation of PAH metabolites in the bile (Krahn et al., 1984). Identification of bile metabolites in this species indicates exposure to aromatic hydrocarbons and verifies that these compounds are bioavailable. Myers et al. (1993) suggest that because of their mobility, benthic fishes are more reflective of exposure to chemical contaminants over a broad geographic range than are measurements of contaminant concentrations in sediments or in the more sedentary organisms. Thus, benthic fishes such as the English sole can act as "integrators" of contaminants from a variety of sources within specific geographic regions, and are therefore useful as indicators of environmental contamination. Nevertheless, a need exists for the development of new indicator species, especially ones having a broad geographic distribution and a non-migratory life style. These species would reflect environmental conditions on a local rather than regional level and could be very useful in evaluating the adverse biological effects, for example, in waters adjacent to specific point source discharges.

Because the effects of exposure to toxic chemical agents are often expressed as characteristic tissue lesions, histopathologic methods are gaining acceptance in pollution monitoring programs. The U.S. Environmental Protection Agency (EPA) has recognized the value of histopathological endpoints as indicators of pollution (U.S. EPA, 1986) and has selected fish liver histopathology as one of several indicators of biological impacts for selected marine dischargers holding 301(h)-modified NPDES permits (Becker and Grieb, 1987). Both the EPA's Environmental Management and Assessment Program (EMAP) (Fournie, pers. commun.) and NOAA's National Benthic Surveillance Project (Varanasi et al., 1989; McCain et al., 1989; Myers et al., 1993) currently use fish liver histopathology in their assessments of environmental pollution in coastal habitats.

Histopathologic evaluations of contaminant effects offer several advantages to agencies responsible for implementing pollution monitoring programs: 1) tissue processing procedures are routine, quick, and relatively inexpensive, 2) effects in multiple target organs are easily and rapidly evaluated, 3) acute as well as chronic effects can be distinguished, 4) mechanisms of action can sometimes be inferred, and 5) correlations with potential higher order effects (ie. at the organismal & population levels) are sometimes possible.

PRELIMINARY DATA

We recently reported high prevalences of hepatic neoplasms in mummichog (Fundulus heteroclitus) inhabiting a creosote- contaminated site in the Elizabeth River, Virginia (Vogelbein et al., 1990). In addition to the liver neoplasms, these fish also exhibited a broad spectrum of preneoplastic and non-neoplastic liver lesions, as well as elevated prevalences of extrahepatic neoplasms including tumors of the exocrine pancreas, bile ducts, vascular system, kidney, and lymphoid tissues. To date, we have seen neoplastic changes in five different organ systems and eight specific cell types in mummichog inhabiting this particular site (our unpublished data).

For the past 2 years we have been funded by the U.S. EPA to conduct morphological, field, and laboratory studies of this new tumor epizootic. To evaluate the mummichog's potential as an indicator of chemical contaminants in urbanized coastal environments, we conducted a histological survey of fish from eight estuarine habitats in Virginia. This study has identified a gradient of pathological responses ascribable to toxicant exposure in this species. Prevalences of hepatic and extra-hepatic proliferative lesions are illustrated for the eight estuarine habitats in Fig. 1a (putatively preneoplastic altered hepatic foci), Fig. 1b (hepatic adenomas (HA), hepatocellular carcinomas (HCC), total altered foci (AF[T]), total hepatic neoplasms (HN[T]), and Fig. 1c (biliary proliferative lesions, (BP), vascular proliferative lesions (VP), pancreatic neoplasms (PN). Highest liver lesion prevalences were found in mummichog from the creosote-contaminated site (AW) in the Elizabeth River, VA, with 86.7 % of the fish exhibiting putatively pre-neoplastic altered hepatocellular foci (AF), and 50 % having hepatic neoplasms (HN) (Fig. 1b). Lowest lesion prevalences were found in fish from a relatively uncontaminated reference site (CI) in the lower York River, VA. A single individual from this locality exhibited an AF, however, no other lesions ascribable to toxicant exposure were observed in this population. In contrast, mummichog from six moderately contaminated sites, five of them in the Elizabeth River and one in the York River, exhibited intermediate lesion prevalences (Figs. 1a, b, c). Fish from two of these sites (SC & CTF) exhibited only AF (Fig. 1b), whereas fish from 4 localities other than the AW site (ERW, ASP, CSY, and RF-SB) exhibited both AF and HN (Fig. 1b). Extra-hepatic proliferative lesions

