BIOMARKERS AND THE PELAGIC ORGANISM DECLINE

Conclusions of the POD Biomarker Task Force
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TABLE OF CONTENTS

	Page
EXECUTIVE SUMMARY	**iii**
PREFACE	**vii**
ACKNOWLEDGEMENTS	**vii**
PARTICIPANTS	**viii**
1. INTRODUCTION	**1**
1.1 Goal and significance	1
1.2 Approach	2
1.3 Scope and perspective	5
2. ANALYSIS: CONCEPTUAL AND FEASIBILITY ISSUES	**5**
2.1 Contaminants of concern	5
2.2 Fish life histories and sampling logistics	8
2.3 Biomarker review and analysis	15
2.3.1 Classification and evaluation	15
2.3.2 CYP1A	16
2.3.3 Metallothioneins	18
2.3.4 Cholinesterases	19
2.3.5 Stress proteins	21
2.3.6 Glutathione biosynthesis and antioxidant enzymes	22
2.3.7 Vitellogenin and choriogenin	26
2.3.8 Immune functions	28
2.3.9 DNA damage	31
2.3.10 Ion regulation	34
2.3.11 Multiple Xenobiotic Resistance	36
2.3.12 Toxicogenomics	39
2.3.13 Conclusions regarding biomarker techniques	44
2.4 Recommended fish condition indicators	45
2.4.1 Introduction	45
2.4.2 Reproductive indicators	47
2.4.3 Physiological condition, growth, and energetics	48
2.4.4 Histology and pathology	49
2.4.5 Stepwise implementation of condition indicators	50
2.4.6 Sampling considerations	50
2.4.7 Population relevance	51
2.4.8 Conclusions regarding condition indicators	52
3. INTEGRATION AND RECOMMENDATIONS	**53**
3.1 Overview	53
3.2 Recommended Tier 1 approach	54
3.3 General approach for Tier 2 investigations	57
3.4 Further technical considerations	58
3.5 Resource and implementation issues	58

4. CONCLUSIONS REGARDING STRATEGIC FRAMEWORK **59**

5. REFERENCES **60**

APPENDICES- Key items discussed at Task Force meeting
Note: Appendices contained in separate file

1-3 Contaminants of Concern
4-7 Fish Life History Diagrams
8-19 Biomarker Summaries

Cover photo of delta smelt: US Fish and Wildlife Service

EXECUTIVE SUMMARY

Four pelagic fish species have declined in the San Francisco Estuary, and the potential role of contaminants in this decline has not been characterized. This document reports the findings of a task force convened to evaluate the potential of biomarker techniques in future assessments of this problem. Biomarkers are sub-lethal, physiologic, and biochemical responses to contaminant exposure that complement other toxicological tools, such as toxicity testing and analytical chemistry. Uniquely, biomarker techniques provide information linking contaminant exposures and adverse biological effects, and they can be applied to field-collected fish as well as fish used in laboratory toxicity tests or *in situ* deployments. The task force critiqued eleven types of biomarkers and formulated strategic guidance regarding applications to the pelagic organism decline (POD).

There were three stages of work for the committee. First, we conducted a conceptual analysis of: biomarkers, fish life histories, chemicals of concern, and fish condition indicators. Fish condition indicators describe aspects of individual health such as reproduction, growth, energetics, and histopathology, which complement biomarkers and can be linked to population condition. Next, we analyzed issues related to the feasibility of implementing specific biomarker studies, and this phase of our work focused on availability of various fish life-stages for investigation. Finally, we concluded by synthesizing the results of each stage, and we recommended a framework for strategic implementation of biomarkers. Committee effort included conference calls, literature review of biomarkers, preparation for and attendance at a two-day conference at Fort Mason in San Francisco, and final synthesis of conference findings during the fall.

The key questions we addressed were: “How can biomarkers be used strategically to determine whether contaminants cause significant stress in the POD species, and how can they be integrated into a larger framework of investigation?” In this regard, some of the most important issues unique to the POD are: geographic, seasonal, and temporal variability of the fish populations and contaminant inputs, difficulty in evaluating responses in such a complex water body, lack of a reference condition, and the need for a rapidly-progressing, problem-solving approach.

Chemicals of concern were evaluated to provide a realistic backdrop for the biomarker discussions. This was relevant because biomarkers are often selected based on the types of chemical exposures that might be observed. Our relatively brief analysis of this topic resulted in the conclusions that there is a poor understanding of the spatial and temporal distribution of contaminants and that there are numerous compounds of potential significance that have not been characterized. This high level of uncertainty implies that biological studies <u>cannot</u> be focused on only one or two categories of contaminants.

Detailed discussion of fish life histories resulted in one strategic observation that has an important influence on the research framework we devised. Upon consideration of various sampling programs and logistics of sampling, it was determined that embryos and larvae of the POD species are very difficult or impossible to capture in the field. Hence, we recommend that investigations on embryos and early stage larvae be conducted primarily in the laboratory using fish obtained from hatcheries. A limited number of *in situ* deployments may also be considered.

We evaluated the strengths and weaknesses of biomarker techniques in detail, also focusing on successful field applications. Critical to our discussion was an evaluation of the extent to which individual biomarkers either reflected chemical-specific exposures or revealed significant sub-lethal effects. Because of the several unique features of the POD populations, we recommend that biomarkers be implemented in an integrated program that includes field and laboratory components.

Fish condition indicators related to reproduction, growth and energetics, and histopathology were also summarized. In the integrated framework, these should be implemented in concert with biomarkers to provide an initial assessment of the general health of the POD species. These measurements are straightforward and can link individual and population health, providing a bridge with ecological assessments and population modeling. While specific alterations in condition indicators can be supportive of a link between contaminants and effects, these indicators, unlike some biomarkers, are not contaminant-specific.

For field studies, we recommend an iterative study design that begins initially with several indicators of the general health of the fish and a small number of exposure-linked diagnostic biomarkers (vitellogenin/choriogenin, CYP1A, and DNA adducts). These would be applied to adult and juvenile fish that can easily be captured in the field. A sophisticated archiving program would also be included so that additional fish tissues and environmental samples could be analyzed for biomarkers and contaminants, respectively, once preliminary data from the fist set of analyses is obtained.

Controlled laboratory studies would emphasize the use of sensitive early life-stages, because these are not easily obtained in the field. Initially, we emphasize resident species toxicity tests using exposures to field-collected samples of water and other media coupled with Toxicity Identification Evaluations (TIE); in addition, a limited number of exposures to chemicals and chemical mixtures may be worthwhile. An archiving program would be used to save tissues for subsequent biomarker studies that become more relevant when chemicals of concern and sources of contamination are better characterized. In addition, some biomarkers are more reliable and easy to interpret when the timing and nature of exposure is well characterized. Hence, the integrated

laboratory studies provide validation of biomarkers that might be applied in subsequent laboratory of field investigations. The suggested laboratory-based studies allow for rapid screening of environmental samples and a limited number of chemicals, and this may significantly reduce uncertainty about what types of chemical exposures are of greatest concern, while also characterizing important effects in early life-stages. When TIE or field biomarker studies uncover effects related to specific toxicants, effect-levels in the laboratory can be compared to contaminant levels measured in the field to make a preliminary hazard assessment that would also inform a second tier of investigations.

Special studies are needed in the POD context to serve as a nucleus for research interest, and to fill gaps in the framework described above. We advise three types of special studies: an examination of neurological and neurobehavioral effects of pesticide exposure on POD species including acetylcholinesterase activity as a biomarker, an evaluation of multiple stressor responses focusing on the interaction between salinity stress and toxicant exposure using biomarkers of ion regulation and multixenobiotic resistance (MXR), and a pilot study applying toxicogenomic techniques to one of the POD species.

Findings from the first tier of investigations, described above, would be used to devise a second tier that might include more extensive use of biomarkers. In particular, data that increase our knowledge of potential contaminants of concern, of the most significant life-stages for investigation, or of the most relevant types of effects would be significant in defining future priorities. For example, findings of extensive histological damage might obviate the need for implementing a suite of biomarkers related to immune status or DNA damage, which could provide further evidence that contaminant exposure is a potential cause of the poor fish condition.

Additional technical considerations must be addressed in a formal study design. These include, but are not limited to, development of: plans for integrated statistical analysis, an analytical chemistry work plan that would be an essential element of all phases of the work, a detailed sampling and analysis plan, and a detailed archiving plan. Consultation with fisheries modelers at this stage might also help to focus sampling strategy, especially the level of effort to be placed on any particular season or geographic locale.

We recommend strongly that biomarkers only be implemented in an integrated framework of investigations; and that if this is done, the studies recommended will be adequate to discern significant contaminant-related effects, within the confines of the best available techniques. Given current constraints on funding, we defined a minimum level of effort for implementation of the suggested framework. We advise a three or four year investigation focused on two of the POD species. These would be selected based on policy concerns as well as the availability of hatchery stock. Studies would be conducted throughout the year

but more intensive sampling in late winter and early spring might be warranted, due to the potential for increased contaminant exposures. This is also a time period when early life-stages of most species are present in the system, again highlighting the utility of laboratory-based studies of the sensitive early life-stages. Suggestions are made regarding the need to develop a scientific consortium to provide adequate leadership for the project.

In conclusion, we recommend biomarkers as useful tools, but that limitations in their use specific to the POD must be recognized. Biomarkers should only be implemented within an integrated portfolio of other approaches. Our hope is that the work of this committee will serve as a useful adjunct to broader efforts within the POD to design a contaminant effects program.

PREFACE

This document presents the findings of a task force created to evaluate the appropriate use of biomarkers to assess contaminant effects on four declining fish species in the San Francisco Estuary. This recent occurrence is referred to as the Pelagic Organism Decline (POD). The Task Force undertook three stages of assessment. First, the team met via telephone conferences during the summer of 2007 to formulate the scope of the project and outline individual responsibilities. Task Force members then summarized state of the science information on biomarkers, contaminants, and POD species life histories and subsequently prepared synthesis tables for the second phase of work, a two day meeting at Fort Mason in San Francisco (August 29-30, 2007). In that meeting, we critically reviewed the items above on the first day and then devoted the second day to developing strategic positions and recommendations regarding implementation of the techniques. In the third stage, writing assignments were completed during fall of 2007, and this document was completed which provides our strategic recommendations on biomarker implementation.

The Task Force was comprised of academic panel members, who were active at every stage of the process, as well as agency advisors, who participated in analysis and production of some aspects of the document, but did not formulate strategic recommendations. The purpose of taking this approach was to conduct an independent academic review that was also grounded in immediate relevance to environmental managers. The members of the expert panel were selected based on three criteria: expertise and productivity in a selected area of biomarker research, experience in integrative or applied research, and communication skills. Each member was excluded from pursuing funding from the California Bay Delta Authority (CBDA) for a period of one year. Dr. Anderson selected the panel members in consultation with agency advisors Randall Baxter and Bruce Herbold with input from contract manager Steve Culberson. In addition, an effort was made to diversify the panel with respect to their previous involvement in San Francisco Estuary research. We selected some experts who knew the Bay Delta system well and others from outside of the area in order to ensure both the relevance and objectivity of our review.

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PARTICIPANTS

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with emphasis on species differences in chemical biotransformation, mechanisms of chemical olfactory injury in salmon, chemical injury to hematopoietic stem cells, polybrominated diphenyl ether-mediated cell injury, zebrafish models in toxicology, and oxidative stress.

Diane E. Nacci is a research scientist and former branch chief in Population Ecology with the US EPA's Office of Research and Development (ORD). Previously, she acted as supervisory senior scientist (Science Applications International Corporation, SAIC) on contract to the US EPA. She is an editor for the journal, Ecotoxicology, and on the editorial board for the Society of Toxicology and Chemistry. She has published many EPA-awarded scientific articles and book chapters, and has participated in the development of several ORD long-term research planning documents. She currently leads multi-organizational research teams at ORD's Atlantic Ecology Division (Narragansett, RI), providing support to the US EPA's Offices of Water and Pesticide Protection.

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Bruce Herbold is a fish biologist with the US Environmental Protection Agency. He helped develop estuarine habitat protection standards adopted by the State Water Resources Control Board in 1995. He was also instrumental in devising the Vernalis Adaptive Management Plan that has been a major aspect of San Joaquin salmon protection since 2000. More recently, he has worked with various CalFed efforts to plan and manage ecosystem resources of the Sacramento San Joaquin Delta. He currently helps coordinate research into the Pelagic Organism Decline (POD) of which this report is a part.

Kathryn M. Kuivila is a research hydrologist with U.S. Geological Survey (USGS) in Sacramento and has been studying the fate of current-use pesticides in the San Francisco Bay Estuary since 1990. She has considerable experience designing sampling studies and interpreting contaminant data collected in the complex hydrodynamic framework of San Francisco Bay estuary and has routinely collaborated with toxicologists to assess pesticide exposure and effects on aquatic organisms. Her research efforts have contributed greatly to understanding pesticide inputs, transport of pesticides during high flow events, and degradation of pesticides within the estuary. She has participated in numerous scientific workshops and panels to assess the fate and effects of contaminants in San Francisco Bay, such as the Interagency Ecological Program's contaminant effects project work team. She is currently the lead scientist for the USGS Toxics Program Pesticide Studies, an interdisciplinary team studying the fate and effects of current-use pesticides in the environment across the nation.

Project Assistant

Linda Righetti Judah works as a part-time consultant to S.R. Hansen and associates and recently completed a teaching credential for positions in high school biology. She obtained her masters degree in biology at San Francisco State University studying phytoplankton community structure. She was the lead for field sampling and logistics for the PEEIR Consortium at UC Davis Bodega Marine Laboratory.

I. INTRODUCTION

1.1 GOAL AND SIGNIFICANCE

The goal of this taskforce is to provide strategic guidance regarding the use of biomarker techniques to quantify effects of toxic substances on four declining pelagic fish species in the San Francisco Bay Delta. Although other stressors such as habitat loss, freshwater flow, and exotic species invasions may impact these populations, the focus of our investigation is on the role of contaminants, with some discussion of other non-chemical stressors. The declining species are Delta Smelt (*Hypomesus transpacificus*), Longfin Smelt (*Spirinchus thaleichthys*), Striped Bass (*Morone saxatilis*), and Threadfin Shad (*Dorosoma petenense*); and these are termed the POD species (Sommer et al., 2005). Concern that contaminant exposure may pose a hazard to the four POD species is pervasive (Moyle, 2002, Bennett, 2005); yet, a strategic and integrative view of how to investigate the issue has not been formulated. This taskforce was asked to address the utility of physiologic and chemical biomarker techniques as one aspect of ongoing efforts to design future contaminant investigations. Uniquely, biomarker techniques provide information linking contaminant exposures and adverse biological effects and can be applied to field-collected fish as well as fish used in laboratory toxicity tests or targeted *in situ* deployments.

In an ideal world, there would be one technique that managers could use to diagnose toxicant effects on fish populations, and this one method would also delineate both the degree and cause of any observed perturbations. Of course, there is no such “perfect” technique; and managers and toxicologists, alike, accept that a portfolio of approaches is needed to discern contaminant effects on wild fish populations. What is not clear in the context of the POD, is what the relative “investment” in different toxicological methodologies should be to ensure the most efficient use of time and money.

What is the potential value of using biomarker techniques in the context of the POD, and what are some of the strengths and weaknesses in comparison to other methods to diagnose and evaluate toxicant effects on fish populations? There are four general types of methods that are widely employed in this characterization: toxicity testing, analytical chemistry, condition indicators and biomarkers. Two of these methods, toxicity testing and analytical chemistry, are used to characterize the presence and potential potency of toxic contaminants. Analytical chemistry provides important information concerning biological exposures to certain categories of chemicals, which can contribute to evidence of ‘probable cause’ for categories of contaminants. Acute toxicity testing is used to characterize effects of sampled water on fish survival. Toxicity results, reflecting the combination of environmental factors and mixtures of contaminants, provide evidence of direct adverse effects on species of concern, or their surrogates. In contrast, two other methods, condition indicators (reproduction, growth, histopathology) and biomarker techniques, are used to describe the health of

individuals, and potentially link adverse changes to contaminant exposures. Biomarker techniques, in combination with condition indicators, can be used to determine long term and subtle effects of contaminant exposure in fish collected from realistic field exposure conditions. They can also provide evidence of significant exposure to a few general classes of toxic compounds. No other methods hold this potential, but the promise of biomarkers cannot be fully realized in all applications. Thus, our strategy describes a hierarchical approach using field and laboratory investigations, as well as high impact "special studies", within which specific suites of indicators and biomarkers are recommended.

In our opinion, biomarkers are complementary to other methods. For example, analytical chemistry, while powerful, must be targeted to categories of known contaminants, and therefore may fail to identify previously unknown chemicals or those for which detection methods are unavailable. In addition, effects of complex chemical mixtures cannot be discerned. In contrast, toxicity tests can be used efficiently to assess direct effects of chemical mixtures, but latent or chronic responses cannot be detected. In addition, realistic exposures that simulate field conditions and multiple stressors are hard to achieve with toxicity tests, and effects observed in test species often do not mirror those observed in resident organisms. Another alternative is to apply condition indicators, such as histopathology, reproductive and immune responses, to resident species, but these approaches lack chemical specificity and are often highly variable. Therefore, none of these techniques can discern causal agents of population decline when used in isolation. A portfolio of approaches is required.

1.2 APPROACH

Our approach was founded on an interplay between conceptual and practical concerns, and these are described below. First, it is critical to delineate the key questions we are addressing:

Question 1: "Can biomarkers be used, strategically, to determine whether contaminants are currently causing significant stress in the POD species?" and

Question 2: "What are the most important factors to consider in applying biomarkers within an integrative study plan that also elucidates the general health of the fish and the role of non-chemical stressors"

Initially, we viewed Question 2 as "outside" our scope of work but found that it was infeasible to provide guidance that was practical without also taking a larger view. This is because it is difficult to put biomarker responses into context or interpret them effectively if fish condition is not known and chemical and physiologic stressors are not well characterized. In addition, biomarkers are limited techniques in many respects, and they should be applied in a specific context to obtain answers that are useful to management.

The initial conceptual approach for the project (Figure 1) involved characterization of chemicals of concern, fish life histories, biomarkers, and condition indicators to create a list of recommended biomarker techniques that could both be applied to the POD species and also be relevant to chemical exposures observed in the San Francisco Bay and Delta. A preliminary list of chemicals of concern was created, because any discussion of biomarkers would not be relevant without example exposures and some knowledge of what chemicals might be present in toxic amounts. Next, fish life histories were summarized and related to geographic and seasonal distributions. In addition, we discussed whether any particular species or life-stage(s) were more likely to be affected by contaminants. To define and critique the biomarkers, we attempted to group them into four categories (diagnostic exposure markers, stress response markers, susceptibility markers, and toxicogenomic techniques), but often learned that many techniques fit in multiple categories; hence we generally discussed the biomarkers individually, describing the pros and cons of each. Our main focus was to critique the biomarker techniques with respect to their effectiveness in discriminating contaminant effects from other stressors.