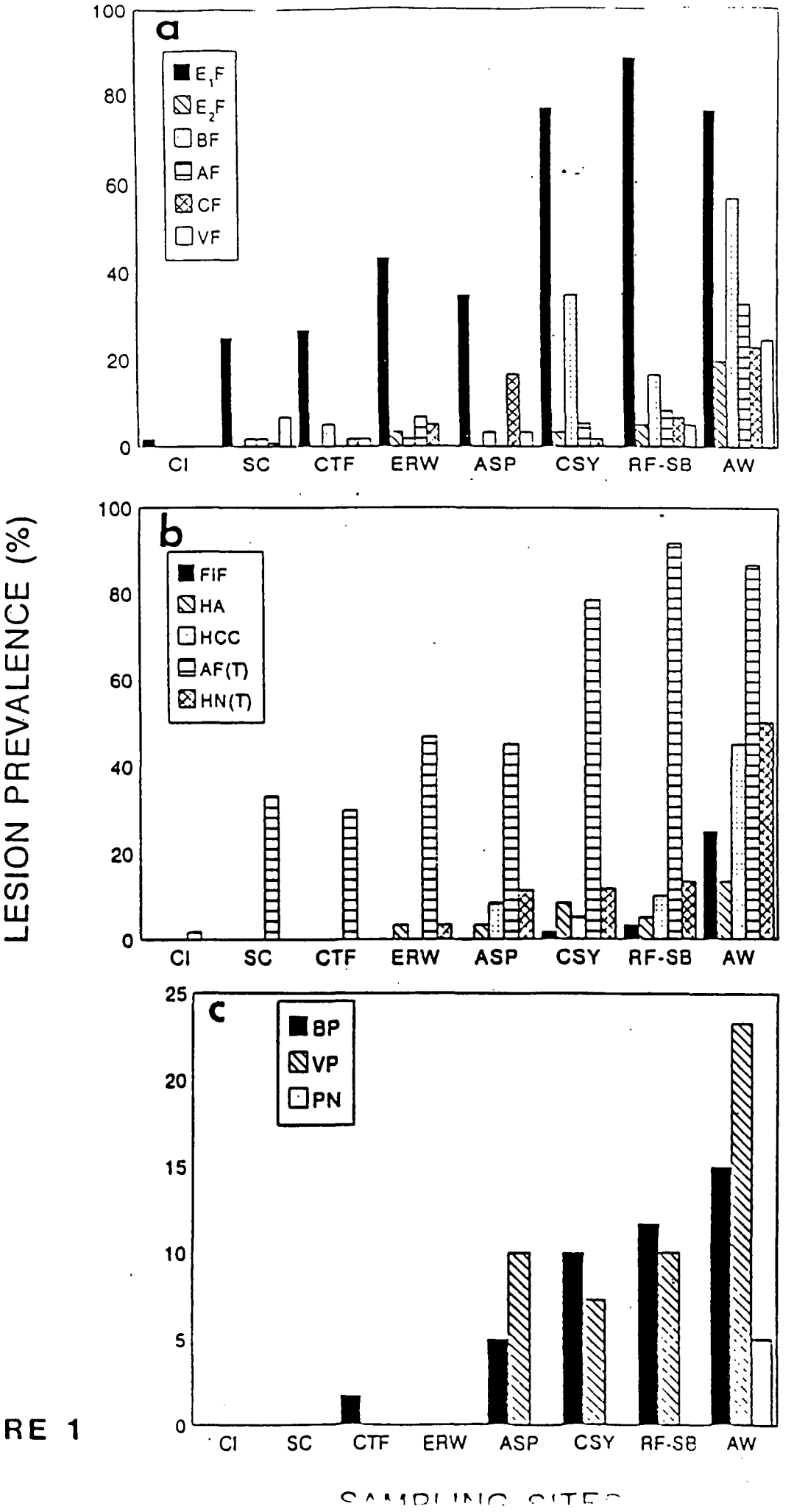

a
E1F
E2F
BF
AF
CF
VF
100
80
60
40
20
0
CI
SC
CTF
ERW
ASP
CSY
RF-SB
AW
b
FIF
HA
HCC
AF(T)
HN(T)
LESION PREVALENCE (%)
c
BP
VP
PN
25
20
15
10
5
0
SAMPLING SITES

FIGURE 1

occurred in mummichog from 5 of the 8 localities examined (Fig. 1c). A broad spectrum of degenerative and regenerative liver lesions also occurs in fish from all stations except the reference site. Morphological studies, characterizing the non-neoplastic liver lesions are in progress. Recent studies indicate that some of these lesions may be equally or even more useful than the pre-neoplastic and neoplastic lesions as indicators of toxicant exposure in feral fishes (Hinton et al., 1992; Myers et al., 1993).

Figure 2a illustrates the positive relationship between total aromatic hydrocarbon concentrations in sediments from the various localities and liver lesion prevalences in resident mummichog. This relationship is even more apparent when the high molecular weight PAH benzo[a]pyrene (BaP) is selected for comparisons (Fig. 2b). We are not implying that the observed liver lesions are attributed entirely to exposure of fish to BaP in the sediments. Obviously, these fish are exposed to extremely complex mixtures of chemicals, the interactions of which are presently not understood. In fact, investigations with rodents indicate that the contribution of BaP to the carcinogenic potency of emission condensates from gasoline and diesel engines, coal combustion processes, and sidestream cigarette smoke is minor and accounts for only 0.17% to 4.0% of the total carcinogenicity (Grimmer et al., 1991). However, our data do indicate that the mummichog is responsive to toxicant exposure and exhibits great potential as an indicator species. Logistic regression analyses are in progress to identify statistically significant differences in lesion prevalences among the different sites.

To aid in validation of this species as a field indicator and to develop methods that will allow us to evaluate toxicogenic and carcinogenic activities of hydrophobic organic compounds, we exposed larval laboratory-reared mummichog to aqueous and particulate fractions of a potent carcinogenic PAH, 7,12-dimethylbenz[a]anthracene (DMBA). We recently examined a 6 mo post-exposure sub-sample from this study and observed a spectrum of toxicopathic liver lesions in the high doses including degenerative and inflammatory changes, nuclear atypia, and putatively preneoplastic altered hepatocellular foci. The majority of the fish comprising this study remain in grow-out and are scheduled to be processed at the end of 1 year (Aug. 1993). These data suggest that the mummichog is responsive to the toxicogenic and carcinogenic effects of DMBA and

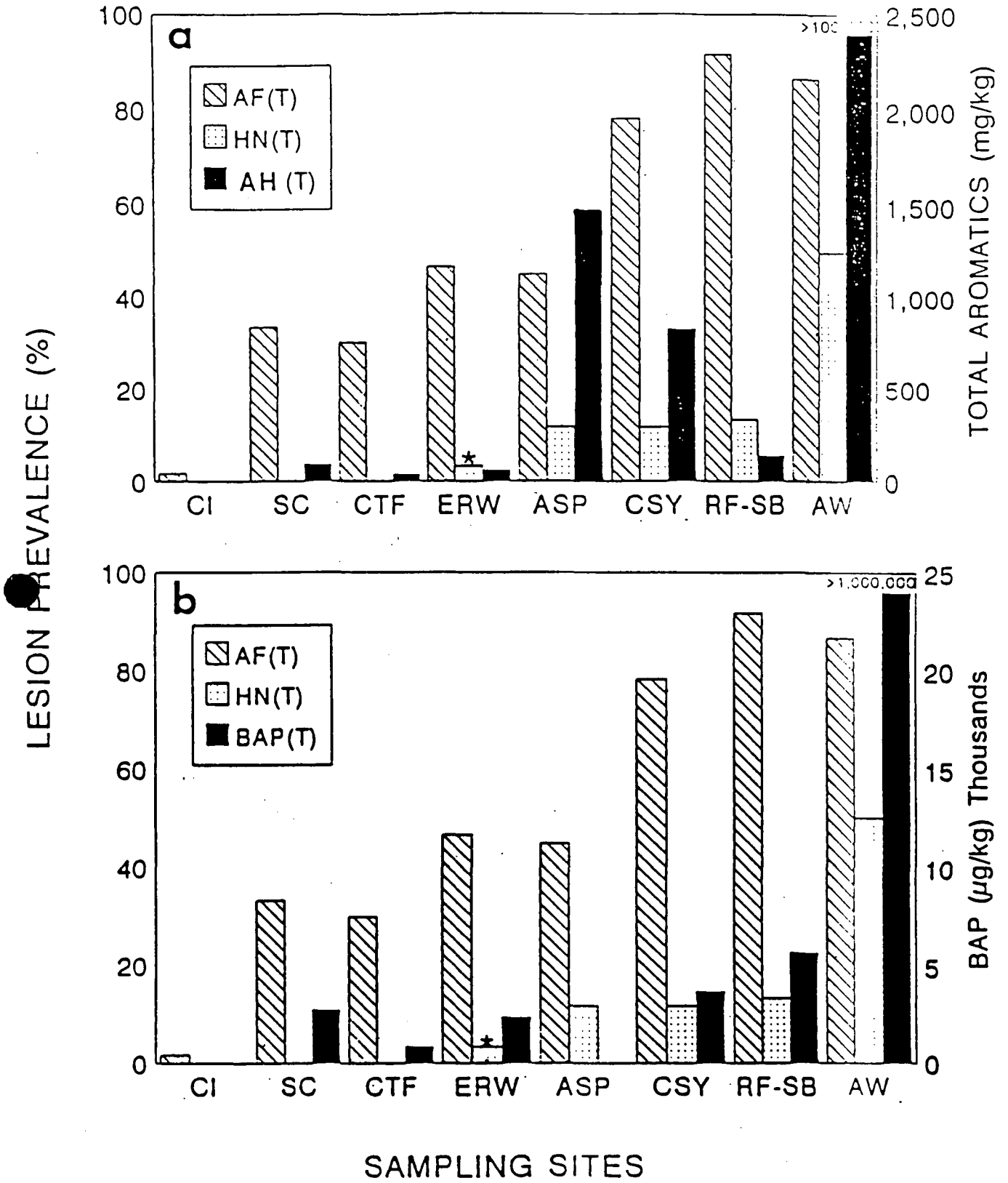

a
AF(T)
HN(T)
AH (T)
>100
LESION PREVALENCE (%)
TOTAL AROMATICS (mg/kg)
100
80
60
40
20
0
2,500
2,000
1,500
1,000
500
0
CI
SC
CTF
ERW
ASP
CSY
RF-SB
AW
b
AF(T)
HN(T)
BAP(T)
>1,000,000
BAP (µg/kg) Thousands
25
20
15
10
5
0
SAMPLING SITES

FIGURE 2

may therefore be an effective new model for laboratory studies of chemical hepatocarcinogenesis, especially evaluations of environmentally relevant compounds such as PAH.