After completing an evaluation of the conceptual issues above, we focused on feasibility issues. This was a significant step, because there was little point in considering alternatives that could never be implemented. Key questions that we addressed at this point were what life-stages of each species can be realistically sampled from the field in adequate numbers and can they be maintained alive until fish are dissected and tissue samples properly preserved? Answering the first question involved a detailed analysis of recent catches by life-stage from existing fish surveys. The second question involved assessing the likelihood of live collection and identification in the field; this was particularly problematic for eggs and young larvae. Another important factor was to determine the amounts and types of tissue that would be needed to execute integrated studies requiring tissue for biomarkers, condition indicators, and chemical analysis. Therefore, the expert panel evaluated and summarized tissue types and amounts for various life-stages and provided reference information to assess how much body mass was available from each species by length and season.

In the final phase of our work (Figure 1), we integrated our findings and formulated specific recommendations based on key “drivers” (see below) that were specific to the POD. This investigation resulted in, not only a review of techniques, but also an integrated approach that reflects our opinion of the best near term strategy for this aspect of the POD contaminants investigations. We envision that the report will be used as a basis for discussion among agency and academic partners while more detailed study designs are being developed. This should enhance the effectiveness of efforts to discern the potential role of contaminants in the POD decline.

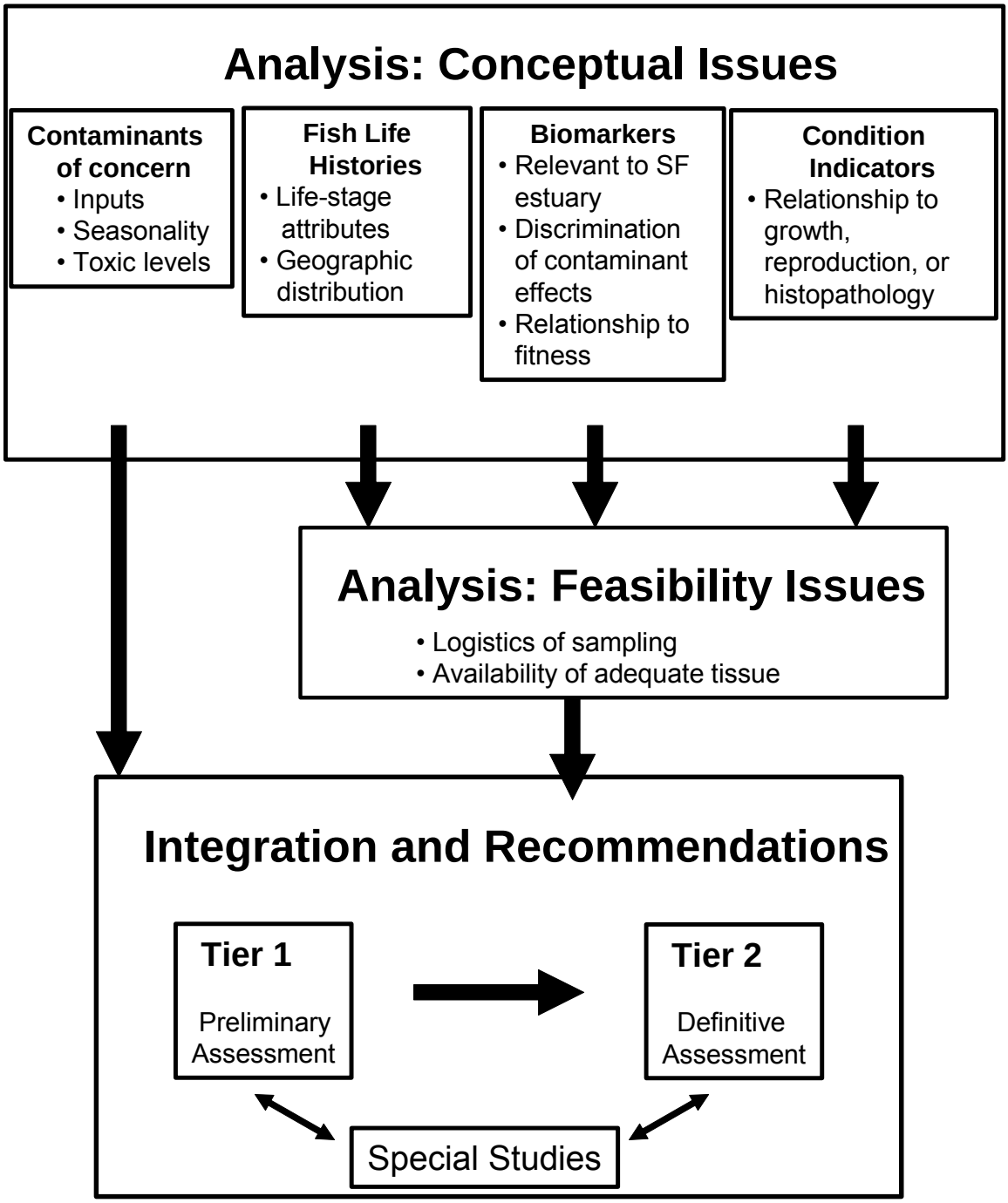


Figure 1. Sequence of analysis and key considerations for the POD Biomarker Task Force

1.3 SCOPE AND PERSPECTIVE

The recommendations of this taskforce apply to the specific issues of the POD decline. Our objective was to formulate a strategic framework recognizing the unique circumstances of the POD. Hence, some of our recommendations would not apply to other projects, such as the development of a general monitoring program or an investigation of a more well-defined contaminants issue.

Some of the most important concerns relevant to the POD were:

- lack of appropriate characterization of contaminants,
- geographic, seasonal, and temporal variability of the fish species distributions,
- difficulty in evaluating contaminant effects on field-collected fish in such a complex hydrologic system,
- lack of a reference condition, and
- the need for a rapid problem-solving approach to stave population declines.

It should be noted that some European monitoring and research organizations undertake a relatively aggressive use of biomarkers and recommend several assays that we did not select for this context (Hagger et al., 2006; Picado et al., 2007). Our approach was highly selective due to the unique issues of the POD, and our recommendations must be viewed as conservative. We hope that our report will be analyzed carefully and that other viewpoints will enrich this attempt to provide a strategic discussion of an urgent resource problem.

Items that were recognized to be beyond the scope of this contract include: a detailed synthesis of contaminants of concern in the San Francisco Bay Delta, a critical analysis of ongoing POD contaminants research, an overview of research publications on biological effects of contaminants in the San Francisco Bay and Delta, an analysis of biomarkers that could be used on invertebrates to elucidate indirect contaminant effects, and the development of a detailed study plan for future POD contaminants investigations.

2.0 ANALYSIS: CONCEPTUAL AND FEASIBILITY ISSUES

2.1 CONTAMINANTS OF CONCERN

Contaminant Identification

In the early conceptual phase of this project (Figure 1), we compiled a list of contaminants of concern (Appendix Tables 1-3) to provide an example of potential exposures. This was important because biomarkers are often selected based on the types of contaminants that are under consideration. Using published summaries of toxicant levels, we concluded that contaminants of

concern in San Francisco Estuary include current-use pesticides, legacy organochlorine pesticides, trace metals, and emerging contaminants. Other contaminants of concern have yet to be identified, but our task was to simply summarize some well known patterns to inform our review of the biomarkers.

Current-use pesticides have established use patterns and are most frequently implicated as the cause of acute toxicity in standard toxicity tests in the watershed (Kuivila and Foe, 1995, Werner et al, 2000). The pesticides of concern include pyrethroid, organophosphate, and carbamate insecticides, herbicides used on rice (molinate, thiobencarb, propanil), and other herbicides (diuron, hexazinone, simazine).

The pesticide inputs to the Delta from the Sacramento and San Joaquin River watersheds and transport from the rivers to the Delta are the best characterized (MacCoy and others 1995; Panshin and others 1998; Domagalski 2000; series of Central Valley Regional Water Quality Control Board technical reports). The occurrence and distribution of pesticides in the Delta and San Francisco Bay watersheds are not as well understood (Foe and Sheipline 1993; Kuivila et al., 1999; Werner and others 2000; Lu 2004). Recently, the Delta is becoming increasingly urbanized as evidenced by an 18% increase in population in the five Delta counties since 2000. In particular, several Delta cities, including Brentwood, Elk Grove, Manteca, Tracy, Rio Vista, and West Sacramento, have grown more than 25% in just the last five years (Eisenstein et al., 2007). This shift from agricultural land to urban area will likely change the type, location, and timing of contaminants into the Delta. Current-use pesticides used in residential areas, pharmaceuticals and antibiotics, and other contaminants associated with urbanization will probably become more important.

Legacy organochlorine compounds and metals were identified from the most recent US EPA 303(d) List for San Francisco Bay and its watershed as well as from the “Pulse of the Estuary” publications (e.g. SFEI, 2006). Compounds of concern for potential effects on aquatic biota were dieldrin, total DDTs, cadmium, and selenium. Chlordane, PCBs, dioxin and furan compounds, and mercury were also included even though these were listed primarily for fish consumption concerns.

Several emerging contaminants are beginning to be detected in San Francisco Bay. In particular, concentrations of one class of flame retardants (PBDEs) have increased markedly throughout San Francisco Bay in both water and sediments (SFEI, 2006). Compounds that have been identified, but with little occurrence data, include: steroid hormones, perfluorinated chemicals (PFCs), 4-nonylphenol, and nitro and polycyclic musks (Oros and David, 2002). These, and other emerging contaminants, are primarily entering the Bay Delta system from urban areas and waste-water treatment plants (WWTP).

Seasonality

Most current-use pesticides are applied during a definitive “season” on specific crop(s) or for a particular purpose. Following application, transport to surface waters can occur via rainfall-runoff, irrigation, release of field water or atmospheric transport. The time interval between application and transport is important since degradation, volatilization, and sorption to soil occurs primarily on the field or application surface. These processes, in turn, will affect the amount and form of the pesticide (dissolved, colloidal, sediment-associated) that is transported to surface water. Current-use pesticides are detected in seasonal patterns that depend on timing of application and transport mechanism. Often, a pesticide is applied in one season and transported to surface water in the same season, sometimes only a matter of days or weeks later. Some of the patterns are clearly defined as to source and transport mechanism, but many patterns are not so clear-cut. Typical seasonal patterns characterized by transport mechanism include the first flush (the first large runoff event in the winter), spring late-rainfall runoff event or tailwater return (direct runoff of irrigation return water), rice-field water release, and summer tailwater return (Panshin et al., 1998; Kuivila and Foe, 2005; Orlando and Kuivila, 2006).

Legacy organochlorine pesticides are typically associated with sediments, so concentrations in the water column would be highest during winter runoff events and during summertime because of wind-driven re-suspension. In contrast, emerging contaminants from urban areas and WWTPs have a more constant source throughout the year, with possible dilution during high-flow events.

Data Gaps

The majority of current-use pesticides applied in the Central Valley have not been analyzed in surface water samples, especially in the Delta. Some have long been of concern, such as ziram and some pyrethroids which are both known to be genotoxic (e.g. Campana et al., 1999). Even less data is available on emerging contaminants. More monitoring is needed to identify the temporal and spatial distributions, especially for sediment-associated contaminants. Monitoring programs need to incorporate an awareness of changing pesticide-use patterns as well as the importance of emerging contaminants when new analytes are prioritized.

Conclusions Regarding Contaminants of Concern

Our task was to summarize some patterns of chemical exposures in the San Francisco Estuary, known to include current-use pesticides, legacy organochlorine pesticides, trace metals, and emerging contaminants, to inform our review of biomarkers. There were five key conclusions by the task force regarding contaminants of concern:

- Although seasonal changes in contaminant exposure are not well understood, late winter through late spring may be the most important seasons for contaminants investigation because of high levels of sediment re-suspension and significant agricultural runoff in wet weather.
- Although some contaminants of concern have been characterized (Appendices 1-3), there is still a poor understanding of the concentration and geographic distribution of contaminants in various media (water, sediment, fish tissues), and these will vary in an unusually complex manner due to the hydrodynamics of the system and the varying chemical fate of numerous compounds.
- Much remains to be done to understand the spatial and temporal variability of inputs from diverse sources including wastewater effluent, point and nonpoint source agricultural inputs, urban runoff, mining inputs and nonpoint source contamination from boating and marinas.
- The potential importance of contaminants that have not been monitored for should be considered. Multi-residue analytical methods are designed to measure many contaminants in a single sample, which restricts analysis to compounds with similar chemical and physical properties. Unmonitored contaminants, that hold the potential for sub-lethal chronic effects, need to be identified by approaches such as those outlined in this document.
- Practical tiered approaches are needed to narrow down this uncertainty. Field sampling design should be based on an understanding of the potential contaminant sources, hydrodynamics, and fish life histories. Whereas, the tiered approaches must also integrate analytical chemistry with toxicity testing and biomarkers moving from routine monitoring into exploratory analyses of new contaminants based on results obtained from toxicity tests combined with TIEs, biomarkers and condition indicators.

2.2 FISH LIFE HISTORIES AND SAMPLING LOGISTICS

In the initial stages of this project (Figure 1), significant effort was devoted to summarizing seasonal changes in the geographic distribution of each species and each life-stage. This was potentially valuable, not only to better understand the ecology of a species, but also to ascertain whether any life-stages were more likely to be affected by contaminants. Also importantly, the availability of certain species and life-stages for live collection or field-preservation (and later analysis) was needed to inform our review of the application of biomarkers. Some of the information we synthesized is encapsulated in Appendices 4-7. After considerable review, we concluded that there was no *a priori* conceptual reason to emphasize particular life-stages for investigation.

Hence, we began to focus on the feasibility of capturing different life-stages of each species for investigation (Table 1). Furthermore, because biomarkers available to answer specific contaminant-related questions are often limited by

Table 1. Seasonal availability by life stage and length for longfin smelt, Delta smelt, striped bass and threadfin shad. Seasons are winter: Dec-Feb; spring: Mar-May; summer: June-Aug; fall: Sep-Nov. Footnotes reference sampling surveys that capture species life stage indicated, and are in no particular order, except if targeted sampling (20) comes first in the list in which case it will be more productive than other surveys.

Season	Longfin smelt stage	Longfin smelt availability	Delta Smelt stage	Delta Smelt availability	Striped bass stage	Striped bass availability	Threadfin shad stage	Threadfin shad availability
winter	Egg	not available field	maturing adult	yes, 50-80mm[4, 6, 7, 20]	juvenile	yes, 80-120mm[3, 5. 6. 7. 20]	adult	yes, 40-110mm[4, 5, 6, 7, 20]
	larvae	yes, 5-20 mm[1, 20]			adult	yes, male 300+ mm, female 600+ mm[20]		
	juvenile-adult	yes, 50-120 mm[4,5,6, 7, 20]						
spring	larvae	yes, 5-20 mm[1, 20]	mature adult	yes, 65-90mm[4, 6, 7, 20]	egg	yes[20]	adult	yes, 40-110mm[4, 5, 6, 7, 20]
	juvenile	yes, 20-40[1, 6, 20]	egg	no	larvae	yes, 5-20mm[1, 20]		
	age-1	variable (good wet-year year-class, poor otherwise)[4,5,6,7]	larvae	yes, 5-20mm, more at large end[1, 20]	juvenile	yes, 20-50mm[1, 5, 6, 7, 20]		
					adult	yes, male 300+ mm, female 600+ mm[8, 20]		
summer	juvenile	yes, 20-60mm[1, 2, 6, 7, 20]	juvenile	yes, 20-50mm[1, 2, 20]	juvenile	yes,20-80mm[2, 5, 6, 7, 20]	egg	maybe[20]
	age-1	yes, 70-100mm[6, 20]	age-1	maybe, 65-95mm[5, 6, 20]	adult	yes, male 300+ mm, female 600+ mm[20]	larvae	yes, 5-20mm[1, 2]
							juvenile	yes, 20-70mm[20, 2, 5, 6, 7]
							adults	yes, 70-110mm[20, 5, 6, 7]
fall	juvenile	yes, 40-80[3, 6, 20]	juvenile	yes, 30-70mm[3, 5, 6, 20]	juvenile	yes, 50-110mm[3, 5, 6, 20]	juvenile	yes, 40-70mm[3, 5, 6, 7, 20]
	age-1	not practical	age-1	maybe, 65-95mm[3, 5, 6, 20]	adult	yes, male 300+ mm, female 600+ mm[20]	adults	yes, 70-110mm[3, 5, 6, 7, 20]

Footnote numbers below indicate sample collection facilitated through particular long-term monitoring survey(s) or targeted surveys: 1. 20mm survey; 2. Summer Townet; 3. Fall Midwater Trawl; 4. Spring Kodiak Trawl; 5. Chipps Island Trawl; 6. Bay Study Midwater or Otter Trawl; 7. State and Federal fish salvage facilities; 8. Adult striped bass gillnet or fyke; 20. Targeted sampling for all larvae and striped bass eggs entails use of a 500 micron mesh plankton net; for small juveniles the use of seines and purse seines is practical; for large juvenile striped bass a large beach seine and setting off shore, for longfin smelt in fall trawling in Central Bay or outside the Golden Gate is possible; for sub-adult and adult striped bass boat electrofishing can be productive.

Sexual dimorphism: none of these fishes exhibit distinct sexual dimorphism; body conformation differences exist among all when mature; longfin smelt males develop elongated fins and tubricles on their head, but this has not been documented in the SF Estuary.

Special collection opportunities: all fishes are available from salvage (longfin and delta smelt from winter and spring seasons only; striped bass and threadfin shad throughout the year); longfin smelt can be caught along the coast by targetted otter trawling and may be available live or preserved from Washington state (Lake Washington) collections; delta smelt exist in culture and a potential surrogate wakasagi is available from Folsom and Oroville reserviors; striped bass are available from culture; threadfin shad are available from upstream reservoir populations.

availibility and amount of appropriate tissues, we also summarized the size of individuals at various stages.

This section describes species life histories, the seasonal timing of each life stage, and the practical availability of each life stage to be collected by current fish monitoring programs or by targeted sampling with available gear types (Table 1). Seasonal length ranges and length-weight relations are presented as an indication of tissue availability and amount per individual (Figure 2). Finally, opportunities for special collections or culture are identified.