RATIONALE FOR CHOICE OF EXPERIMENTAL ANIMAL

Several attributes suggest that the mummichog may be an effective indicator of chemical pollution in estuarine environments of the Eastern Seaboard of the U.S.. This small fish has a wide geographic distribution, inhabiting coastal marshes from Nova Scotia to Florida (Bigelow and Schroeder, 1953). It is one of the most abundant intertidal marsh fishes along the east coast of the U.S. and thus, relatively easy and inexpensive to collect. A very important attribute is that local sub-populations are non-migratory, exhibiting a summer home range on the order of 30-40 m (Lotrich, 1975) and greatly restricted winter movements (Fritz et al., 1975). This, coupled with the demonstrated responsiveness to chemical toxicants (Vogelbein et al., 1990, our unpublished data), suggests that native mummichog integrate a broad range of environmental insults and can thus be used to evaluate potential adverse biological effects in waters in close proximity to point source discharges. The mummichog is extremely hardy and will tolerate broad fluctuations in environmental variables such as temperature, salinity, dissolved oxygen, etc. This has created the widely held view that this fish is not effective for environmental monitoring and toxicity testing. **THIS VIEW IS NOT SUPPORTED BY OUR RECENT FINDINGS. We suggest that hardiness and sensitivity to chemical toxicants are unrelated. In fact, several closely related cyprinodontid fishes have been recommended as "sensitive" carcinogen assay organisms (Courtney and Couch, 1985; Koenig and Chasar, 1985). The hardy nature of this fish is, in our opinion, a positive attribute in the context of pollution monitoring. Because of its adaptability and hardiness, we would expect to find mummichog in even the most heavily contaminated environments (as already demonstrated for the creosote-contaminated AW site). Less hardy, more "sensitive" species would probably not survive long-term exposures and might disappear entirely from many chemically contaminated habitats.**

The mummichog is currently the only known species exhibiting neoplasia in polluted environments that

also is amenable to laboratory culture and experimental manipulation. It is therefore the best available model system to verify the putative cause-effect relationship between contaminant exposure and the development of liver pathology in feral fishes.

OBJECTIVES

Based on results of our previous field and laboratory studies, we hypothesize that the mummichog, Fundulus heteroclitus is an effective histopathological indicator of chemical contamination in coastal embayments along the Eastern Seaboard of the U.S. Further, we hypothesize that the high prevalences of idiopathic liver lesions occurring in populations from industrialized environments are caused by chronic exposure of the fish to xenobiotic chemical contaminants, in particular a suite of high molecular weight polycyclic aromatic hydrocarbons, including those identified by EPA as priority pollutants. The objective of the proposed study is to conduct field and laboratory studies that will test these hypotheses and validate this small estuarine teleost as an effective field sentinel and laboratory model.

EXPERIMENTAL DESIGN

FIELD STUDIES

We propose a liver histopathological analysis of mummichog from ten different localities on the Eastern Seaboard of the United States including areas in the Hudson River (i.e. New York Harbor, Arthur Kill, and Passaic River), Delaware Bay, Chesapeake Bay, and the Savannah River in Georgia. Specifics of site selection will follow discussion with EPA scientists and will reflect available knowledge of chemical contamination in these waterways, however, we would like to include several EMAP sampling stations in our design. Each of the broad geographical regions that we plan to sample will also include at least one relatively uncontaminated reference site. Our general goal is to select habitats that are contaminated with major classes of hydrophobic chemicals originating from different industrial processes. Because sediments are known to be efficient integrators of hydrophobic contaminants, we further propose to correlate results of the sediment chemical

analyses with liver histopathologic responses in mummichog native to these localities. Analyses will identify specific classes and concentrations of hydrophobic organics such as PAH, PCB, polar hydrocarbons, pesticides, and trace metals. In addition, we propose wide spectrum sediment chemical analyses that will flag high concentrations of unknown compounds. We will attempt to identify these unknowns by mass spectrometry.