Longfin Smelt

Field ecology and sampling: Longfin smelt (*Spirinchus thaleichthys*) spawn first in the year of the four POD fishes. Primarily 2-year old fish begin laying adhesive eggs as early as December and as late as June at water temperatures of 7.0 – 14.5 ºC (Wang, 1986). Exact spawning locations have not been identified, so eggs are not available from the wild (Table 1). Collections of early-stage larvae suggest that most spawning takes place within tidal fresh to slightly brackish water in the Delta (Wang, 2007). Embryos take 40 days to hatch at 7 ºC (Dryfoos,1965), hence about a month after fertilization embryos hatch into buoyant pelagic larvae. Larval (5-20mm) abundance peaks in the water column February through April when outflows disperse them downstream from the western Delta through eastern San Pablo Bay in low outflow years and from Suisun Bay to central San Francisco Bay in high outflow years (Table 1). Larvae are readily available to plankton nets but not sampled by current surveys until spring (Table 1). Juvenile distribution initially mimics that of larvae, but they soon move to deep-water channels to avoid water temperatures in excess of 20°C (Rosenfield and Baxter, 2007). Juvenile abundance is strongly and positively related to freshwater outflow during the larval period; thus, numbers of juveniles and pre-adults (age-1) available for capture will vary based upon flow conditions in the year they hatched. Both age classes are captured well by current surveys but are less available in fall. Length, and to a lesser extent weight, vary widely within a season (Figure 2).

Other options and culture: Longfin smelt inhabit estuaries and coastal areas northward along the Pacific coast of North America to Prince William Sound, Alaska (Moyle, 2002). Individuals might be obtained from other populations such as those of the Columbia River or Lake Washington, Washington State. A small population persists in Humboldt Bay (Pinnix et al., 2005; Wallace, pers. comm., 2007. Current culture techniques for delta smelt are likely also adaptable for longfin smelt (Joan Lindberg and Brad Baskerville-Bridges, pers. comm., 2007), allowing local propagation of test organisms. However, similar to striped bass and delta smelt, longfin eggs and young larvae (<45 days) will likely require special handling and new assay methodology.

Delta Smelt

Field ecology and sampling: Delta smelt (*Hypomesus transpacificus*) mature and spawn after a year of life beginning in late February and ending in June (Wang, 1986). As water temperatures warm past 7°C in spring, adults release adhesive eggs periodically

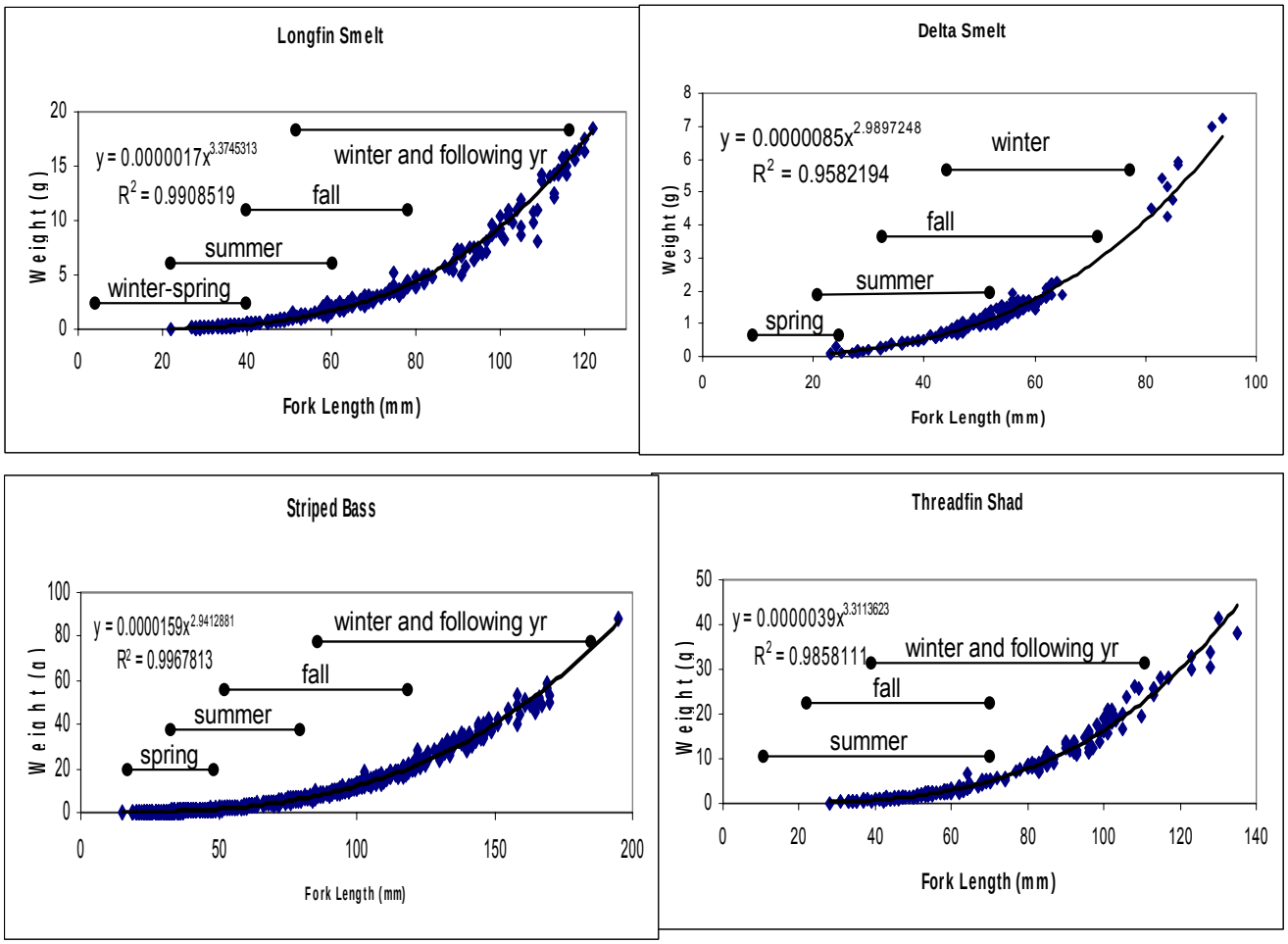


Figure 2. Length-weight relationships for longfin smelt, delta smelt, striped bass, and threadfin shad. Equations are all in the form weight = $a(FL)^b$ where weight is in grams and length measured as fork length (FL), is in millimeters. Horizontal lines depict approximate seasonal size range (winter: December-February; spring: March-May; summer:June-August; fall:September-November) until water temperatures surpass 20°C (Bennett 2005). Delta smelt spawning locations are not known, so eggs are not available from the wild (Table 1). Under laboratory conditions incubation requires 11-13 days at 14.8-16.0°C (Mager et al., 2004). Upon hatching, larvae are buoyant for the first three days, then will slowly sink without active swimming or upwelling currents. Swim bladder inflation occurs approximately 60-70 days post-hatch (approximately 20mm SL), at which time they are able to position themselves in the water column (Mager et al., 2004). Larval abundance (Table 1) in the water column peaks between April and May,

and they are readily caught during this period (Bennett et al., 2002; Dege and Brown, 2004). Early stage larvae have been found in Cache and nearby sloughs in the northern Delta and within the northeastern and central Delta. Once in the water column, they are dispersed downstream and many rear at the freshwater-brackish water mixing zone during spring and summer (Dege and Brown, 2004). It is known that rearing in low salinities improves survival. The spring mixing zone location varies with freshwater flow, ranging from the western Delta in low outflow years to western Suisun Bay or Carquinez Strait in high outflow years, and delta smelt distribution shifts similarly (Sweetnam, 1999). The distribution of juveniles in the summer typically extends from far eastern San Pablo Bay and the Napa River in high flow years, upstream throughout Suisun Bay and the large sloughs of Suisun Marsh. This distribution shifts eastward into the Delta and compresses during low outflow years. By fall, juveniles and pre-adults are distributed from eastern Suisun Bay and Marsh into the western Delta, where they remain until increased outflows attract them upstream. They mature and spawn in upstream reaches such as the upper Delta and in the Sacramento River above Rio Vista, and less frequently in Suisun Slough in Suisun Marsh or in the lower Napa River. All life stages from large larvae (15-20mm) to adults reside in the water column favoring mid-depths to the surface and are readily caught by fish monitoring surveys (Table 1). Length-weight relations are depicted in Figure 2.

Other options and culture: Delta smelt are endemic to the upper San Francisco Estuary, and no other populations exist. Successful culture of delta smelt has taken place for over a decade and with advanced planning all life stages might be available as test subjects. Currently the Delta Smelt Culture Facilities at Byron are reliably producing eggs, larvae, juveniles and adults for experimental use (Baskerville-Bridges et al., 2005). They are in the process of expanding and improving facilities to maintain thousands of culture-derived adult fish as experimental stock and brood stock, as well as more juveniles and larvae.

Transport of eggs and yolk-sac larvae (<5 days old) is successfully accomplished in bags of oxygenated water maintained at a constant temperature and insulated from physical shock. Similar methods can be used for older larvae (10-18mm) and juveniles (>30mm). Care must be taken that transfer and receiving water temperature and chemistry are initially similar. Delta smelt are reared in 0.2-2 ppt salinity and cooler temperatures than in the wild to reduce disease. Eggs can be transported and used in assay work with good survival, but stage duration is short – 10-14 days depending upon temperature. Newly hatched or yolk-sac larvae can be transported with good survival and used in short-term tests (stage lasts 1-6 days).

Stages not recommended for laboratory studies include young larvae at first feeding (5-10 days, about 6mm) which require low light levels, increased turbidity (algae), and live food to successfully feed and survive and18-25 mm larvae which enter another sensitive period.

Striped Bass

Field ecology and sampling: After over-wintering in the Delta, mature striped bass *Morone saxatilis*), age 3 and older, ascend the Sacramento and San Joaquin rivers in spring, preparing to spawn when water temperatures surpass 14°C. This typically occurs in April (Moyle, 2002). Spawning peaks in May and early June at temperatures of 15-20°C, and it will cease above 21°C. Females broadcast large numbers of semi buoyant eggs into riverine and tidal currents among a group of males who respond by releasing milt. Fertilized eggs hatch in 48 hours at 19°C; after which, undeveloped larvae require an additional 7-8 days of development before they become mobile. Spawning takes place primarily in the Sacramento River from Isleton to Butte City and the San Joaquin River and its sloughs from Venice Island down to Antioch (Moyle, 2002). Sacramento River larvae are carried into the Delta and Suisun Bay; whereas, those in the San Joaquin River often do not experience net downstream currents but remain re-suspended by tidal currents near where they were spawned. Eggs and larvae were successfully collected historically by gear still available for use (Honey et al. 2004), but only larvae >10mm are well captured by gear in current use (Table 1). Larvae disperse farther downstream in high flow than in low flow years (Dege and Brown 2004). As they grow, larvae and juveniles move toward the bottom and the shoreline and remain well caught by fish monitoring surveys (Table 1). Juveniles are typically found throughout the northern, central, and western Delta as well as throughout Suisun Bay and Marsh. In high flow years, they are often distributed into San Pablo Bay and the Napa and Petaluma rivers (Greiner et al., 2007). Juveniles retain and broaden this distribution with growth and mobility. Juveniles are captured in large numbers by trawl surveys into their second year of life, after which they must be targeted by special sampling efforts until adults. Adult fish are captured in spring either prior to or during their spawning run by fish monitoring surveys, but must be targeted by special sampling in other seasons (Table 1). The relationship between length and weight for juveniles to 180mm is depicted in Figure 2.

Other options and culture: Currently there is no reliable supply of cultured striped bass eggs and larvae in California other than Dave Ostrach (UC Davis), and his culture facilities are newly established and have suffered survival and production problems in the past 1+ years of existence. He has addressed past problems and anticipates better production success in 2008. Striped bass also live and reproduce in some upstream reservoirs within the Sacramento-San Joaquin watersheds, the closest being New Hogan Reservoir on the Calaveras River east of Stockton.

Transport of eggs, yolk-sac larvae 1-4 days old and larvae >14 days old (feeding and healthy) can be successfully accomplished in bags of oxygenated water maintained at a constant temperature and insulated from physical shock. Care must be taken that transfer and receiving water temperature and chemistry are initially similar. Eggs can be transported and used in assay work after water hardening 12-24 hr post-fertilization and should be used immediately for assays or maintained suspended. Newly hatched or yolk-sac larvae can be used for 24-48 hr tests, but must be able to feed and fill airbladder shortly after the 48 hr period. Use of larvae 5 to 14 days old may require

some method development but larvae >14 days old, once they have begun feeding and are healthy, can be transported and used in assays. Survival in transport improves for this age group if Instant Ocean® is added at 3-5 ppt, or if growth to 30 mm in length (45 days) is allowed before transport and use.

Threadfin Shad

Field ecology and sampling: Threadfin shad (*Dorosoma petenense*) can mature and spawn at the end of their first summer, but most mature in their second summer. Spawning commences when water temperatures exceed 20°C, usually from late April through August, peaking in June or July (Moyle 2002). Adhesive eggs are broadcast around floating or partially submerged objects and are not collected by current surveys. The embryos hatch in 3-6 days, and larvae immediately assume a planktonic existence. Larvae are found throughout the Delta and are captured well by fish monitoring surveys (Table 1). Juveniles form dense schools, and though most abundant in freshwater of the eastern Delta and the lower San Joaquin River from Stockton downstream, they commonly disperse into brackish water of Suisun Bay and Marsh, and uncommonly even into San Pablo Bay (Greiner et al., 2007). Juvenile and adult threadfin shad concentrate in surface waters and are readily caught by fish monitoring surveys (Table 1). The best growth and survival occur in waters where summer temperatures exceed 22-24°C and not become colder than 7-9°C in winter (Moyle, 2002). The population in the Sacramento-San Joaquin Delta experiences heavy die-offs every winter when the water cools to 6-8°C (Moyle, 2002). The relationship between length and weight for threadfin shad through their second year of life is depicted in Figure 2.

Other options and culture: Threadfin shad is currently being cultured in several southern states as a forage fish (e.g. http://www.aces.edu/dept/fisheries/aquaculture/threadfin-shad.php; http://www.americansportfish.com/threadfinshad.html) and is available commercially as a test subject. Threadfin shad also exist in some upstream reservoirs within the Sacramento-San Joaquin watersheds, the closest being New Hogan Reservoir on the Calaveras River east of Stockton.

Conclusions Regarding Fish Life Histories and Sampling Logistics

An understanding of species ecology and potential contaminant exposure patterns provides essential information concerning the likelihood that species and life-stages will be affected by contaminants. As importantly, the availability of certain species and life-stages for live collection or field-preservation, and later analysis, is needed to inform our review of the application of biomarkers. The committee reached five principle conclusions regarding the need to sample as well as the feasibility of working with various fish life-stages:

- In the toxicological literature, it is often noted that early life-stages are more sensitive to toxicants than juveniles and adults. Consequently, despite the numerous challenges, early life-stages cannot be ignored. If laboratory

investigations are to be based on use of hatchery cultures, it is crucial that hatchery efforts obtain adequate support.

- Embryonic stages can only be obtained from the field for striped bass, and these collections are limited in number. Hence, for the POD species, investigations must be conducted using embryos obtained from hatchery cultures and exposed to field water or sediment samples in the laboratory. A limited number of carefully planned *in situ* exposures might also be considered. Much of the activity would have to be conducted in the winter and spring, because this is when embryos and larvae of the majority of POD species are in the estuary.
- Larvae are rarely caught alive in field sampling. Therefore, any biomarkers used on field-collected larvae must be compatible with fixed tissues. Alternatively, larvae from a hatchery could be exposed to field water samples as described above.
- It is generally advised to implement biomarker research in a program of integrated study, and adequate numbers of fish and tissue quantities are needed. Specifically, tissue is needed for biomarkers as well as condition indicators and chemical analyses. In a variety of instances, we have found that this becomes practical when fish are greater than 50cm in length, but this is a guideline not a rule, as many techniques can be applied to very small amounts of tissue.
- Large numbers of fish can be obtained from salvage screens at south Delta water export facilities. These should be utilized for tissue sampling. Although some bias may occur with this sampling approach, it is important to recognize that useful information on fish condition, sex ratios, and some biomarkers can be obtained. Delta smelt are already sampled in this manner, but the approach could be extended to other POD species.

2.3 BIOMARKER REVIEW AND ANALYSIS

2.3.1 Classification and Evaluation

Biomarker endpoints are associated with biological response pathways that are invoked by exposure to chemicals with similar mechanisms of action. When taken together with other information, potentially including tissue chemistry, condition indicators, and laboratory correlations, these biomarkers may provide evidence that chemical exposures are contributing to adverse biological responses. Some of these biomarkers can be linked diagnostically to recent exposures to specific categories of chemicals. For example, changes in levels of metallothioneins can be indicative of metal exposure, acetylcholinesterase enzyme activity is indicative of organophosphate and carbamate pesticide exposure, and CYP1A expression is indicative of planar aromatic hydrocarbon exposure. Other biomarkers such as glutathione concentrations and stress protein induction can sometimes provide evidence of stress response that is not linked uniquely to a single category of stressor. However, results from controlled studies may suggest correlative relationships between changes in these indicators and exposures to specific categories of chemicals. Indicators of susceptibility to contaminant response include methods related to ion regulation and multixenobiotic response. It is important to reiterate that many of the biomarkers we review in this document may be markers of

exposure, effect, or susceptibility depending on the application. In addition to the more traditional biomarkers, new technologies are now contributing to the development, application, and accessibility of toxicogenomic techniques.

The first step in our analysis of biomarkers (Figure 1) was to summarize the strengths and weaknesses of each technique, with emphasis on their potential to discriminate contaminant effects in relatively large-scale field investigations. These discussions, as well as a description of the biological basis of each technique, are presented in Appendix Tables 8-19 and the text below. After each biomarker was reviewed, we analyzed the feasibility of implementing the technique with respect to availability of appropriate tissues and life-stages.

2.3.2 CYP1A

Introduction

Biotransformation is a critical process in the toxicology of most environmental toxicants. In an attempt to enhance the elimination of non-polar, lipophilic toxicants, various enzymes radically alter the chemistry of absorbed toxicants making them more water soluble and readily excretable. This process involves two phases. The initial phase I enzymes tend to add or expose polar atoms within toxicants; whereas the Phase II enzymes add large bulky water soluble endogenous compounds to toxicants that may or may not have undergone Phase I processes. One of the predominant Phase I enzyme systems is the Cytochrome P450 monoxygenase (CYPs) which are a multi-gene family of enzymes that occur in nearly all plants and animals. One of the most common and highly conserved is the CYP1A subfamily (Appendix 8). Since CYP1A is an enzyme that is transcriptionally regulated by the Aryl hydrocarbon receptor (AhR) which is activated by planar aromatic hydrocarbons (see below), measurements can be made of the protein by immunoblotting or the transcript by qPCR as well as by enzymatic activity. Each can be measured in embryos, larvae (including fixed tissues by Immunohistochemistry) and juvenile tissues. The enzyme is expressed in all tissues, but is in greatest concentrations in the liver. Excellent reviews have been published on the natural history, function and regulation of this subfamily of enzymes and their potential use in biomonitoring (Bucheli and Fent, 1995, Stegeman and Hahn, 1994, Van der Oost et al., 2003).

Strengths

CYP1A expression is increased primarily through activation of a cytosolic receptor known as the aryl hydrocarbon (Ah) receptor, which eventually serves as its own transcription factor initiating CYP1A mRNA expression. Ah receptors have a relatively selective binding region and prefer planar aromatic hydrocarbons as agonists (Hahn et al., 2005). Thus, expression of CYP1A, which has a typically low basal expression rate, can be used as a biomarker of exposure to various planar aromatic hydrocarbons ranging from PCBs and dioxins to numerous PAHs.

Although most of the metabolites resulting from CYP1A catalyzed reactions are hydroxylated derivatives of parent compounds, the reaction often involves the formation

of reactive electrophilic intermediates which may undergo nucleophilic attack by critical macromolecules such as sulfhydral groups of proteins and amine moieties of nucleic acids. Because of the enhanced possibility of protein or DNA adduction which may result in cellular dysfunction, induction of CYP1A has also been proposed to be used as a biomarker of adverse effect.

Applications

The predominant application of CYP1A is as an indicator of exposure to planar aromatic hydrocarbons. However, some studies have shown relationships between CYP1A expression and reproductive alterations following exposure to PCBs or TCDD (Cook et al., 1997; Teraoka et al., 2003). CYP1A catalytic activity in embryos and eggs has been used to determine toxicity equivalent factors (TEFs), which allow calculations of threshold values for dioxin-like compounds to adversely affect development in eggs of salmonids (Cook et al., 1997). These data further suggest that CYP1A expression may not only be used as a biomarker of exposure, but also of adverse effect, especially when applied to eggs or embryos (Nacci et al., 2005).

Weaknesses/Caveats

Expression of CYP1A is extremely variable among individuals, and some organisms have been shown to induce multiple isoforms from this family. Numerous allelic variants have been observed in an inbred strain of rainbow trout (Buhler and Wang-Buhler, 1998), as well as wild Atlantic tomcod (Wirgin, et al., 1991), which not only complicates nomenclature but may also have significant implications for biomarker studies. For example, evaluation of Tomcod recalcitrant to CYP1A induction in PCB contaminated sites would potentially lead to an underestimation of exposure (Yuan et al., 2001). Another caveat focuses upon methods of detection. Since CYP1A is an enzyme, activity is typically measured using the substrate ethoxyresorufin which is O-de-ethylated (EROD) to a fluorescent product, resorufin. However, since EROD activities are generally measured using liver homogenates, which may accumulate numerous CYP1A substrates, EROD activity may be inhibited by residual substrates or in some instances, metals. Therefore, CYP1A protein or mRNA should also be measured to validate negative EROD results especially when animals are taken from sites known to be heavily polluted by classical CYP1A substrates such as PCBs or PAHs, or metals.

In order to maximize signal to noise ratios, it is suggested that male or sexually immature animals be used to conduct studies. These animals should be of the same size and developmental stage. CYP1A as well as most inducible protein systems within the cell are greatly affected by circulating hormones. For example, CYP1A is typically down regulated by estradiol. Hence, signal-noise ratios are normally reduced in sexually mature females when circulating levels of estradiol are enhanced (Stegeman and Hahn, 1994).

Summary/Recommendations

In summary, CYP1A is a relatively sensitive, and therefore rapidly-responding biochemical endpoint of exposure. In combination with other indicators, it may provide

some indication of adverse effects in aquatic organisms. Although CYP1A has demonstrated strong correlations with exposure to particular planar aromatic hydrocarbons, elevated expression should not be equated with toxicity. Induction demonstrates Ah receptor activation, which may or may not lead to toxicity. With regard to the POD, livers from feral adults can be readily evaluated either through trawling, seining or bycatch at pump screens. Livers can be fixed, and immunohistochemistry can be evaluated. Alternatively, livers or embryos and larvae from field or laboratory studies can be placed in liquid nitrogen or dry ice upon sampling and stored at -80 deg C for enzyme activity (EROD), ELISA or qPCR. Thus, this method can be used for feral fish or laboratory exposures. Linkages between induction and development of embryos and larvae can be readily evaluated in laboratory studies. Since expression tends to be low if undetectable in naïve fish, measured expression can be used in a tiered approach for specific chemical analytes (i.e. planar aromatic hydrocarbons) for confirmation of exposure.

2.3.3 Metallothioneins

Introduction

Metallothioneins (MTs) (Appendix 9) are low molecular weight cytosolic proteins of which 30% of the residues are cysteine. Because of this large thiol concentration, MTs have the ability to bind many transition metals and act as free radical scavengers. Several field studies have indicated that MT expression in adult organisms strongly correlates with transition metal content from metal contaminated sites (For review see Kling and Olsson, 2005). Since MTs are highly conserved in structure and transcriptionally regulated, measurement of protein expression and transcript can be carried out with ELISA and qPCR, respectively. Indirect methods of sulfhydral content can also be used. The liver is the primary site of residence, but kidney and gill also express measurable content. Expression can be measured in embryos and larvae, but not in fixed tissues.

Strengths

Expression of MT is transcriptionally up-regulated in response to exposure to metals. Thus, induction can be measured at the level of mRNA by quantitative PCR. Expression of MT is also observed as part of the generalized positive acute protein stress response associated with cellular injury. Thus, MTs can also be induced by extracellular inflammation, starvation, and oxidative stress resulting from disease or chemical exposures (Kling and Olsson, 2005).

Applications

Since metal deposition can occur in areas besides target organs, MT can be used to evaluate bioavailable metals within target organs such as gill, liver, and kidney (Palace et al., 2003). Several investigators have indicated that simple tissue residue measurements of metals would provide the same information as MT and be a better indicator of exposure and possibly effect. However, in laboratory studies using channel catfish, comparisons of various whole-animal endpoints such as growth, liver weight and condition factors with hepatic MT expression and copper content (after a 10 week

exposure to copper), it was determined that MT protein expression consistently exhibited stronger correlations to each endpoint than did hepatic copper content (Perkins, et al., 1996). In addition, strong correlations were observed between lipid peroxidation and MT in trout chronically exposed to zinc and copper (Farag, et al., 1995). These data suggest that MT may also be useful as a biomarker of effect, but only effects mediated by MT-binding metals. An excellent review of field studies using MT is presented in Van der Oost et al. (2003).

Weaknesses/Caveats

While several studies have shown relationships between MT expression and metal bioavailability, other studies have failed to demonstrate relationships primarily due to influences from non-metal inducers, sexual development or seasonality (Stegeman et al., 1992). Significant developmental and gender-related differences in MT expression have been observed within fish (Olsson, 1996). Regarding the latter, sexually mature female fish again may be poor choices to conduct biomarker studies with MT because they possess enhanced hepatic and serum concentrations of the zinc-containing egg yolk precursor protein, vitellogenin. Consequently, in certain species such as trout, MT levels tend to be repressed by estradiol in sexually mature females (Olsson, 1996). Since MT can be an indicator of acute as well as chronic stress, it is imperative that other acute phase proteins be measured to verify an acute stress condition (see HSPs below). Moreover, handling of animals should be minimized in order to enhance the signal to noise ratio between exposed and control samples. This is especially important in conducting cage studies where animals should be properly acclimated.

Summary/Recommendations

In summary, MT is not a useful measurement of metal bioavailability in the field. However, when used in controlled exposures (i.e. caged animals) in well-studied species (i.e. trout, channel catfish, turbot, mussel, oyster, crab) in conjunction with other endpoints, specifically measurements of oxidative damage, a better indication of metal effect and exposure may be obtained. Overall, extreme caution should be used when attempting to apply MT as a bio-indicator of stress. Although several laboratory and aquaculture-based studies indicate relationships with stress, feral variability and the documented incidence of adaptive expression of MT in animals surviving long-term exposure to stressors limits its usefulness as a consistent stress endpoint. Thus, given a limited budget, metal analyses are probably a more cost effective indicator for feral animals. However, utilization of caged animals or exposure of embryos in a laboratory setting may provide better indications of cellular stress. Given the potential inputs of copper and mercury (each of which are excellent inducers of MT) in San Francisco Bay, this may be something to pursue if additional resources are available.

2.3.4 Cholinesterases

Introduction

Acetylcholinesterase (AChE) (Appendix 10) catalyzes the hydrolysis (and hence inactivation) of the neurotransmitter acetylcholine (ACh) in cholinergic nerves (Ecobichon, 1996). Inhibition of AChE resulting in over-accumulation of ACh and

prolonged electrical activity at nerve endings comprises a key mechanism of toxicity for organophosphorous (OP) and carbamate insecticides that are major use insecticides currently. AChE inhibition by these compounds occurs through the formation of a covalent bond between the active site of the enzyme and the insecticide. The consequences of AChE inhibition depend upon the nervous tissue affected and include cardiovascular, respiratory and gastrointestinal dysfunction (parasympathetic autonomic tissue), hyperactivity, lethargy and unconsciousness (central nervous system), and muscle weakness, respiratory collapse and paralysis (somatic motor nerve fibers). Activities can be measured in rapidly frozen, whole tissue homogenates of embryos and larvae (non-fixed). In juveniles, brain, blood and muscle are the best tissues. In addition, for larger fish, such as adult striped bass, noninvasive techniques are available to sample blood.

Strengths

AChE and other cholinesterase activities (such as butyrylcholinesterase which preferentially hydrolyzes butyrylcholine) occur in brain, muscle, red blood cells and plasma. While not functional in nervous system physiology, activities in blood cells and plasma provide useful surrogate markers for nerve fiber AChE activity and can be measured non-destructively. Measurement of AChE in brain tissue and of cholinesterase activity in blood has been employed to monitor exposures to fish and wildlife to OP and carbamate insecticides with good success (Fulton and Key 2001). The utility of this metric is enhanced by its relationship between mode of toxic action and by the difficulty of measuring tissue levels of these compounds themselves, which is complicated by the number of compounds that may occur at a given field site and by their frequent rapid metabolism in animals. Methodology for measurement is relatively simple as AChE activity can be measured by using Ellman's reagent (DTNB) and acetylthiocholine as substrate.

Applications

Examples of applications for acetylcholinesterase in field settings are provided in Fulton and Key (2001). Given the important role of acetylcholine in neurological function, disruption of its breakdown can be utilized to assess neurological and behavioral endpoints. Reductions of acetylcholinesterase activity in fish from OP-polluted sites was observed in muscle tissues of brown trout and flounder in Newfoundland, Canada (Payne et al., 1996). Muscle activities in the three-spined stickleback from parathion polluted streams Germany was also diminished (Sturm et al., 1999, 2000). In a more recent study, Whitehead et al. (2004) examined acetylcholinesterase activity in fish caged in the San Joaquin River and tributaries as well as in fish exposed to field-collected water in the laboratory. Exposures were timed to coincide precisely with runoff of dormant spray pesticides from agricultural fields in the Central Valley. Despite the precise timing of the investigation, low organophosphate and carbamate levels were detected and consequently acetylcholinesterase induction was not significant.

Weaknesses/Caveats

Variability in enzyme activity measurements can be substantial because inhibition of cholinesterases by OPs is generally irreversible, but inhibition by carbamates is

reversible. That is, typically over a timeframe of hours, the carbamate is released by the enzyme, thereby regenerating active enzyme, even in a moribund animal. Therefore, cholinesterases are considered more reliable markers for exposures to OPs than to carbamates. Since the enzyme activity is based upon the oxidation of thiol groups from acetylthiocholine and Ellman's reagent (DTNB), metals tend to disrupt the assay. Enzyme values should also be normalized to length of the organism as recent studies by Rodriguez-Fuentes et al. (2007) indicated in flatfish collected near reference and oceanic wastewater outfall sites in Southern California. Site specific differences were observed in acetylcholinesterase activities in muscle but size-dependent differences were also observed and shown to correlate with activity.

Summary/Recommendations

Cholinesterase measurements within blood and plasma are a simple non-destructive method that evaluates the bioavailability of organophosphate or carbamate pesticides. Species-specific laboratory testing to establish thresholds of inhibition relative to neurological or behavioral endpoints (See Scholz et al., 2006) can be utilized to link exposure to effect. Kinetic issues should be considered in the case of false negatives within field sampling. Initial evaluations in brain, blood, and muscle of fish from pesticide contaminated sites in winter through spring would provide evidence of bioavailability of OPs or carbamates during critical spawning periods. Laboratory studies using field-collected water during winter-spring can be carried out using embryonic stages to determine whether linkages exist between enzyme activities and development.

2.3.5 Stress Proteins

Introduction

Stress proteins, originally known as heat shock/stress proteins (Appendix 11), are a non-specific group of positive acute phase proteins, which serve several protective and homeostatic functions within the cell. The number and exact size of heat shock proteins are specific to both tissue and species. Except for the highly inducible proteins of the HSP70 family (specifically HSP72), all of these proteins are present in low concentrations under normal conditions in most organisms studied (Iwama et al., 1998). Proteins of the HSP 70 and 60 families are primarily chaperone proteins that are up-regulated during proteolysis and aid cells in the folding and transport of newly synthesized proteins. They have been characterized in several fish species and consistently respond to numerous organic and inorganic chemicals (Iwama et al., 1998).

There is compelling evidence suggesting HSP70 may be a useful bioindicator of general cellular stress due to proteolysis. Although the induced synthesis of the protein is transient, the turnover is much less rapid and the proteins tend to accumulate upon continued cellular stress (Van der Oost, 2003; Geist et al., 2007). In addition, the kinetics of induction appear to be longer following chemical-induced stress compared to heat-induced stress, and recovery is not achieved until several days following exposure to metal, presumably due to accumulation of the metal in the cell (Stegeman, et al. 1992). Since HSPs are transcriptionally regulated, qPCR as well as immunoquantitative

methods can be used in rapidly frozen but not fixed embryo, larval, and juvenile tissues (preferably the liver, but also the gill).

Strengths
Each protein is highly conserved and antibody as well as nucleotide probes to mammalian forms have been shown to recognize homologous forms in numerous fish and invertebrate species (Stegeman et al., 1992). Evidence supporting HSP70 as a biomarker of effect was also observed in β-naphthoflavone (BNF) treated rainbow trout, in which altered metabolic status of the liver as evidenced by lower phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase and 3-hydroxy-acyl-coA dehydrogenase activities correlated to hepatic HSP70 expression. This study also showed that HSP70 in this species was not modified by handling stress (Vijayan et al., 1997). Relationships between HSP70 expression and ovarian follicular apoptosis were observed in white sucker exposed to bleached kraft pulp mill effluent (Janz et al, 1997). Seasonal differences in HSP70 expression have also been observed (Iwama et al., 1998).

Applications
As an acute stress protein, measurement with appropriate baseline and calibration in tissues such as gill and liver, cellular stress due to proteolysis can be evaluated. Responses in embryos and larvae can also be assessed under laboratory conditions following exposure to site water or specific agents of concern

Weaknesses/Caveats
Overall, significant gaps remain in characterizing basal activities of piscine HSPs especially regarding potential susceptibility, gender-related, and developmental differences. Consistent relationships between HSP expression and adverse effects of reproduction and growth in feral animals have not been observed.

Summary/Recommendations
While HSPs have shown relationships to adverse effects in controlled-exposure situations, relationships in the field have been less consistent. It is recommended that these proteins only be used in conjunction with other acute phase protein markers (i.e. MT) to verify effect in controlled exposure settings. Thus, if embryos and larvae are exposed to site-collected water, HSPs could be used and potential linkages to development evaluated.

2.3.6 Glutathione Biosynthesis and Antioxidant Enzymes

Introduction
A number of disease etiologies or chemical toxicities occur via a process termed *oxidative stress*. Oxidative stress occurs in all living organisms when small molecules termed *reactive oxygen species* (or their by-products) cause cell and tissue injury (Winston, 1991; Kelly et al., 1998). Oxidative stress is experienced to varying degrees when the antioxidant defenses present in cells and tissues are overcome by pro-oxidant forces. The process of oxidative stress contributes to physiological aberrations such as

atherosclerosis, cancer, neurodegenerative diseases and also ageing (Winston, 1991). Exposure to certain types of environmental contaminants, including pesticides, metals, and PAHs may cause oxidative stress in aquatic organisms (Winston, 1991; Kelly et al., 1998; Livingstone, 2001; Pandey et al., 2003; Banni et al., 2005; Farombi et al., 2007). Aquatic organisms contain the major antioxidant enzymes which comprise antioxidant defenses, although there can be quantitative differences in the normal levels of these constituents as well as their induction among the various aquatic species (Kelly et al., 1998). The fact that often the responses in fish exposed to these pro-oxidant xenobiotics can be similar to those of mammals suggests similarities in the health consequences of exposure to these compounds. Accordingly, the levels of these antioxidants have been used in the field as an indicator of chemical-induced oxidative stress (Livingstone, 2001; Pandey et al., 2003; Banni et al., 2005; Farombi et al., 2007).

Some of the common antioxidant enzymes and antioxidants (Appendices 12 and 13) include:

1. *Superoxide dismutases (SODs)***,** enzymes that are present in cells that scavenge particularly damaging reactive products, superoxide ($^{\cdot}O_2^-$), by a rapid dismutation biochemical reaction.
2. *Catalase*, an enzyme located in the peroxisomal compartment of cells that detoxifies another toxic pro-oxidant,*hydrogen peroxide* (H_2O_2). Both superoxide and hydrogen peroxide are common toxic products produced in higher concentrations during the process of cellular oxidative stress.
3. *Glutathione peroxidases* (GPx), are several different enzyme forms that catalyze the reduction of organic peroxides, which are potential free radical forming species within the cell. There are several cytosolic, selenium-containing GPxs as well as an extracellular form. These enzymes also detoxify H_2O_2 and may be important for maintenance of low intracellular levels of H_2O_2..
4. *Glutathione (GSH),* is a small molecular weight thiol, and not an enzyme, that is found in relatively high concentration in all cells. The main functions of GSH include providing reducing power needed by glutathione peroxidase enzymes to carry out the reduction of organic peroxides and also as a cofactor and conjugation reactions. These reactions are largely protective and detoxifying. GSH can directly reduce a number of reactive oxygen species, and during this process, GSH is oxidized to a more non-active oxidized form termed *oxidized glutathione* (abbreviated GSSG). Relatively high ratios of the reduced to oxidized forms (GSH/GSSG) are maintained in normal cells, but these ratios can be reduced under oxidative stress (Conway et al., 1989). The total depletion of cellular GSH during oxidative stress is rare, but when it occurs can have a significant impact on toxicity (Monroe and Eaton, 1988). Also, exposure to certain chemicals can increase tissue GSH concentrations (Dickinson et al., 2004; Iles and Liu, 2005; Lee et al., 2006). This occurs through a two enzyme pathway involving the enzymes *glutamylcysteine ligase* and *glutathione synthetase*. Of the two enzymes, *glutamylcysteine ligase* levels are often co-elevated with GSH on exposure to some chemicals and metals (Dickinson et al., 2004; Chen et al., 2005; Iles and Liu, 2005). Studies of GSH biosynthesis in aquatic animals are limited, and most of the data involving chemical exposures and perturbations to GSH, GSH/GSSG ratios levels and the activity of the glutathione-associated enzymes (Gallagher and Di Giulio, 1991;

Winston, 1991; Gallagher et al., 1992; Pandey et al., 2003; Farombi et al., 2007; Prieto et al., 2007).