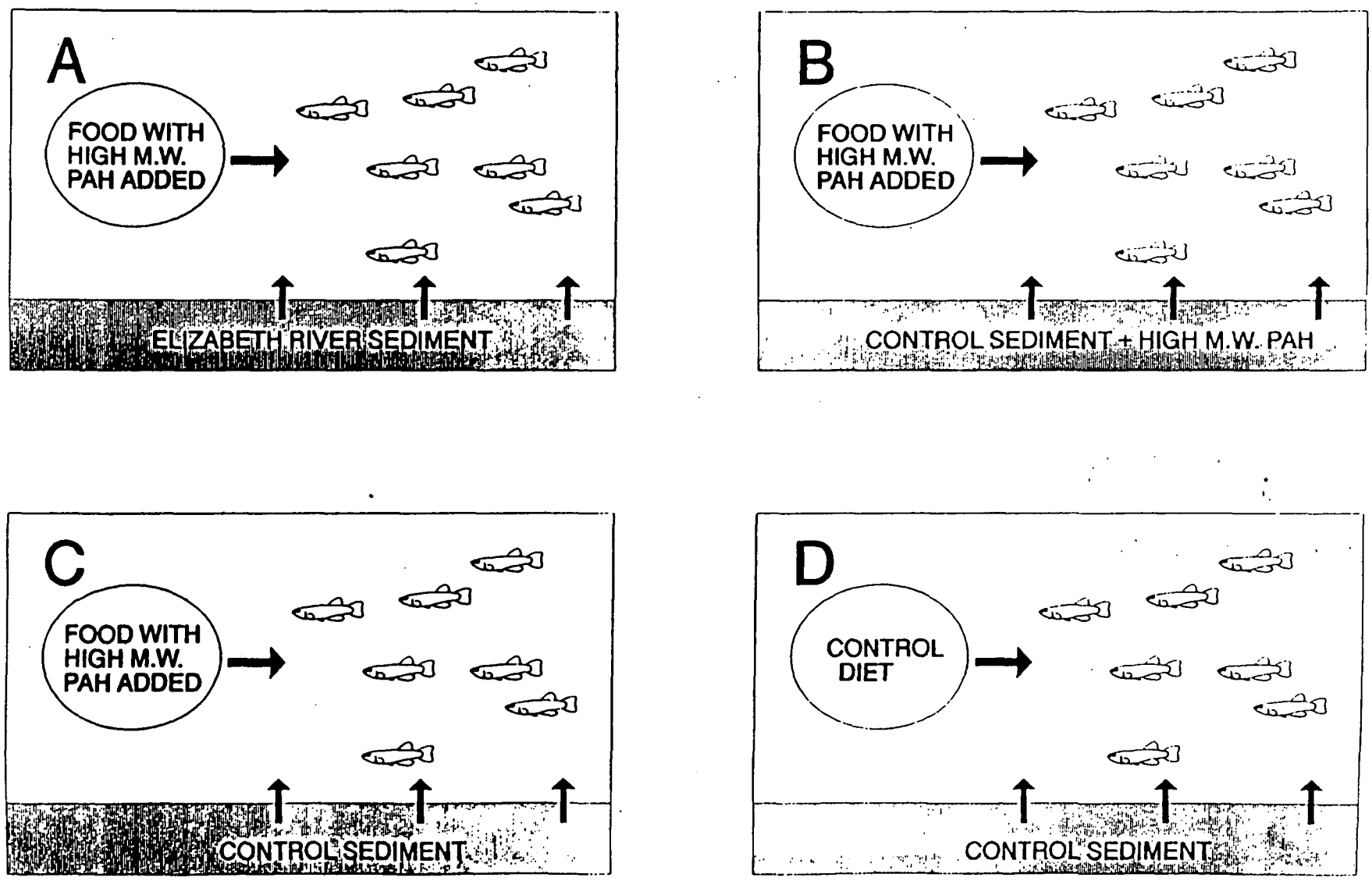
LABORATORY STUDIES

We propose to conduct four chronic (1 year duration) sediment and dietary exposures with mummichog in order to evaluate toxicogenic and carcinogenic activities of complex mixtures of environmentally relevant hydrophobic organic chemicals. We are proposing a stepwise series of laboratory exposures to an increasingly narrow set of chemicals extracted from contaminated sediments in order to evaluate the role of the higher molecular weight PAH fraction in the etiology of liver pathology. Fish in treatment A (Fig. 3) will be exposed to a diluted Elizabeth River sediment, and fed a diet amended with a high molecular-weight aromatic fraction of the Elizabeth River sediment extract. Whole sediment extract is not appropriate for inclusion in the diet, because of large concentrations of low molecular-weight PAH such as naphthalene and its alkylated derivatives which are not as likely to accumulate in food organisms and may also cause acute toxicity at high dietary concentrations. This treatment will provide exposure via gills, skin, and alimentary tract. Treatment B will use the same high molecular-weight extract in both sediment and in the diet. Treatment C will consist of dietary exposure to the high molecular-weight extract alone, and treatment D will serve as a control, with fish maintained over an uncontaminated reference sediment and fed a unfortified control diet.

METHODS

Fish Collection: Because our recent studies in Virginia indicate that liver lesion prevalences are influenced by age and season, and that highest prevalences occur during the fall and in the largest fish, we will collect the field samples during September and October 1994. Adult fish (TL >75 mm) will be collected using standard

FIGURE 3. EXPERIMENTAL DESIGN FOR LABORATORY EXPOSURES



baited minnow traps. Sixty fish (30 males and 30 females) will be obtained from each of the 10 sampling sites. Fish will be shipped live by overnight mail to VIMS, where they will be necropsied and processed for histopathologic evaluation within 5 days of capture.

Animal Necropsy/ Histology Methods: Fish will be anesthetized with tricane methanesulphonate (MS-222), sexed, measured to the nearest mm (SL and TL), and weighed to the nearest 0.001 g. Visceral organs will be removed and carefully inspected. The liver will be separated from other viscera, weighed (0.001 g), and photographed. Gross morphological characteristics of the liver (i.e. color, texture, size, shape, lesions) will be recorded. Livers will be cut with a razor blade into 5 slices and placed in Bouin's fluid. Selected liver tissues and lesions will be sub-sampled and processed by routine methods for electron microscopic analysis (Hayat, 1989) because morphologic studies of mummichog lesions, although not emphasized in this scope of work, will continue in collaboration with EPA investigator Dr. J.W. Fournie. Otoliths will be removed and stored for future aging studies. The remaining viscera, head, and section of body containing the kidney will be fixed and archived for future studies. Livers will be processed by routine methods for paraffin histology (Luna, 1968). Briefly, after 48 h of fixation, tissues will be rinsed to remove all traces of fixative, dehydrated, and embedded in paraffin. Sections 5μm in thickness will be cut on a rotary microtome, adhered to glass slides, and stained with hematoxylin and eosin. All five liver slices will be embedded in the same block so that five different levels of the liver can be evaluated simultaneously. Diagnoses will be based on lesion nomenclatural systems developed in our prior EPA study (CR 818165-01-0).