When the protective antioxidant parameters (*i.e.* glutathione, antioxidative enzymes) are overwhelmed during the process of oxidative stress, the organism may shows signs of damage via the appearance of metabolic byproducts reflecting *oxidative damage*. These metabolic byproducts include oxidized pigments such as lipofuscin, peroxidized cell membranes and their ensuing membrane breakdown products (*i.e*. lipid peroxides, malondialdehyde) and also oxidative DNA and protein damage. Lipid peroxidation is more routinely measured in field studies to reflect chemical-induced oxidative damage.

Strengths

Numerous studies in mammals and fish have demonstrated that antioxidant enzymes are often up-regulated, particularly in response to toxicant exposures. A major strength of these markers is that they can link chemical exposures to adverse effects *(i.e. chemical-induced oxidative stress)*. The occurrence of oxidative stress can relate to the health status of the organism and has been linked to the pathogenesis of a number of diseases. Furthermore, if performed correctly, the antioxidant biochemical assays are specific, and typically are not as technically challenging to carry out as the genomic approaches described below.

Applications

A host of field studies have evaluated the impacts of oxidative stress in natural populations. The preferred approach has been assessment of lipid and DNA damage combined with changes in the levels of antioxidant defenses to serve as a means of detecting exposure to xenobiotics that induce oxidative stress. For example, elevated antioxidant enzyme parameters associated with increased lipid peroxidation have been observed in F*undulus* exposed to complex mixtures of organic chemicals in the Elizabeth River, Virginia (Bacanskas et al., 2004). Similar results have been reported for bivalves (Banni et al., 2005). Because the response of the organism may be both xenobiotic- and also tissue-specific, it is also important to examine several endpoints related to oxidative stress and several tissues. For example, Otto and Moon (1996) compared brown bullhead (*Ameriurus nebulosus*) collected from a system polluted with PCBs to bullhead collected from a relatively non-polluted site. Fish from the polluted site had extremely high PCB concentrations in muscle compared to fish from the non-polluted site, and SOD activity was increased in the kidney of fish from the polluted site. However, the activity of another antioxidant enzyme, catalase, was lower in the kidney, and there were differences in the trend of expression of other enzymes. Studies such as these highlight the importance of tissue selection when evaluating oxidative stress in feral organisms.

The use of a comprehensive suite of markers within a single target tissue can sometimes yield results that are difficult to evaluate biologically. For example, McFarland et al. (1999) investigated site differences in liver antioxidant parameters in brown bullheads in a PAH-contaminated and a control site in Ohio (McFarland et al., 1999). In that study, the levels of the enzymes glutathione reductase, glutathione *S*-

transferase, and glutathione peroxidase were measured, but no site differences were present. In contrast, other antioxidant parameters such as SOD, catalase, and total tissue glutathione levels appeared to correlate with environmental exposure to PAHs. In another study, 11 biochemical markers of aquatic pollutants were evaluated in the livers of chub caught at several sampling sites of a river containing various pollutants (Machala et al., 2001). The results were tested against analytical data of concentrations of organochlorine compounds, polycyclic aromatic hydrocarbons (PAHs), and heavy metals. The biochemical markers of oxidative stress, including *in vivo* lipid peroxidation and *in vitro* production of reactive oxygen species, did not correlate with the concentrations of the contaminants. In contrast, glutathione-dependent enzymes formed a suitable battery of exposure biomarkers (Machala et al., 2001). These studies are among many that illustrate the complexities associated with drawing meaningful conclusions of differential antioxidant responses observed in the field. As discussed below, for application to the POD, it will be important to establish a profile of antioxidant parameter and oxidative damage modulations in POD species in the laboratory that could be a benchmark for chemical exposures in the field.

Weaknesses/Caveats

Problematic to the POD is that there has been very little attention given to biological and environmental influences on antioxidant enzyme expression and GSH biosynthesis in fish. Studies in other species indicate that these antioxidant defenses are dependent upon species, age, nutritional status and disease state. In particular, dietary and nutrition status can markedly influence glutathione levels (Wu et al., 2004). The influence of biological and environmental variables on antioxidant enzyme expression and GSH status are critical research needs. It is likely that dissolved oxygen, as well as temperature and nutritional status influence the expression, catalytic activity, or inducibility of these enzymes. On review of the aquatic literature, there also appears to be a significant degree of variation in the basal activities of antioxidant enzymes across species. Another source of uncertainty is the lack of knowledge of species differences in the inducibility of these pathways under oxidative stress. Finally, the presence of oxidative damage byproducts (lipid peroxides, etc) can be present as result of bacterial infections and nutritional abnormalities (Prieto et al., 2007).

Summary/Recommendations

There is considerable evidence indicating that antioxidant enzymes are altered in many fish species exposed to chemical pollutants in the field. When viewed collectively, however, the field studies also indicate species differences in responses. Particularly problematic in the context of the POD are the lack of studies delineating the effects of biological variables (age, disease status) or environmental variables (dissolved oxygen, starvation, etc) on antioxidant enzymes in striped bass, threadfin etc. It is also likely that the antioxidant aforementioned variables are likely to be influenced by age, and disease status. Accordingly, these markers may not be effective in the field context of elucidating the role of chemical exposures in the etiology of the POD. However, these indicators could be useful to help in the assessment of fish health status, especially if used in the context of a comprehensive suite of biochemical, histological, and physiological indices and including control animals. It is also important to note that the

accurate measurement of these cellular markers of oxidative damage can be influenced by sample handling and methodology employed. Accordingly, the laboratory conducting these analyses should have experience with these techniques. Given the constraints described above, substantial research is required for effective use of these techniques. Based upon the aforementioned caveats, the following recommendations are provided for the use of these markers should they be utilized:

- Establish dose-response curves to model oxidative stress-producing environmental chemicals in the laboratory using POD, and not surrogate species. These studies should address the fact that inhibition of each of these enzyme systems leading to *lower levels* of enzyme expression or GSH concentrations may occur under oxidative stress.

- For eventual use of antioxidant parameters in field situations, determine how natural fluctuations in oxidative responses occur in the POD species, as well as effects of biological variables (sex, reproductive status, age, nutritional status) and also the most relevant environmental variables (i.e. dissolved oxygen, salinity, and temperature, etc) on these parameters.

- Use oxidative stress markers in the context of a comprehensive suite of biochemical, histological, and physiological indices.

- Use antioxidant enzymes and GSH status with markers of oxidative damage, such as oxidatively damaged DNA, proteins or lipids to better link exposures or stressors with tissue injury.

2.3.7 Vitellogenin and Choriogenin

Introduction

Vitellogenin (Vtg) (Appendix 14) is an egg-yolk precursor protein produced in the liver of oviparous animals through the activation of estrogen receptors by endogenous and xenobiotic estrogens (Tyler et al., 1996). As the final expression of protein encompasses the entire Hypothalamus-pituitary-gonad (HPG) endocrine axis, it is an excellent indication of estrogenic activity within an animal (Hiramatsu et al., 2005). Expression of the protein in male or juvenile sexually immature animals has been utilized to monitor surface waters for the occurrence of estrogenic compounds. Since the protein is synthesized in the liver and transported to the oocyte, blood or liver can be sampled to measure expression of the protein or mRNA. Measurements can be made in embryo and larvae (including fixed tissues) by immunochemical methods as well as by qPCR.

Choriogenins (Chgs) are the precursors of the fish egg envelope, the chorion. Like Vtg, these proteins are produced in the liver in response to estrogens. Two Chgs are common in fish and these are Chg L and Chg H (Murata et al., 1997). Chgs have been used as indicators of endocrine disruption (Celius et al., 2000; Arukwe et al., 2000;

Scholz et al., 2005; Anderson et al., 2006; reviewed by Arukwe, 2001). Both Chg and Vtg have been measured in fish mucous (Meucci and Arukwe, 2005), and this represents a promising easy method for sampling large numbers of fish.

Strengths

Since expression is specifically controlled through the estrogen receptor, both Vtg and Chg are excellent indicators of estrogen exposure or alteration of endogenous estrogen metabolism or the HPG axis, particularly in male oviparous animals. For many species, measurements can be made utilizing ELISAs or western blots using commercial kits or antibodies, or by qPCR for mRNA. Protein can be measured in blood plasma so non-destructive sampling can be carried out in sexually dimorphic species (Anderson et al., 2006). The presence of the proteins in the blood is typically a much longer response than mRNA expression in the liver, and therefore is probably more practical for field sampling of fish. In addition, ELISA analysis for protein is similar in sensitivity as mRNA detection in some studies, and perhaps less variable between individuals (Celius et al., 2000). It should be noted that Chgs possess a highly conserved "ZP domain" (Kanamori et al., 2003) and typically, antibodies to Chgs will cross-react with Chgs from other species. This is a real strength of the use of choriogenins as a biomarker in poorly characterized species. Consequently, the antibody should recognize Chg of Striped bass and delta smelt. Vtgs are typically more unique between phylogenetic groupings of fish, and it can be expected that antibodies for species previously not studied may need to be developed.

Applications

The primary use of this biomarker is to evaluate biochemical feminization of male or sexually immature fish. Livers, blood/plasma or mucous from feral animals can be utilized. Blood/plasma provides the best signal:noise ratio of response and can be utilized for non-lethal sampling in larger animals. If vitellogenin is detected in male or immature animals, the estrogen receptor has been activated. This may occur by the xenoestrogen or by endogenous estrogens (i.e. estradiol) which have accumulated due to shunts in synthethic pathways (i.e aromatase) inhibition of degradative pathways, or augmentation of the hypothalamus-pituitary-gonadal axis (i.e. gonadotropins). For reviews of these markers in field studies, see Hiramatsu et al. (2005). Recent studies in Southern California have indicated consistent expression of Vtg in male flatfish collected near wastewater outfalls without significant impacts on gonadal histopathology, development, gender ratio, or abundance (Roy et al., 2003; Rempel et al., 2006; Deng et al., 2007). In contrast, a recent study by Kidd et al. 2007 demonstrated induction of Vtg in male fathead minnows in a lake that had been treated with the synthetic steroid, ethinylestradiol prior to the complete extinction of this species within two years. Clearly in this species, induction of Vtg was an early warning for the population collapse. Another application for these tools is their use in identifying causative agents of feminization in New York City wastewater as well as sediments from wastewater outfalls of Los Angeles and Orange Counties, Vtg was employed in bioassay-guided fractionation (TIE) experiments coupled with analytical chemistry (Sapozhnikova et al., 2005; Schlenk et al., 2005). Results indicated nonyl phenol isomers in active fractions of NYC wastewater, but no sex steroids. Similarly, in southern California, steroids were

also not observed in biologically active fractions. Currently, a similar study funded by CalFed is being carried out in the Central Valley and Sacramento Delta (Schlenk, personal communication).

Weaknesses/Caveats

While the protein is ubiquitous in oviparous animals, measurement in the laboratory can be impaired due to species-specific sequences in protein structure. Consequently, care must be taken when using commercial kits for ELISA measurements to be sure the detection antibody in the kit specifically measures vitellogenin in the species of interest. Although expression of Vtg is an excellent indicator of bioavailable exposure to estrogenic or feminizing compounds, its relationship to impaired reproduction or growth is not consistently demonstrated in the field, but is more frequently observed in controlled exposures in the laboratory. mRNA expression may occur over a shorter timeframe than protein expression and thus could be more limited in use in field sampling programs.

Summary/Recommendations

Expression of Vtg and Chg in male or juvenile animals is an excellent indicator of exposure to estrogenic/feminizing influences in the field. Relevance of these biomarkers to reproduction, growth and population declines is still unclear in most species and thus requires synthesis with endpoints such as gonad-somatic index or other fitness metrics. Since it is likely that Chg antibodies will recognize multiple species, it is recommended that samples of blood from field-collected adult males and sexually immature animals be obtained and evaluated for Chg first. These measurements need to compared with higher order endpoints such as gonadal histopathology or gender ratios to determine whether exposure is linked to adverse population effects. Vtg can also be measured and should be considered an important alternative as Chg assays are refined, but development of species specific antibodies for ELISA or sequence information for qPCR analyses may involve added time. In general, protein measurements may be most useful on field fish because proteins remain in the blood longer; however, mRNA expression may be most sensitive in laboratory studies.

2.3.8 Immune Function

Introduction

A functional immune system is necessary for survival, and may be impaired by chemicals and other stressors. The immune system of fishes, like other vertebrates, is a complex, interacting system comprised of non-specific (innate) and specific (adaptive) components. The innate component is an important first line of defense, characterized by responses that are broad, immediate, and independent of prior exposure. Important cellular components include phagocytic cells, which ingest foreign agents and stimulate lymphocytes (and other immune cells) to respond to pathogens, and cytotoxic lymphocytes, which kill tumor and virally-infected cells. The cellular component of innate immunity provides an important defense against bacterial, viral, and protozoan stressors (Dautremepuits et al., 2006). Adaptive immunity is characterized by

recognition and memory of specific antigens, including those associated with pathogens and parasites (Warr, 1997). Antibodies (immunoglobulins), generated in response to unique antigens, appear in peripheral circulation at a relatively slow rate compared to innate responses, but may persist (Dautremepuits et al., 2006). Some studies have linked alterations in the adaptive immune system (i.e., poor antibody production) to reduced disease resistance (Arkoosh and Collier, 2002). However, the innate immune may be more important than adaptive immunity for ensuring bacterial resistance in fish (Maule et al., 1996).

Immune system impairment (Appendix 15) is defined empirically by increased susceptibility to infectious disease (under controlled conditions) and associated with unusual and high rates of infection and/or parasitism in field-collected fish. Immunological impairment in fish is often assessed by quantifying factors associated with innate (e.g., lysozyme) or adaptive immunity (e.g., total immunoglobulins, IgMs). It is less often assessed using functional assays, such as phagocytosis or respiratory burst, which assess functionality of resting rather than activated immune cells (e.g., review Kollner et al., 2002). However, a framework of static indicators (instantaneous concentration or activity), functionality tests, and standardized challenge routines provide a comprehensive assessment of fish immune status and function (e.g., Zelikoff, 1998, Zelikoff et al., 2000, Kollner et al., 2002).

Strengths

There are well-established and more exploratory (i.e., genomic) biomarker and assay methods to assess immune function in live field-caught, explanted, or laboratory-exposed juvenile and adult fish (e.g., Dean et al., 1982; Arkoosh et al., 2005), including striped bass (e.g., Polonio et al., 2000). Fish blood and lymphoid tissues (anterior kidneys and spleen) are useful, even from fish as small as 5 cm length. The most promising biomarkers are robust and broadly applicable, and do not require the development of species-specific markers. As measures of innate immune function, plasma and lymphoid lysozyme and lymphoid cyclooxygenase-2 (COX-2) are unaffected by general stress and serve as sensitive indicators of the physiological and functional state of lymphoid phagocytes in all vertebrates (e.g., Frederick et al., 2007). Thus, lysozyme (e.g., Beckham et al., 2005) and COX-2 activities (e.g., Frederick et al., 2007) may provide good indicators of innate immunity in field collected fish. Specific and total immunoglobulin (IgM) levels are not overtly modified by handling stress and thus are reliable indicators of adaptive immune function status at the time of capture (Frederick et al., 2007). Evidence of inadequate immune response (disease) and severe reductions in immune biomarkers could suggest, but are not diagnostic of, chemical immunomodulation; study design requires concurrent, similar samples (species, life stage) from unstressed controlled or field conditions to assess relative impairment in field-collected fish.

Applications

Many studies have shown that exposure of fish to several categories of chemicals alters immune function and/or disease susceptibility via direct or indirect (general stress) mechanisms (e.g., Fries, 1986, review; Yada and Nakanishi 2002). For example,

altered immune measures and disease response have been observed following exposures to heavy metals including mercury, selenium, copper, and PCBs (e.g., Rice et al., 1996, Frederick et al. 2007). PCBs have been shown to have direct effects on the adaptive immune system and to decrease resistance to bacterial (Arkoosh and Collier, 2002) and viral (Spitzbergen et al., 1988) pathogens. PAHs suppress immune function in many fish species examined (e.g., Weeks and Warinner,1986; Arkoosh et al., 1991, 1996; Carlson et al., 2002, 2004). A number of field studies have shown alterations in immune function of chemically-exposed fish by measuring aspects of innate and adaptive immune status concurrent with measurements of condition and other general indicators of health (review Hyland et al., 2003). One particularly comprehensive series of studies suggest that with appropriate validation of immune biomarkers can contribute to an understanding of the relative importance of chemical exposures to fish population declines. Specifically, the incremental risk to wild Pacific salmon populations of Puget Sound (WA, USA) associated with immunological impacts from PCB and PAH exposures was estimated using a stepwise progressive ecological risk assessment approach, beginning with assembling information supporting a logical argument linking contaminants and effects (McCain et al., 1988), characterizing field exposure (McCain et al., 1990; Stein et al. 1995) and laboratory dose-responses (Arkoosh et al 1991, 1994, 1998, 2001), then integrating this information using a risk characterization (Arkoosh and Collier, 2002) and a predictive disease model (Loge et al., 2005).

Weaknesses/Caveats

Alterations in molecular and cellular immune components support interpretations of immune impairment in the field and can provide mechanistic links to chemical exposures. However, impaired immune function is not diagnostic of any single stressor because many categories of chemicals, general stress and natural factors can also influence immune alterations. For example, temperature, salinity, and dissolved oxygen changes may modulate immune function indirectly via a general stress response (review Yada and Nakanishi, 2002). Spawning may also be a period of increased vulnerability to disease and other stressors, although much of research in this area reflects semelparous (and therefore, aged) fishes (e.g., salmon, Schreck, 1996). Explorations in immunogenomics research using model species such as zebrafish (e.g., van de Sar, 2004) and salmonids (e.g., Merilainen et al. 2007, and other references from this group, using salmonid species) are promising but are currently limited by the availability of immunogenomic tools.

Summary/Recommendations

Stressors including chemicals can alter immune function and reduce fish survival, so immune biomarkers are recommended as indicators of pollution effects in large biomonitoring projects (Haggar et al. 2006). Furthermore, in conjunction with histopathological indicators of increased infections and infestations, biomarkers provide powerful indicators of altered biological function that have been correlated with chemical exposures. However, development and laboratory validation of immune biomarkers has begun for only a few fish species (but include striped bass), so informative comparisons must be made among populations or control groups. Several biomarkers can be

assessed using small samples of blood/lymphoid tissue from field-collected fish (e.g., Frederick et al. 2007), and some have general applicability for many fish species (Frederick et al., 2007, Haggar et al. 2006). Considering these attributes, the most promising biomarkers that can be applied to POD include measures of innate immune function, plasma and lymphoid lysozyme and lymphoid cyclooxygenase-2 (COX-2) and total immunoglobulin (IgM) levels. Thus, we recommend that when condition indicators (histopathology) provide evidence suggestive of immune alteration, then immune biomarkers, such as these, be applied to field-collected fish. Taken together with experimental work using species such as striped bass linking specific chemical exposures and immune alterations, these results will contribute to understanding mechanisms of contaminant effects on POD. Based on precedence with Pacific salmon (albeit representing a great deal of work) and with appropriate context, immune biomarkers have good potential to assist in understanding the relative contribution of chemicals to declining fish populations, including species of the San Francisco Estuary.

2.3.9 DNA Damage

Introduction

Genotoxic agents interact with and modify DNA. If these changes are not repaired, cell function may be affected, and following replication, alterations become fixed as mutations associated with carcinogenesis and poor viability of germ cells (review, Monserrat et al., 2007). DNA damage to sperm cells has been correlated with reduced fertility in invertebrates (Lee and Steinert 2003), chromosomal damage to abnormal development of sea urchins (Anderson et al., 1994), DNA adducts to decreased growth in tadpoles (Sadinski et al., 1995) and DNA content variation to frequency of pollutant tolerant fish (Theodorakis et al., 2000). DNA damage is recognized as important (e.g., ICES 2004), and several biomarkers have been developed that measure specific aspects of DNA damage in fish. These biomarkers are generally categorized as those measuring changes to DNA structure/integrity or chemical alterations in DNA components.

Methods typically used to assess DNA integrity in cells of aquatic species can include cellular and molecular approaches (Appendix 16). Two commonly used techniques are the single cell gel electrophoresis (SCGE) or comet assay and the micronucleus test (MT). The comet assay measures single- and double-strand breaks as well as alkali-labile sites and excision repair events (Singh et al., 1988). Micronuclei represent small cellular chromatin bodies that are produced following cell division from damage to the spindle apparatus or chromosome breaks (Schmid, 1975). Both of these methods are responsive to genotoxic exposures and have been applied to field-collected fish (e.g., Russo et al., 2004). Flow cytometry is also used in fish to estimate DNA integrity as variation in nuclear DNA content, which represents post-DNA repair DNA damage (e.g., Farag et al., 2006) and has been applied in numerous field studies with rodents and some aquatic species (Theodorakis et al., 2000). Anaphase aberrations have been evaluated in embryos of aquatic species, including herring in Prince William Sound.

Another category of methods indicate specific types of DNA damage related to a causal agent. These methods measure specifically altered components of DNA such as oxidatively modified or DNA-adducted nucleotides. This method is diagnostic of specific adduct-producing agents such as PAHs and has been used for the detection of bulky adducts in fish following laboratory and field exposures (e.g., Nacci et al., 2002; Akcha et al., 2004; Lyons et al.. 2004). DNA adducts are persistent lesions and hence are good indicators of long term exposures.

Strengths

Of those biomarkers that measure DNA integrity, the comet assay has been used frequently to measure DNA strand breaks in tissues of aquatic species, using cells that occur singly, such as nucleated blood cells, or cells dissociated from tissues of interest, such as gill, liver, and sometimes, sperm cells. It is simple and accessible, and has been validated in the laboratory with known genotoxic chemicals or physical agents (e.g., Nacci et al. 1996; review Lee and Steinert, 2003), as well as other chemicals, including pesticides such as endosulfan (Pandey et al., 2006), and metals including chromium (review Farag et al., 2006). Using the Comet Assay, Whitehead et al. (2004) demonstrated potent genotoxic activity in the San Joaquin River in two years of parallel field and laboratory studies which included Ames assays to further validate genotoxic activity in the water. Other field studies have observed reduced DNA integrity in sperm of environmentally exposed fish (Rempel et al., 2006) and livers of fish exposed to known endocrine disrupting (estrogenic) chemicals (Rodriquez-Ariza et al., 1999). Several studies have shown the utility of applying, to the same sample type, both the MT and comet assays, which provide complementary information and can sometimes be used to infer genotoxic mechanisms (e.g., Amado et al., 2006 a,b). Of those biomarkers developed for specific types of DNA damage, i.e., oxidatively modified or DNA-adducted nucleotides, DNA adducts provide documentation of specific, bio-effective exposures (to important target molecules), and this approach has been best developed for PAHs. The standard methodology, while specialized, uses small samples, is very sensitive, and is recommended for international biomonitoring (ICES 2004;Hagger et al. 2006,). When used together, DNA integrity assays as well as measurements of chemically-modified bases may provide complementary information because these endpoints reflect different processes with varying temporal characteristics (e.g., Flammarion, et al. 2002).

Applications

For fish, nucleated red blood cells (erythrocytes) are often used, and sufficient samples can be obtained for strand break and MT even from larvae >= 5 cm (e.g., small fish, e.g., sticklebacks with sizes ranges from 3 – 6 cm). However, gill, liver, and kidney have also been used in small adult fish (e.g., Pandey et al., 2006); and these tissues might better reflect contaminant exposures (Brown and Steinert, 2003). Factors such as sex, size, and seasonal influences, can affect apparent DNA integrity (Wirzinger et al., 2007). Fish species vary in their background levels of DNA damage, and their sensitivity to genotoxic damage (Rodriguez-Cea et al., 2003). Species variation and variation among tissues and cell types is related to such factors as DNA repair capacity, metabolic activity, and antioxidant factors, which contributes to differences in test results

for similar exposures (Lee and Steinert, 2003; Akcha et al., 2004; Farag et al., 2006). The DNA ^{32}P post-labeling technique is a sensitive, reproducible, but technically specialized method used for the detection of bulky adducts in fish following laboratory and field exposures (e.g., Nacci et al., 2002; Akcha et al., 2004; Lyons et al,. 2004). An alternate, easier but less sensitive method using immunofluorescent antibodies for PAH adducts has been used in limited studies (Machella et al., 2005). In addition, oxidative DNA damage, assessed as 8oxodG (7, 8-dihydro-8-oxo-2'-deoxygaunosine), has been used to detect DNA damage in livers of fish exposed to dieldrin and estrogenic chemicals (Rodriquez-Ariza et al., 1999). While methodological difficulties have plagued the detection of for DNA oxidative damage, an immunoperoxidase method uses small volumes of fixed or frozen cells, and has been laboratory-validated on several marine organisms (Machella et al., 2004).

Weaknesses/Caveats

While chemical exposures can certainly contribute to increased DNA strand breaks in fish tissues, technical artifacts and natural factors including sex, age, season, and condition can affect results. Therefore, results are relative to control or reference fish, and are not diagnostic of specific chemical exposures unless caution is taken in parallel field and laboratory studies and with well established technique for each species. For example, attempts to apply the Comet Assay to delta smelt were not successful due to high and variable background levels in this species (Anderson, unpublished) which were not observed in another local species, the Sacramento Sucker (*Catostomus occidentalis*) (Whitehead et al., 2004). Biomarkers of specific DNA damage, which can potentially link exposures to effects, require specialized detection methods (i.e., ^{32}P post-labelling). When used together, DNA integrity measures, such as SCGE, and measurements of chemically-modified bases often do not correlate because these endpoints reflect different processes with varying temporal characteristics (e.g., Flammarion et al., 2002). However, these processes may be linked mechanistically; for example, DNA strand breaks can result from bulky (e.g., PAH) adducts, or DNA oxidative damage. For almost all assays except DNA adducts, results are not meaningful without some knowledge of the time course of exposure since the kinetics of DNA damage and repair must be characterized.

Summary/Recommendations

In a review in aquatic organisms (Ohe et al., 2004), genotoxicity was measured most frequently as mutations at the chromosome level (micronuclei), DNA adducts (^{32}P post-labeling), and DNA strand breaks (comet assay), in that order. Although relatively few biomarkers have undergone the standardization and quality assurance procedures necessary for their incorporation into international monitoring programs (Hagger et al., 2006), bulky adduct formation has been recommended by the International Council for the Exploration of the Seas (ICES) as a measure for marine fish (Hagger et al., 2006; adapted from ICES, 2004). This endpoint is diagnostic of biologically-effective exposure from causative agent, usually PAHs, which are linked to tumor incidence in fish. In addition other specific DNA adducts, such as those reported for triazine pesticides (see Bodin et al,. 2004), may be important for POD. We recommend that bulky DNA adducts in field-collected fish could provide useful information about the magnitude of exposure

to ubiquitous polyaromatic hydrocarbons, which has been correlated with reduced fecundity in some fishes. Further development targeting specific adduct-producing chemicals is required before other specific DNA adduct detection methods could be recommended. DNA strand break methods (including the Comet assay) and micronuclei techniques have been recommended as promising, but because measurements are relative, the lack of reference populations for some species is problematic. Therefore, we concur with other investigators that these cannot be recommended without careful screening and prior method development for each species. (Hagger et al. 2006; adapted from ICES 2004). Anaphase aberration analysis is a laborious cytogenetic technique but it may have some use in determining whether abnormal embryo development in a POD species is associated with contaminant exposure. Flow cytometry is an intriguing technique, and while it is a specialized method, it has shown promise because data can often be generated rapidly in field studies (Theodorakis et al., 2000). However, to our knowledge, it has not been applied in estuarine fish and would have to be considered a special research opportunity.

2.3.10 Ion Regulation

Introduction

The ability of organisms to regulate their ionic homeostasis is key to survival in aquatic systems. This is particularly important in estuarine organisms that experience large fluctuations of salinity on a daily, monthly, or seasonal basis. The changes that occur in fish as they move between fresh and salt water are well understood for a subset of species (reviewed by Karnaky, 1998). Since the gills of fish are the first tissue to respond to changes in salinity with rapid adaptations in permeability (cellular junctions), ion pumps, and transport mechanisms, they have been one of the primary focal points for adaptations and impacts of stressors on the ability to undergo these physiological changes (reviewed by Evans, 1987) (Appendix 17). The physiological functions of gills include: A) carrier-based ion exchanges such as apical Na^+-H^+, Na^+-NH^{4+}, or Cl^--HCO^{3-} exchanges, basolateral Na^+-K^+ or Na^+-NH^4 exchanges, and basolateral NaCl + KCl cotransport; B) transport enzymes such as basolateral Na/K-ATPase and intracellular carbonic anhydrase; C) paracellular pathways; and D) vascular hormone receptors (e.g. catecholamines) (Evans, 1987; Madsen et al., 1994; Karnaky, 1998).

The general model for osmoregulatory adjustment includes proliferation of chloride cells that secrete Cl^- and are mitochondria-rich. The chloride cell has an associated Na/K ATPase in order to excrete excess salts derived from drinking and passive diffusion (Karnaky, 1998). In most teleosts, this is considered the “rate limiting” step and often takes up to 5 days for increases in ATPase activity and chloride cell proliferation (Madsen et al., 1994). A few marine species show increases in chloride cells when they are moved to lower salinity, but this is uncommon. It appears that species that move back and forth from fresh and salt water lack classic acclimation patterns with regards to chloride cells and Na/K ATPase changes. Striped bass for example are one of these species where changes in activity occur over many days but the amount of Na/K ATPase protein remains constant (Madsen et al., 1994). Another estuarine fish, the mudsucker also shows no change in chloride cell numbers (although there are

differences in cell size) or Na/K ATPase in 1.5 ppt seawater, thereby being ready for rapid re-entry into seawater when the tide returns (Yoshikawa et al., 1993).

The gill is a tissue that is a key target of chemical contaminants (Lichtenfels et al., 1996). Gill Na/K ATPase activity is an excellent indicator of saltwater adaptation as well as a target of contaminants (Evans, 1987). ATPase activity has been shown to be inhibited by a variety of xenobiotics including metals (Jagoe et al., 1996; Stagg et al., 1992; de la Torre et al., 1999), pesticides (Sancho et al., 2003), and endocrine disrupting chemicals (Lerner et al, 2007). The Na/K ATPase assays (Appendix 17) are very routine and the archived gills can be stored frozen until used in the assay. Homogenates of gill are made, and the supernatant used for assessing Na/K ATPase activity (Uchida et al., 1996). Typically, a Luciferase assay is used where the firefly protein is luminescent in an ATP-dependent manner. Since a variety of ATPases can function in the luciferases assay, total luciferase is determined, and the percent of that activity in the presence of oubain (a specific Na/K ATPase inhibitor) is determined. The amount of oubain-sensitive luciferase activity is the Na/K ATPase activity. The activity of the ATPase is determined and expressed as activity/mg of protein. The assay is sensitive and can be conducted on many individuals within a single analysis.

Gill chloride cells can be quantitated using histology. However, a quantitative analysis is difficult due to three dimensional parameters and the need for serial sectioning. Some have suggested that a mitochondria probe may be useful as chloride cells are mitochondria rich. However, actual activity of Na/K ATPase is a better indicator of osmoregulatory capabilities than chloride cell nember and is certainly less labor intensive.

Strengths

A major advantage of using Na/K ATPase activity is that tissues can be archived and analyzed at a later time. The assay is straightforward and is quantitative based on the amount of protein present in homogenates. While altered salinity will affect enzyme activity, as long as this is accounted for, detection of contaminant-induced changes in Na/K ATPase activity can be readily conducted. Of particular relevance to estuarine species, are the effects of contaminants on their ability to osmoregulate. As such, the use of this biomarker may be very important in species within the Delta.

Applications

A number of monitoring studies have included gill Na/K ATPase activity as a biomarker in fish (Stagg et al., 1992; Jagoe et al., 1996; Hamann et al., 1999; de la Torre et al., 2005). In one study (Garcia-Santos et al*.,* 2006), Na/K ATPase activity was not found to be decreased following contaminant exposure, however most other studies (field and certainly laboratory exposures) have shown that this major osmoregulatory mechanism is impacted by contaminants.

Measurement of Na/K ATPase activity can be made on gills from adults, but also on embryos and larvae. In addition, a method for determining Na/K ATPase in embryo/larval homogenates can include the use of fluorescently conjugated oubain

(FITC-oubain) (McCormick, 1990, 1993). The fluorescent oubain binds to the Na/K ATPase irreversibly, and the level of fluorescence can be compared to reference or control individuals. This method can use fluorescence microscopy and thus can be conducted with very few individuals or small tissue samples. While the FITC-oubain has to be added to fresh tissue, the washed tissue can be fixed in paraformaldehyde for later analyses.

Weaknesses/Caveats

Obviously, the normal level of Na/K ATPase will be variable between species and may not be upregulated (e.g. striped bass) in some species. It is critical to compare the salinity history among sites if one is to compare fish with respect to ATPase activity, because salinity alone could be responsible for any differences observed. While it may be possible to measure the amount of ATPase activity per unit of Na/K ATPase protein using specific antibodies in order to address if contaminants are specifically inhibiting enzyme activity or expression of the protein, this approach has not been developed at this point in time. Furthermore, environmental factors that subtly alter ATPase activity in fish from the field are not well characterized, and therefore the use of Na/K ATPase activity solely as a biomarker for contaminants cannot be recommended at this time.

Summary/ Recommendations

Since the POD species of concern are estuarine and must adapt in freshwater and saltwater, Na/K ATPase activity and chloride cell numbers are quite relevant in the overall understanding of the POD decline. Certainly, an understanding of the ability of the three other species, other than striped bass, to adapt to salinity changes is important in the overall health assessment of fish as well as to determine their sensitivity to chemicals that are known or suspected Na/K ATPase inhibitors. Some level of Na/K ATPase activity screening associated with routine collections of fish, taking into account temporal and spatial factors, could be important. However, at present, the use of this biomarker should be restricted to laboratory exposures using site water or chemicals of interest or should be used in special studies to investigate interactions between contaminant exposure and salinity stress. Na/K ATPase activity as a biomarker of chemical exposure should only be included in an integrated study where exposures are determined or in experimental studies using targeted outplants or laboratory exposures with site water. Nevertheless, metals and pyrethroids are well known to perturb ion channels and fluxes and therefore, assessing ion regulation is certainly relevant to POD species.

2.3.11 Multixenobiotic Resistance

Introduction

Numerous aquatic organisms protect themselves from natural and anthropogenic toxins by employing cellular efflux mechanisms, termed multixenobiotic resistance (MXR, Appendix 18) (Kurelec, 1992, 1995, 1997; Smital and Kurelec, 1998; reviewed by Bard, 2000, Moore et al., 2004**)**. These “first line of defense” mechanisms appear to be very important, particularly in developing embryos where other detoxifying mechanisms may not be active (Hamdoun et al., 2002, 2004; reviewed by Hamdoun and Epel, 2007).

The importance of these mechanisms in management is supported by the EPA Office of Research and Development (Daughton and Ternes, 1999). MXR is achieved through the use of transport proteins that belong to the family of ATP binding cassette proteins (ABC). These transport or efflux proteins are located in cell membranes and function by pumping exogenous and endogenous toxins out of cells (6). P-glycoprotein is a common efflux protein in mammalian tumor cells responsible for tolerance to chemotherapeutic drugs (6**)**. The most common efflux protein found in aquatic organisms is P-glycoprotein (Pgp). Pgp glycoprotein provides MXR in mussels, clams, oysters, snails, sponges, worms, decapods, and fish (Bard, 2000). A second type of efflux protein found recently in some organisms is multidrug resistance-associated protein (MRP) (Sauerborn et al., 2004; Hamdoun et al., 2004; Minier et al., 2006). Cancer research has led to the discovery of chemicals capable of competitive and non-competitive inhibition of Pgp and MRP, and these have been employed in studies with aquatic organisms. While each efflux pump is responsible for the removal of specific compounds from cells, there is some overlap in the substrate and inhibitor specificity of Pgp and MRP (Ambdukar et al., 1999). Components of wastewater effluent including PPCPs are known substrates and/or inhibitors of MXR efflux proteins (Bard, 2000; Smital et al., 2004).

MXR has been used successfully as a biomarker for polluted environments (Minier et al., 1999) and is known to be inducible when organisms are exposed to contaminants. MXR induction has been observed in fish exposed to oil constituents and the induction correlates with P450 enzyme upregulation (Bard, 2000).

Dye Accumulation Assays. Assessment of MXR (Appendix 18) has been at the levels of transport or efflux activity, protein expression, or mRNA expression. The majority of the use of MXR activity (dye accumulation assays) has been conducted on organisms using gills, blood cells, or early life stages (Bresler et al., 1999; Hamdoun et al., 2004; Luckenbach and Epel, 2005; Pain and Parant, 2007). In these assays, a fluorescent dye such as calcein-AM or rhodamine-B are added with or without a specific transporter inhibitor, and dye accumulation as quantitated using fluorescence microscopy or homogenizing tissue and determining dye levels using a plate reader of spectrofluorometer. Data from these experiments show relative activity differences (less dye accumulation, more efflux activity) as well as identify transporter type when specific inhibitors are used.