Laboratory Studies: Chronic exposures of mummichog will require a series of range-finding tests to determine levels of sediment and dietary contaminants that will preclude acute morbidity and mortality. These tests are planned for summer 1993. To further reduce occurrence of potential acute toxicity during the definitive study, we propose to use 10 day old F_1 larvae of fish obtained at the Atlantic Wood site in Virginia's Elizabeth River. These fish appear to be resistant to the acute toxicity of the sediment contaminants (C. Horton and W. Vogelbein , unpublished results). Continuous flow-through exposures will be conducted in glass aquaria on a

wet table supplied with filtered, temperature controlled (25°C) York River water. Our experimental design is outlined in Fig. 3. We will evaluate several different diets to minimize potential problems with palatability of food amended with high molecular weight PAH. Fish will be sampled intermittently for histopathologic evaluation according to the schedule outlined in Fig. 4. This approach will maximize the information available to us regarding potential etiology and histogenesis of developing toxicopathic liver lesions.

FIGURE 4. SAMPLING STRATEGY FOR LABORATORY EXPOSURES

TIME	0	1wk	2wk	4wk	8wk	12wk	6mo	12mo
TANK SIZE	---------20 gal----------		----------55 gal----------			---------70 gal---------		
FISH PER TREATMENT	280	240	220	200	180	150	120	60
SAMPLE TAKEN	20	20	20	20	20	30	60	60

Statistical analyses: Statistical significance of differences in lesion prevalences in feral fish from reference and test sites and laboratory fish dosed by different treatments will be evaluated by logistic regression analyses. This approach permits examination of the influence of multiple risk factors on the probability of disease occurrence. It has been applied to retrospective epidemiological studies (Breslow and Day, 1980) and has recently also been used to examine the epizootiology of liver and kidney lesions occurring in feral English sole, flathead sole, starry flounder, hornyhead turbot, white croaker, and black croaker in chemically contaminated west coast sites (eg. Myers et al., 1993).

Field Collection and Preparation of Chemistry Samples: A single sampling effort at each of selected field sites will be used to collect surface sediment grab samples. Probable sites will be selected, though considerable latitude will be exercised during the actual collection, so that unusually sandy or otherwise anomalous samples

may be avoided. Sediment samples will be taken with a pre-cleaned Ponar grab. The grab sampler will be washed with copious amounts of water and pesticide grade methanol between stations to prevent contamination of the samples. The upper 2 cm of the intact grab will be removed from five grabs at each station location and mixed in a solvent rinsed stainless steel bucket to produce a composite sample. This composite sample will be homogenized and placed in three precleaned quart glass jars with teflon lined lids, one for organics analysis, the second for metals, TOC, and grain size, and a third for duplication in case of sample loss. Sample storage jars will be washed, acid rinsed, baked and solvent rinsed prior to use. Samples will be stored on ice in the field and immediately frozen when returned to the laboratory. One site sample will be collected in sufficient amount to permit sub-sampling, and divided into three well-mixed replicates, each of which will be separately analyzed to enable the estimation of variance due to analysis.

Chemical Analysis-Organics: Frozen sediment samples will be thawed, placed in clean stainless steel trays and lyophilized in a freeze-dryer fitted with a nitrogen bleed valve to prevent contaminant backstreaming during vacuum operation and venting. All labware which contacts the samples will be scrupulously pre-cleaned and baked. Weighed dry samples of about 100 g will be spiked with internal reference standards and Soxhlet-extracted with methylene chloride for 48 hours. The extract will be concentrated by rotary vacuum evaporation and cleaned up by gel permeation chromatography to remove large biogenic molecules. The resulting aliquot will be separated by polarity into aliphatic, aromatic, and polar fractions by micro-liquid chromatography on silica gel by gradient elution with hexane, methylene chloride, and methanol. The efficacy of the aliphatic/aromatic hydrocarbon separation is routinely monitored with standards and flow parameters adjusted as required. These separation techniques will also be used to produce fractionated extracts from sediments collected at the Atlantic Wood site in the Elizabeth River, Virginia. Composition of these extracts will be verified by gas chromatographic analysis prior to use in laboratory exposure experiments.

Liquid-chromatographic fractions will have the solvent exchanged to hexane-toluene and be concentrated for gas chromatographic separation with fused silica capillary columns coated with DB5 or

equivalent crosslinked silicone polymer stationary phase. Standard analytical conditions use helium carrier gas at constant pressure, a splitless injection technique, and oven temperature programming at 6° C per min from 75° C to 300° C. Flame Ionization Detectors (FID) will be used to detect hydrocarbons, and Electrolytic Conductivity Detectors (ELCD) will be used for analysis of halogenated compounds. Detector responses are digitized and stored for further analysis using a HP3350 Laboratory Data System. Standard integration algorithms are used for peak integrations, but Relative Retention Index programs are used for peak identification and quantification using internal standard methods from raw retention time and peak area data. Daily injections of standard mixtures are monitored to ensure instrument performance and precision. Aromatic fractions will be further analyzed by electron impact and negative chemical- ionization mass spectroscopy to confirm the identity of suspected aromatics and halogenated species and to attempt the identification of selected unknowns. Routine sediment parameters will be measured for comparative purposes, including grain size, total organic carbon (TOC), and moisture content.