Protein Expression. The level of Pgp or MRP protein in tissue can be readily measured in tissues using specific antibodies to the transporters that are commercially available (Eufemia and Epel, 2000, Hamdoun et al., 2002; reviewed by Bard, 2000). These antibodies have been used for biochemical detection using electrophoresis and western blotting as well as immunocytochemistry. While the western blotting technique is useful for total MXR protein, it does not always relate to total MXR dye efflux activity (discussed below). Immunocytochemistry has been routinely used on fish tumors and anti-Pgp ("C219") nicely labels the tumors (see Bard, 2000 for review). However, this is a property of cancer cells and is not related to MXR functioning to remove environmental xenobiotics.

mRNA Expression. Detecting Pgp or MRP gene expression is straightforward and has been used in a number of studies (Luedeking and Koehler, 2004; Luedeking et al., 2005). Using specific cDNA probes for Pgp or MRP, levels of the transcript can be quantitated by PCR or real-time PCR assays. Again, the transcript level does not necessarily relate to increased dye efflux. Increases in MXR responses are typically long-lived since increases in numbers of membrane transporters and this is believed to last for days to weeks. However, inhibition of transporters by environmental chemicals can be very transient and reversible such that washing in clean water removes tissue level changes in inhibition. There is also evidence that upon exposure to contaminants, MXR activity is increased, but that in some cases, this increase is actually depressed in chronic exposures (ICES WGBEC Report, 2006).

Strengths

MXR activity has been shown to increase in organisms from polluted habitats. The efflux activity, protein levels, and mRNA can all be used and as such, can be included in a larger program where samples are archived and the physiology assessment (dye accumulation) cannot be conducted with fresh tissue. The assays are very easy to conduct since probes (antibodies, cDNA) are either available commercially or can be constructed. While not providing direct chemical exposure data, overall health and stress levels of the organisms can be assessed using MXR. When combined with exposure data or tissue chemistry, MXR can be directly linked to exposure. For embryos, dye accumulation assays are relatively easy to conduct and can be conducted with site water in laboratory settings or in outplant experiments. MXR activity is an excellent tool to determine chemical defenses as it may be one of the only mechanisms for dealing with contaminants at early stages of development.

Applications

MXR has been and continues to be used in many European field-monitoring studies particularly in invertebrates (Smital and Kurelec, 1998; Bresler et al., 1999; Kurelec et al., 2000; Minier et al., 2002; ICES WGBEC Report, 2006; Minier et al., 2006; reviewed by Minier et al., 1999; Bard, 2000; Moore et al., 2004; Pain and Parant, 2007). It has had only limited use as an indicator of exposure in fish from the field to date, primarily due to a lack of study.

Weaknesses/Caveats

It is well known that the MXR response is also part of the generalized cellular stress response. Like heat shock proteins, MXR can be induced by stresses such as salinity and temperature (Eufemia and Epel, 2000; Minier et al., 2000; see Bard for review). As such, salinity and temperature comparisons must be know for different sites used in any monitoring program. mRNA and protein assessments may not always yield the most useful information. While induction of these is easy to measure and can be used as an exposure biomarker, it is clear that protein can exist in the cytoplasm of cells prior to being transported to the cell surface; this cytoplasmic protein is a “sink of future MXR activity”, but only the direct measurement of efflux activity will be a measure of an adaptive response or chemosensitization of organisms to contaminant exposure. As

such, western blots may show little difference between sites; however, efflux activity could be very different based on how much transporter has been inserted in the membrane. Therefore, it is recommended that efflux activity be the primary MXR assay, followed by protein and then mRNA.

Summary/Recommendations

For purposes of determining if POD species have been exposed to contaminants, MXR as a biomarker will not be very useful. However, as part of an integrated program that includes physiological status of organisms, salinity and temperature history, contaminant loads, and other factors, MXR could be useful as an indicator of susceptibility and innate protection against xenobiotic insults. MXR assessment could also be very useful when site water is collected and assessed for biological activity in the laboratory. In particular, MXR assays would inform managers whether chemical substrates for the transporters are present. Since these compounds can act as chemosensitizers (enabling other contaminants to accumulate in cells and be more toxic; Kurelec, 1997; Kurelec et al., 2000), a knowledge of this activity in site water could be important. Finally, MXR is one of the few defense mechanisms that is used by very early life stages, and functional MXR as well as an inhibition of MXR in early life stages from the field or exposure to site waters is likely to be a predictive tool. Assessing the MXR activity of fish embryos in reference or laboratory waters versus site waters would enable detection of the presence of MXR substrates in the water in question. These substrates could function as toxicants or as chemosensitizers. This MXR attenuating activity could be coupled with fractionation and TIE efforts using small volumes of water and the embryos.

2.3.12 Toxicogenomics

Introduction

Genomics-based markers refer to the process of measuring a characteristic profile within thousands of genes in tissues that are generally thought to be indicative of a pathway of toxicity of drugs or chemicals. The term *toxicogenomics* refers to the use of *gene expression* profiles as biological endpoints in toxicological studies (Denslow *et al.*, 2007). In this document, we will use the term *genomics* interchangeably with the terms *transcriptomics* and *toxicogenomics,* to refer to gene expression studies in the context of the POD. In contrast, there are other *-omics* based technologies such as *proteomics*, which refers to the measurement of tissue protein profiles, and *metabolomics,* which refers to assay of biological metabolite profiles in a matrix (Khoo and Al-Rubeai, 2007). The *-omics* are sometimes referred to as *global profiles* because they are intended to measure the entire transcriptome, proteome, or metabolome in a particular target tissue of the organism under study. However, this is often inaccurate, as it is presently impossible to accurately detect and identify all small molecules in a given tissue.

While all the *-omics* methodologies are being exploited to some degree by aquatic toxicologists, studies involving *genomics* approaches to assess chemical exposure and effects in aquatic organisms comprise the majority of research effort. Accordingly, this discussion will focus on the utility of genomics in assessing the potential role of

chemical exposures in the POD. Genomics approaches for biomonitoring are based on the assumption that measurable gene expression effects are temporally, spatially, and mechanistically related to either chemical exposures or adverse effects in an organism. With regards to the latter, this is accomplished by linking the *-omic* profile to a response that has been linked to fish condition, such as tissue damage, histopathological abnormalities, endocrine alterations, cancer, etc. These types of phenotypic anchoring studies (because they anchor genome markers with a particular phenotype such as tissue pathology) are more prevalent in the biomedical literature than in aquatic studies.

The main tools for the study of genomics are the DNA microarray technologies (Appendix 19), which are often referred to "DNA chips". These techniques allow researchers to measure the full complement of activated genes that encode mRNAs within a tissue. Currently, there are a number of platforms for aquatic species including fathead minnow, Atlantic halibut, catfish, flounder, *Fundulus*, *salmonids* (trout and salmon), bivalves (*Mytilus* and others) as well as zebrafish and several flounder species (Denslow, 2007; Douglas 2007; Matsuyuma, 2007; Volker, 2007; Venier, 2006). The preceding is not an all inclusive list, as recent advances in high throughput sequencing are leading to the rapid development and applications of microarrays from other species. Quantitation of microarray data is initially carried out through a series of informatics steps involving quality control analysis, background subtraction and data normalization (Wilkes et al., 2007). The data outputs for most automated quantitation packages include programs such as Microsoft Excel, and these are used concomitantly with data modeling and data interpretation with computational biology methods. The term computational biology refers to the rapidly evolving area where methodologies from computer science, mathematics, and statistics are applied to address fundamental problems in biology (Ji and Wong, 2006). Typical microarray data analysis may include such approaches as principal components analysis, gene expression mapping, pathway and cluster analysis. These processes are an integral part of the microarray experimental analysis and interpretation process and especially important in drawing conclusions regarding the biological significance of the microarray data.

Strengths

Toxicogenomics holds promise as a means of potentially capturing an integrated snapshot of the entire biology of response to a toxic substance in a given tissue at a given dose, and at a given time-point (Denslow et al., 2007). The comparisons of these gene expression 'snap-shots' from chemical exposures have the potential to identify molecular biomarkers of effects or susceptibility and also differences in responses to toxic exposures across aquatic species. An application of the latter would be the identification of potentially resistant and sensitive species within a stressed ecosystem (Douglas, 2006), and thus a potential application to the POD. Depending upon the genes involved, transcriptional changes can be extremely sensitive to chemical exposures and are observed before later stage effects such as tissue pathology are manifest. In addition, mRNA information can provide information upon the specific biochemical pathways that are affected by chemical exposures, thus potentially providing a linkage among exposure and early effects. Changes in dramatically-altered genes (up- or down- regulated), in particular, can lead to identification of new

biomarkers if carefully validated in controlled laboratory studies. Finally, if used correctly, microarray studies can help uncover new mechanisms of toxicity of chemicals.

Applications

The majority of the toxicogenomic studies involving chemical toxicity in fish have been laboratory-based and using dose-response exposures with single compounds. These laboratory-based studies are numerous and will not be addressed here. In general, these studies often demonstrate altered gene expression patterns consistent with the mechanisms of action of the chemicals under study (i.e. metals, PAHs, PCBs etc.), thus providing somewhat of a basis for the application of microarray studies in the field.

A review of the literature indicates a paucity of papers regarding the use of microarray technologies in assessing or delineating effects of pollution in field studies. In one study, Venier *et al.* used marine bivalves of the genus *Mytilus* as biosensors of coastal pollution in microarray applications. The authors developed a cDNA microarray specific for *Mytilus galloprovincialis*, which generally revealed transcriptional profiles of mussel tissues exposed to crude oil mixtures of environmental relevance (Venier *et al.*, 2006). In that study, the authors reported tissue-specific changes in gene expression indicating the importance of evaluating different tissues for microarray analysis. Further testing of the most responsive organ, the digestive gland, allowed correct classification of mussels treated with mixtures of heavy metals versus organic contaminants in the laboratory. Similar analyses made a distinction possible between mussels living in a polluted lagoon (Italy) in a petrochemical area and mussels inhabiting less polluted areas close to the open sea (Venier et al., 2006). The presence of gene markers tracing organic contaminants in mussels from the polluted area was generally consistent with the extent of chemical contamination, and thus has relevance to the POD. In this regard, the study could be considered an example of a successful application of microarrays to aquatic field studies. However, the authors indicated that additional work was necessary in order to understand how much gene expression profiles truly identified the signatures of pollutants in mussel tissues. As with other field studies (and not a direct criticism of the Venier study), there was little information regarding how the gene expression patterns reflected adverse health effects from the exposures, or what the added benefit provided by microarrays was relative to the analytical chemistry data alone.

In another field study, William et al investigated the effects of pollutants on an environmentally relevant local fish species (European flounder) using a 160-gene custom microarray. After optimizing the microarray process, hepatic mRNA was isolated from flounder caught in the polluted Tyne River and flounder from a relatively unpolluted estuary. Although the authors identified a number of differentially expressed genes between female flounder from the two sites, the changes were not significant due to high inter-individual variation. In males, 11 transcripts were found to be significantly different (Williams et al., 2003). However, problematic was the fact that follow-up studies using quantitative PCR confirmed the significance of the array results for only several genes. Although the Williams study provided a link between traditional single-gene biomarker studies and the utility of microarray studies using environmentally sampled wild fish, it underscored the problems associated with interindividual variability

and confirmation of microarray data (Williams et al., 2003). As with the bivalve study described above, it is also unclear on how the flounder microarray data provided a link among chemical exposures and adverse effects in the flounder, or how the data discriminated other non-chemical stressors.

Weaknesses/Caveats

Implicit in the approach of using toxicogenomics in biomonitoring studies such as the POD are several assumptions, including that; 1) a particular chemical or group of chemicals produces their adverse effects through common mechanism(s) that are observed in other species, 2) these changes in gene expression resulting from chemical exposures can be quantitated and validated in the laboratory and are similar to those observed in the field under complex conditions, 3) the chemicals present in the field cause quantitative changes in gene expression that are based on the chemicals mode of action and that can be discriminated from other parameters such as dietary stress, physiological condition, disease state, or other environmental or physiological condition, 4) sampling and analysis procedures are carefully validated so that artifactual changes are not observed (*i.e*., false negatives or false positives), 5) that appropriate bioinformatics analyses and gene normalization procedures are applied and the results are validated by quantitative PCR, and 6) there is not an unusually high degree of variability in the expression of genes among individuals being studied. Violation of these of assumptions can end up resulting in a lack of specificity and variability in genomics responses.

The application of toxicogenomic approaches in the field has been seriously hampered by technical challenges surrounding these basic assumptions (see the case studies below). A problem with the immediate use of toxicogenomics in assessing the role of chemical pollution in the POD is the uncertainty associated with species differences in responses. For example, species such as striped bass or threadfin shad may respond differently than other closely related species when confronted with the same dose of a particular chemical. This could raise uncertainty with the use of surrogate species in these studies. An example is the marked species sensitivity among some closely related salmonids to the carcinogenic effects of the natural toxicant aflatoxin B_1 (Eaton and Gallagher, 1994). Accordingly, it would be important to develop microarrays specific for POD species.

Summary/Recommendations

Although results from studies using toxicogenomic approaches in aquatic animals are encouraging, they reinforce the conclusion that there are significant challenges associated with standardization, reproducibility and relevance, especially in field studies involving complex stressors that likely affect gene expression. In essence, genomics approaches in aquatic animals are still at a relatively early stage of development when compared to platforms involving genetically defined mammalian strains. These issues are further complicated by lack of knowledge of gene regulation in fish and extrapolations across species, both of which can muddy data interpretations. Furthermore, there can be problems with internal laboratory reliability, as well as

differences in measurements across laboratories that can raise questions regarding accuracy and reproducibility.

Despite these aforementioned limitations, there is potential for the eventual use of genomic approaches in the POD. Such applications would need more research and development to achieve these goals, and these efforts will require time and resources associated with validating these technologies. Some specific recommendations for the extension of microarray technologies to the POD include:

- The generation and validation of array platforms that involve oligonucleotides or cDNA probes that are specific for the POD species; or, alternatively but less desirable, the careful scrutiny and validation of array platforms based on closely related species (i.e. largemouth bass arrays for studying striped bass). Such applications may require genomic sequencing advances for the prominent POD species. These studies would also require a review of the modes of action of the potential chemicals present in the delta region so that appropriate target tissues (i.e. liver, brain, gonads) are selected, and also are validated in laboratory studies.

- Because of the suspected roles of a number of environmental stressors in the etiology of the POD, it is important to design studies addressing the influence of environmental and physiological contributions on the altered gene expression profiles observed. Some relevant stressors that could affect gene expression profiles potentially associated with the POD are dissolved oxygen, salinity, infectious disease, and starvation.

- As discussed, a key challenge in using *-omics* technologies is validating that a particular pattern of gene expression change is strongly correlated with an adverse response in the whole organism. Accordingly, it is critical to establish direct linkages among the adverse responses of physiological significance in target POD species and gene expression. For example, laboratory studies could be conducted using model chemicals that produce adverse effects such as reproductive injury (endocrine disruptors) and establishing specific patterns of gene expression that might be indicative of this process (*i.e.* modulation of a particular hormonal pathway such as estrogen, androgen, thyroid hormone biosynthesis).

In summary, laboratory-based microarray studies specific for target aquatic species being studied show promise with regards to the generation of altered mRNA profiles that can be associated with chemical exposures and their potential effects. However, the usefulness of these approaches for the POD is limited due to technological issues such as the lack of specific microarray platforms for POD species, and knowledge of the effects of biological and environmental variables on gene expression profiles. It is imperative to have appropriate reference (control) animals of similar strain, age and sex. Also problematic is the high level of variability likely to be encountered in field sampled fish and the large numbers of arrays needed for adequate statistical power and

discrimination. Such experiments are likely to be expensive. There could potentially be less variability in strains of POD fish reared in captivity and exposed to environmental samples in the laboratory or in field enclosures, and this could be an application.

2.3.13 Conclusions Regarding Biomarker Techniques

Key conclusions of our technical review of chemical and physiologic biomarker methods are:

- Most of the biomarkers can be adapted for use with tissues of juveniles or adults, which are easily obtained from the field. However, very few can be applied to larvae from the field, because these are rarely obtained in a viable state; and furthermore, few of the biomarkers are applicable to fixed tissues (principally immunologic techniques). Nevertheless, biomarkers can easily be implemented using embryos and larvae obtained from hatcheries and exposed in the laboratory.

- Few biomarkers are recommended for immediate implementation in large-scale field surveys. In our opinion, vitellogenin/choriogenin can be used as an indicator of exposure to feminizing (estrogenic or anti-androgenic) compounds, and we recommend CYP1A for exposure to planar aromatic compounds and DNA adducts for exposure to PAHs. (Acetyl)cholinesterase activity would be useful only when specific exposures to organophosphate and carbamate pesticides are under investigation and time series data can be obtained.

- Biomarker techniques must be applied in an integrated portfolio of methods including condition indicators and analytical chemistry. In the context of the POD, the most significant application of biomarkers is to contribute to a weight-of-evidence approach to the question, “Is it plausible that contaminants are causing significant stress in POD species?” Therefore, any discussion of biomarkers must be considered in the context of the potential payback of using other methods, such as toxicity testing. An interplay between field and laboratory observations is often needed.

- When an iterative approach is used, some biomarkers become more useful as the contaminant issues are better defined. For example, if histopathology reveals poor organ condition, disease or cancer in fish, then it would be important to develop and implement additional immunologic and DNA damage biomarkers for targeted investigation. These can be related to fitness parameters and provide evidence that contaminants are related to poor fish condition.

- Metallothionein, stress proteins, and antioxidant enzymes are not recommended for large-scale field studies; however, these could be useful in targeted investigations of fish health when a number of experimental issues are considered. Given that other techniques are likely to produce more directly relevant results quickly, use of these techniques is a low priority.

- Biomarkers of susceptibility include multiple xenobiotic resistance (MXR) markers and also responses related to ion regulation. We recommend that these be considered for special studies to elucidate potential multiple stressor effects, prinicipally interactions between salinity stress and contaminant exposure. MXR is upregulated in the presence of organic contaminants such as pesticides and in some cases, heavy metals. Ion regulation can be inhibited or affected by a number of pesticides (organophosphates, pyrethroids) as well as heavy metals that are channel blockers.

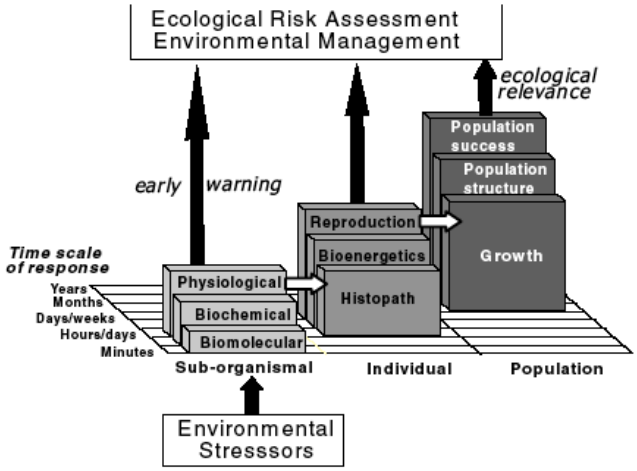
- Toxicogenomics techniques are potentially the wave of the future, but an initial investment of resources to develop and validate methods is required and, hence, decreases the immediate relevance to the POD decline. We make the tentative recommendation that a pilot study be initiated for a single POD species.