Metals Analysis: Aliquots of the sediment homogenates will be analyzed for chromium, copper, manganese, zinc, aluminum, arsenic, cadmium, nickel, lead and mercury by atomic absorption spectroscopy. Sediments will be digested by the same techniques used when analyzing sediments in previous Chesapeake Bay Monitoring Programs. Each sediment homogenate sample will be analyzed in triplicate and appropriate blanks and a standard reference sediment (SRM 1646) will be analyzed with the environmental samples. All glassware will be thoroughly detergent washed and rinsed with deionized water, followed by successive washings with 1:1 HCl and 1:1 HNO_3. Final rinsings will be performed with semiconductor grade deionized water. All glassware and other materials used for processing samples will be capped or covered prior to use to prevent contamination from laboratory particulates. All sediment leachings and standard solutions will be prepared with "Trace Metal Grade" HCl and/or HNO_3. Acid blanks will be periodically checked for contamination. Standard solutions of each element will be prepared from sequential dilutions of commercially available AAS standards (Fisher) and stored in polyethylene bottles. Estuarine sediment SRM 1646 from the NIST will be used for reference values to check recoveries in the leaching procedure. A Perkin-Elmer Model 1100B Atomic Absorption

Spectrophotometer will be used for all determinations via flame (Cr, Cu, Mn, and Zn), graphite furnace (Al, As, Cd, Ni, and Pb), and cold vapor reduction (Hg). Calibration curves will be generated from prepared standards for every instrument run. Instrument drift will be checked after every 10-15 samples during each run by measuring the concentration of a standard solution used to prepare the curve. New working curves will be prepared if the standard concentration reported is not within 10% of the expected value.

Sediment samples will be digested in accordance with procedures followed previously when analyzing samples for EPA sponsored monitoring of the Chesapeake Bay. Each sample will be placed on a large watchglass and inspected to remove foreign materials (twigs, etc.). Samples will be dried overnight at 102 °C or until a constant weight is obtained. Dried samples will be thoroughly ground in mortars to a homogeneous consistency and transferred to glass vials for archival storage.

Samples on the order of 1 g will be transferred to 150 mL polyethylene beakers, after which 1 mL of deionized water and then 10 mL of aqua regia will be added. The beakers will then be covered with watchglasses and refluxed on hot plates for two hours, with two additional 5 mL portions of aqua regia added to prevent evaporation to dryness. After cooling, 15-20 mL of water will be added, and the samples quantitatively transferred to 50 mL polyethylene centrifuge tubes for centrifugation. The resulting supernatant solution is then quantitatively transferred into 100 mL volumetric flasks. The residual sediment will be washed an additional two times and the resulting wash added to the leachate solution. After dilution, the solutions will be transferred to polyethylene bottles for storage prior to analysis. Acid blanks and SRM 1646 will be processed in an identical manner. Each environmental sample will be processed in triplicate.

<u>% Moisture Analysis</u>: Sediment samples will be thoroughly thawed and mixed to provide as homogeneous a consistency as possible. Samples on the order of 1 g will be placed on acid washed and oven dried watch glasses and weighed to the nearest 0.1 mg. Samples will be dried at 110° C overnight (18 hr) and reweighed. A second weighing after an additional 4 hr drying will be made to insure that all moisture has been removed.

Again, samples will be analyzed in triplicate and the mean concentration and variance reported.

Grain Size Analysis: The technique used for separating sediment samples into gravel, sand, silt and clay fractions will follow the procedure of Carver (1971). A 50 g aliquot of each homogenized sediment sample will be wet sieved to separate the sample into a coarse and fine fraction. The coarse fraction will be further separated into gravel and sand fractions by dry sieve separation. The fine fraction is then separated into silt and clay by pipette analysis. Select replicate samples of the sediment homogenates will be analyzed blind by the analyst to assure that good precision is maintained throughout the procedure.

Sediment Total Organic Carbon Analysis: Aliquots of the sediment homogenates will be analyzed for total organic carbon (TOC) by a high temperature combustion technique using a Carlo Erba NA1500 analyzer. To prepare sediment samples for TOC analysis, 10% HCL will be added to a small portion of the dried sample. If effervescence is observed, another aliquot of the sample will be weighed into a tared dish. Acid will be added to this sample in small amounts until no effervescence is seen. The sample will then be dried and analyzed for carbon using the Carlo Erba Analyzer.

QA/QC Procedures:

Sampling Procedures: Drs. Vogelbein and Unger will conduct all environmental sampling for this project to assure uniform procedures are used at all locations. Sediment samples will be labeled at time of collection, and remain in the custody of project personnel throughout analysis. Aliquots of environmental sediment samples will be retained frozen until the project final report is approved by EPA.

Calibration Procedures: The VIMS Analytical Protocol is an internal standards method, relying on addition of a known amount of surrogate standards to samples prior to extraction. These surrogate standards are prepared in concentrated form from reputable sources each year, and aliquots are diluted for working standards on a regular basis. Standard solutions are stored in the dark at -20°C. The VIMS Analytical Protocol also relies on

a relative retention index (ARI) system of seven marker compounds for qualitative identifications. A pre-weighed known mixture of these compounds are analyzed by GC-FID each day to confirm constancy of response factors, retention times, and peak shape.

Data Reduction, Validation etc.: Chemical data will be collected using an HP 3350 Lab Automation System. This system will be used to integrate samples and further process the raw data using custom computer programs for qualitative and quantitative analysis. Data collected for this project will be backed up and stored on magnetic tape. Additional hard copies of chemical data will be retained by both W.K. Vogelbein and M.A. Unger.

Internal QC Checks: In addition to daily analysis of the marker standard mixture, reagent blanks will be analyzed with each group of samples to ensure that no compounds that are derived from the laboratory procedure interfere with, or are reported as part of, the sample. In addition, the NIST reference sediment #1941 will be analyzed for PCBs and PAHs to verify the accuracy of the methodology. If unsuitable contamination is discovered in a blank sample, then analysis of environmental samples will stop until the laboratory contamination is rectified. Routine preventive maintenance is preformed on all analytical equipment by Institute personnel and logs are maintained for each instrument. Daily analysis of GC standards assures proper performance of equipment. If any step of the analytical procedure is found to be out of expected limits, ie. retention times are off, contamination in procedural blanks, low recovery of surrogate standard, then analysis of environmental samples will be stopped until the problem is rectified. Additional aliquots of all environmental samples will be kept frozen and available for reanalysis in the event of sample loss during the analytical procedure.

RESULTS AND BENEFITS EXPECTED

The proposed field studies will provide managers with a cost-effective method, applicable on both a local as

well as a regional level, that can be used to link adverse health effects in a native fish species with sediment contamination. It can also be used to identify specific localities within shallow-water, near shore habitats that are heavily impacted and that may require remediation. Laboratory exposures will provide direct evidence that liver pathology observed in feral fishes from chemically contaminated habitats is caused by chronic exposure to xenobiotic chemical contaminants. They will also provide a detailed understanding of the histogenesis of toxicant-induced liver lesions in a feral species and important information regarding developmental relationships between the different types of lesions. These studies will provide critical information required for validation of this species as a field sentinel and form the methodologic basis for future studies examining potential synergistic, antagonistic, and passive promotional effects between individual as well as broad classes of hydrophobic organic contaminants.

RELATIONSHIP OF PROPOSED STUDIES TO OTHER WORK

The proposed scope of work is a logical extension of EPA Contract 818165-01-0 in which we have characterized the liver and exocrine pancreatic proliferative lesions occurring in feral mummichog, conducted an epizootiological survey of mummichog inhabiting chemically contaminated Virginia environments, and elicited toxicopathic liver lesions in fish exposed to the potent carcinogen DMBA. The proposed studies are critical in our stepwise approach to validating this species as a field sentinel and laboratory model and are related to studies that have been conducted with English sole, winter flounder, and Atlantic tomcod. However, unlike to these species, the mummichog is amenable to lab culture and experimental manipulation. This suggests that where prior studies have been unsuccessful or only marginally successful in examining cause and effect relationships in the laboratory, we have the highest likelihood of being able to demonstrate these relationships.

ADDENDUM TO EPA PROPOSAL: Validation of the Mummichog, Fundulus heteroclitus as a Histopathological Indicator of Pollution along the Eastern United States by Vogelbein et al. Virginia Institute of Marine Science, Gloucester Point, VA 23062.

The Gulf killifish, Fundulus grandis inhabits coastal marshes of the southern U.S., ranging from Florida into Mexico. It replaces the mummichog, Fundulus heteroclitus in northern Florida and is very similar to this species with respect to taxonomy, habitat preference, life history, and physiology. Despite a lack of specific data regarding its' migratory habits, it is highly probable that the Gulf killifish, like the mummichog, has a restricted home range. It therefore may serve as an effective indicator of chemical contamination in the Gulf of Mexico. In order to expand the original scope of work to include a histopathological examination of a shallow-water estuarine teleost native to the Gulf of Mexico, we recommend the following modifications to our proposal:

1. That the proposed field study sites in New York (Hudson River) and Delaware (Delaware Bay) be eliminated and replaced by a liver histopathological evaluation of feral Gulf killifish, Fundulus grandis from 4 sites in the northern Gulf of Mexico (3 chemically contaminated sites including 1 creosote-polluted site if possible, and 1 uncontaminated site). Specific sites will be selected following evaluation of available sediment contaminant data and discussion with EPA scientists.

2. That experimental exposure studies be modified to include an evaluation of the Gulf killifish without compromising existing experimental design or cost. We suggest that:

a. sample size of the original mummichog exposure study be reduced from 280 to 150 fish/treatment and that only the six and twelve month sampling times be retained. This will essentially eliminate the information obtained on the histogenesis of the developing liver lesions but allow us to retain the original experimental design.

b. a parallel study using the identical experimental design be conducted with F. grandis, and that experimental animals and sediments for this study be obtained, if possible, from a creosote- or PAH-contaminated Gulf coast environment. For the original study we have proposed to expose offsping of AW mummichog because they are resistant to the acute toxicity of the AW sediments, and therefore more likely to survive long term sediment exposures. Ideally, we would want to use a resistant population of Gulf killifish as well.

c. monies saved by reducing the scope of the field studies will be allocated to conduct chemical analyses required for the extended laboratory exposures.