2.4 RECOMMENDED FISH CONDITION INDICATORS

2.4.1 Introduction

Biomarkers provide information diagnosing chemical exposures with realized or potential effects on individuals. In contrast, condition indicators provide information regarding individual fish health that is designed to contribute directly to assessments of population health, such as altered population dynamics, abundance, and distribution (see later discussion on population relevance). Furthermore, changes in some condition indicators, when consistent with known effects of specific chemicals, can contribute additional evidence to diagnoses of chemically-mediated effects. Thus, in combination, information from biomarkers and condition indicators can be used to provide evidence linking chemical exposures to population declines.

Specific Indicators of individual organism condition have been recommended for evaluating the health of fish populations, as well as for diagnosing the stressors present in aquatic systems (Teh et al., 1997; Adams et al., 1999; reviewed by Adams and Greeley, 2000 and Adams 2001). These represent an important and easily implemented suite of measurements. This section provides a brief overview of recommended condition indicators and discusses basic aspects of their implementation.



Hierarchial response of organisms to environmental stressors illustrating the more sensitive early warning indicators at the lower levels of biological organization and the slower-responding but more ecologically relevant indicators at the higher levels of organization (from Adams and Greeley, 2000). The individual responses can be key indicators of organismal health.

Typically, indicators of individual health include three categories of response: reproductive success, growth and energetics, and histology and pathology (Table 2). Reproductive indicators can be directly linked to population level impacts, and these indicators are often more sensitive to contaminants than other parameters. Such indicators include deviations from normal spawning frequency and normal age at spawning, gonadosomatic index (GSI), gamete quality and numbers, fertilization success, occurrence of abnormal embryo and/or larval development, endocrine disruption (sex ratios and ovotestes), and histological/pathological condition of gonads. Some of these indicators can be included in any routine monitoring program (GSI, age at spawning, and gender ratios), while others could require the ability to mature and induce spawning in fish (fertilization success, embryo abnormalities, and larval malformations). Growth and energetic indicators include assessments ranging from routine hepatosomatic index (HSI), weight/length relationships and age structure (otolith growth rings) to more complex indicators such as bioenergetic markers (RNA/DNA ratios, glycogen, and adenylate charge). Histological and pathological indicators include tissue and organ lesions (ovotestes, organ damage, and dysfunction), basic blood chemistry, presence of pathogens, glycogen levels (energy reserves and food availability), inflammation, specific immunocytochemical markers, tumors, macrophage aggregates, apoptosis or programmed cell death (carcinogen expoure), and lysosomal instability (an indicator of decreased ability of tissues to detoxify contaminants).

To reiterate, it is essential to combine condition indicators with biomarkers and analytical chemistry to develop the linkages between exposure and effect. For example, fish from reference and contaminated sites may both share signs of decreased health, but usually only a subset of indicators are unique to contaminated sites (Teh et al., 1997). When little is known regarding the contaminants present or levels of fish exposure, as in the case of the POD, it can be more difficult to identify relationships between condition indicators and stressors. Much of the basis for assigning specific indices of organism health to pollutant exposure has been through statistical correlations with exposure and with single-chemical laboratory studies; such conclusions must be developed cautiously but are feasible in a highly integrated study plan.

Table 2. Recommended condition indicators for fish populations

INDICATOR
Reproduction • Spawning frequency • Age at spawning • GSI • Gamete numbers and quality • Endocrine disruption (sex ratios, ovotestes, etc.) • Histology of gonads (tumors, cell death, etc.) • Fertilization success • Abnormal embryo and larval development
Growth and Energetics • Age-length relationships (otolith growth) • HSI and other organ/somatic ratios • RNA/DNA ratios
Histological and Pathology • Tissue and organ lesions • Pathogens • Glycogen levels • Inflammation • Histochemistry and immunocytochemistry (specific biochemical responses, expression of specific antigens, etc.) • Apoptosis • Lysosomal membrane instability

2.4.2 Reproductive Indicators

Both reproductive output of adults and successful recruitment can be a direct mechanism for decline in populations of fish. The impact of contaminants on fish reproduction has been described in models that predict population-level impacts (Landahl et al., 1997; Hussam, et al., 2005; Murphy et al., 2005). Parameters used in these models include age at first spawning, spawning frequency, GSI, fecundity (oocyte and sperm size/numbers), egg, embryo, and larval viability, endocrine disruption indicators, etc. The advantage of employing reproductive parameters for assessing individual effects is that reproduction is directly linked to population parameters. In addition, some biomarkers are easily linked to reproduction.

Clear linkages between contaminant exposure and reproductive success exist for fish in the field, including PCBs (Barnthouse et al., 2003; Feist et al., 2005), DDTs (Feist et al., 2005), and synthetic estrogens (Kidd et al., 2007). Recently, indicators of reproductive

impairment have been routinely used across many species. These include the occurrence of poor reproductive output, ovotestes and decreased sperm function. (Arwuke, 2001; Sole' et al., 2003; Anderson et al., 2006), as well as skewed sex ratios (reviewed by NOAA, 2002). For assessing reproductive impacts associated with effects on early life stages, laboratory studies have been extremely useful in assessing effects of exposure to site water as well as individual chemicals (Luckenbach et al., 2001; Stark et al., 2003). Many life-stages of fish are impossible to collect or to identify. As such, reproductive indicators will always require integration of field collections as well as laboratory/*in situ* outplant approaches. These studies are critical since early life-stage effects (not just mortality but alterations in condition) are a major effector of fitness of the population. Poor embryo/larval fitness in the F_0 animals can impact F_1 adult fitness ranging from reproductive output to susceptibility to stressors (Meyer and Di Giulio, 2003).

2.4.3 Physiological Condition, Growth, and Energetics

In general, fish condition reflects ability to maintain and grow, and is correlated with survival and reproduction (review Kammenga and Laskowski, 2000). While certainly influenced by natural factors, changes in condition may be some of the most important indirect effects of contaminant exposure (e.g., Munnkittrick and Dixon, 1988, Woodward et al.,1995; Smolders et al. 2003). For example, allozyme changes suggest that alterations in glycolysis metabolism are sometimes a compensatory response in fish to long-term, sublethal contaminant exposures (e.g., Kramer et al., 1992). While it may be difficult to establish causal relationships, it is reasonable to infer that condition reflects the integrated effects of stressors, and that poor condition exacerbates stressor effects. Therefore, indicators of condition and energetics provide important context to interpret and predict the impact of chemicals on fish populations.

Simple measurements of fish size, in total (length and weight) and on organ-specific basis, provide informative indicators of fish condition, easily measured on freshly caught or preserved fish. For example, liver: body (weight:weight) ratio or hepatosomatic index are often used as indicators of stored reserves (and see reproduction below). Otolith analysis is a common technique used to estimate fish age, which in combination with fish size may be used to calculate growth rates and infer environmental quality (e.g., Able et al., 1999; Rose et al., 2005). For example, individual fish growth rates have been employed to compare habitat quality among different juvenile nursery areas (e.g., for Atlantic croaker, Searcy et al., 2007). In addition to information about fish growth, chemical analysis of otolith rings can link specific habitats to realized growth (e.g., Thorrold et al., 2001), and potentially document temporal exposures to some metal contaminants. RNA/DNA (R/D) has become a standard method for estimating growth rates in larval fish (review Ferron and Leggett, 1994; Buckley et al., 1999). A generalized R/D - temperature - growth rate equation which can be applied to any species of larval fish has been developed and is currently under validation (Buckley et al., submitted). To date, there are still no RNA specific dyes available, so unless sample size is severely limited (i.e., << 5 cm larvae), standard fluorochrome methods are appropriate and have been extensively tested (Caldarone et al., 2001).

Various forms of energetic assessments have been used to demonstrate the cost of chemical exposures on fish (e.g., Mattson et al. 2001, using rainbow trout). Bioenergetics indicators such as scope for growth, which have been used to assess aquatic invertebrates especially mussels (e.g., Widdows et al., 1995), are used less frequently in fish, perhaps due in part technical difficulties (review Lawrence et al. 2003). Other bioenergetics indicators, such as adenylate energy charge (AEC), the ratio of ATP to total adenine nucleotides (sum AMP, ADP, ATP), have sometimes been used with fish (e.g., review Mayer et al., 1992, review Kammenga and Laskowski, 2000). Similarly, energy budgets have been calculated using measured concentrations of glycogen, protein, and lipids in whole body homogenates of small fish (i.e., zebrafish, in Smolders et al., 2003). More generally, indicators of condition and nutrition, such as serum triglycerides, have been measured with a suite of biomarkers to assess the relative contribution of chemicals to ecological endpoints in risk assessment (i.e., Adams et al., 1999). Exploratory studies are now investigating genomics as an approach to assess simultaneously genes known to be related generally to energetics (e.g., Merilainen et al., 2007, using trout, Roling et al., 2006, using the small estuarine fish, Atlantic killifish).

2.4.4 Histology and Pathology

Histopathology has been used for many years as a biomarker of stress, including contaminant exposure (Hinton et al., 1987; McCarthy and Shugart, 1990; Bernet et al., 1999). While not an early warning biomarker, histological lesions are typically medium- or long-term indicators of stress and adverse fish health. A variety of tissues are typically included in histological assessments including liver, kidney, gonad, digestive tract, muscle, gill, neurological tissue, etc. Neoplastic and non-neoplastic lesions are assessed and quantitated in order to develop a health index for an individual or a population (Meyers et al., 1990, 1991, 1994; Bernet et al., 1999). In addition, simple investigations of pathogenicity and chemical-specific diagnostics (immunocytochemistry, apoptosis, lysosomal stability) can be integrated into routine monitoring studies with some planning that includes appropriate tissue archiving.

It is important to recognize that histopathology, as a tool for detection of contaminant stress, can only be used as “pollutant-associated” rather than “pollutant-induced” lesions (Schwaiger et al., 1997). An important consideration is the differences in responses of various species in the same water system. For example, Schwaiger et al., (1997) demonstrated that brown trout showed clear differences in histopathology between those individuals exposed to a moderately polluted river as compared to a more severely polluted one. However, responses in loach from the same systems did not reflect the variation in contaminant loadings. In this study, contaminant levels were known for each system; and thus, exposures could be quantitated.

Natural stressors are known to induce histological lesions (Teh et al., 1997); and while histological changes in polluted sites are readily observed, mechanistic linkage of tissue damage to exposure may not always be direct. For example, observed lesions could be

directly (chemical-induced cellular alterations), indirectly (weakened immune system), or not associated (pathogens, energetics, nutrition, etc.) with the presence of contaminants. As such, histopathology is extremely useful for assessing the health of individuals and populations; however, it is not a direct indicator of contaminant exposure without integrated assessments of site water chemistry, tissue chemistry, responses in laboratory experiments, and other biomarkers of exposure or effect.

2.4.5 Stepwise Implementation of Condition Indicators

Field studies have shown that histological and other health biomarkers are useful when known pollution exists and environmental chemistry and companion biomarker data are available (Teh et al., 1997; Ham et al., 1997; Adams et al., 1999; Triebskorn et al., 2002). For example, histopathology can be considered a tool that can be used to diagnose disease states (specific pathogens and lesions) and physiological condition. In addition, it can be combined with histochemistry, immunocytochemistry, and molecular probes to obtain tissue- and even cellular-specific information about contaminant exposure.

Implementation of condition indicators within a field monitoring program can be undertaken in a phased approach. For example, preliminary screens for pathogens and lesions can readily be conducted with tissues processed in initial collections. These same samples (appropriately archived), if positive, can be analyzed further in a second phase using specific antibody probes and numerous other assays that are more diagnostic. For example, lysosomal stability, apoptosis, and specific immunological staining all can directly indicate contaminant exposure and effects. The indicators in this first step are easy to implement as part of an initial condition screen of fish, and the subsequent follow up measurements can be rapid and less expensive if planning for such follow up work includes sample archiving. Evaluation of growth and energetics can also be phased, with age-length relationships and somatic indices measured first, and RNA:DNA ratios measured subsequently on frozen or freeze-dried tissues (Caldarone et al., 2001)

2.4.6 Sampling Considerations

Archiving for biochemical condition indicators usually requires freezing of freshly dissected tissue in liquid nitrogen or on dry ice. Generally, this approach, as well as fixation in preservatives, will be suitable for archiving tissues for analysis of condition indicators as well. This means that with an integrated study design, the same samples may be used for multiple purposes if the tissues are of sufficient quantity. Ideally, much of this sampling can be conducted as part of a routine monitoring survey.

For example, entire larval and juvenile fish can be fixed or frozen for later histology processing or biochemical analysis, while adult tissues can be dissected and placed in fixative or frozen. Stabilized individuals or tissues can generally be stored for months, such that targeted processing may be possible depending on the importance of different collected individuals from select stations. For otoliths, fish heads can be fixed or frozen

for subsequent dissection of the otoliths. For long term trends in populations, it is advisable to initiate routine condition assessments and this should be continued over time in order to track trends that may be seasonal, annual, or decadal. It should be noted that while some reproductive indicators can be used with archived tissue, gamete and embryo viability can only be assessed on spawning adults and may be difficult to determine unless these adults are readily available.

It is not uncommon for hypotheses regarding different stressors to evolve over time, and appropriately archived samples will enable testing of these new hypotheses. While most of the common condition indicators described above may not provide direct information on specific stressors, the information gathered is extremely useful for assessing declines or increases in organismal health as related to population levels, and can be integrated with other environmental parameters such as analytical chemistry and biomarkers.

2.4.7 Population Relevance

As state above, measurements from condition indicators, and some biomarkers which are correlated with vital rates (survival and reproduction), can link effects on individuals to population-level effects (e.g., Barnthouse et al., 2007). Knowledge of species life histories provides a foundation for understanding ecological mechanisms by which contaminants affect populations. First, the concept of concurrence of life-stages and seasonal or location-specific stressors is critical to understanding contaminant exposures (see Appendices 4-7). However, toxicological and demographic variation among species will affect how these exposures translate into population effects. Toxicological variation results in differences among species in chemical effects on survival and reproduction (vital rates). Differences among species in life history traits, such as number of offspring, age at maturity, and longevity, will result in differences in population-level effects of vital rate alterations (Stearns, 1992; Caswell, 2001). Because of these differences among species, the susceptibility of populations to chemical stress is dependent on demographic structure, as well as toxicological sensitivity (e.g., Barnthouse et al., 1990; 2007).

Demographic models provide a quantitative mechanism to incorporate life-stage related stressor responses (e.g., Caswell, 2001). For example, small changes in survival have a greater impact on populations of long-lived species such as striped bass than short-lived species such as delta smelt. Barnthouse and colleagues (1990) compared hypothesized similar pollutant responses in populations of menhaden (a small, short-lived fish species) versus striped bass, and showed increased risks from chemically-mediated mortalities to the longer-lived, striped bass. However, small changes in early life-stage success have a greater impact on populations of species with relatively low fecundity such as delta smelt than those with higher fecundity. Therefore, species such as delta smelt might be more susceptible to nursery ground low-level chemical exposures since early life-stages are often most sensitive toxicologically and in this case are also demographically important. Thus, demographic modeling provides a useful framework to integrate single or multiple stressor effects throughout the life cycle

into projected population responses that take into account variation in life history characteristics among species (e.g., Winemiller and Rose, 1992). Importantly, population projections can be tested for concurrency with field data to infer the relative contribution of one or more stressors to population condition or trend (e.g., as has been done with San Francisco Estuary fish and other species, Rose, 2000).

In summary, the use of biomarkers and condition indicators that are correlated with vital rates (survival and reproduction) when taken together with other information, such as tissue chemistry, biomarkers of exposures, and laboratory or field experimental correlations, may provide evidence that chemical exposures (alone or in combination with other stressors) are contributing to population declines. Initially, the POD should place emphasis on biomarkers that are either chemical specific and reveal important exposures or are more clearly related to fitness effects such as growth, reproduction, and survival. Population modelers should be consulted when study plans are being developed, but a detailed modeling effort does not need to be implemented in the first year. The focus should remain on simple questions of identifying contaminant exposures and potentially significant detrimental effects at the individual level. If effects are revealed, toxicologists and ecologists can target key emphases for the models such as an emphasis on growth effects vs. reproduction.

2.4.8 Conclusions Regarding Condition Indicators

The Task Force reached three main conclusions regarding condition indicators and their relevance to biomarker implementation. These are:

- Condition indicators are easily sampled in ongoing monitoring, and they provide a good first approximation of whether a population is under stress; however, they are not contaminant specific. Hence, integrated analyses of biomarkers and analytical chemistry are needed to link changes in fish condition to contaminant exposure as well as to fish declines.

- Three types of condition indicators are recommended for implementation with biomarkers. These include parameters related to reproduction, growth/ energetics, and histology/pathology.

- A well-conceived archiving plan is needed to implement a stepwise approach for condition indicators, which ultimately increases cost-effectiveness while preserving options for more complex analyses.

3.0 INTEGRATION AND RECOMMENDATIONS

3.1 OVERVIEW

For any large-scale contaminants investigation, there is a dynamic tension between the proportion of resources that are invested in controlled laboratory studies and the proportion invested in field sampling. In the case of the POD, there is a need for both types of investigations to be undertaken in an integrated and iterative study design. The advantage of field studies of both condition indicators and biomarkers on POD species is that potential effects of long term and chronic exposures, as well as responses to multiple stresses, are integrated. In essence, it is vital to get into the field and "Ask the Fish" what is really happening. Laboratory exposures of POD species to either field water samples or to selected chemical compounds are also an essential complement, because these allow for:

- many compounds and samples to be screened rapidly to assess relative risk and further refine study objectives,
- toxicity identification procedures to be used to identify unknown compounds,
- characterization of effects on early lifestages that cannot easily be discerned in the field,
- controlled exposures and timecourse studies that improve the interpretation of biomarker responses.

A cost-effective combination of different approaches, therefore, provides multiple benefits that are often not widely recognized. In addition to the topics above regarding field and laboratory approaches, it is important to note that integration among laboratories provides additional resource advantages. For example, more effective use of fish tissues is a distinct advantage of carefully planning and executing an integrated program. When dissections from field or laboratory studies are performed by one team, then tissues can be shared and archived for follow-up studies more effectively. Variability associated with sampling error is also diminished. Accordingly, scientific team integration must also be a critical part of the planning from the outset, especially given the fact that molecular and enzymatic approaches necessitate specific tissue isolation and storage protocols. Unfortunately, such considerations are often afterthoughts and lead to losses of opportunities. When chemistry and biological effects and laboratory and field approaches are all thoroughly integrated in multiple laboratories, then increasingly novel statistical approaches and experimental procedures can be used to discern multiple stressors.

The strategic recommendations of this taskforce are represented in a tiered framework that is based on "drivers" that are specific to the POD and the overriding need to implement biomarkers and condition indicators in a highly integrated program. The drivers are:

- a requirement for more extensive characterization of contaminants of concern which necessitates both field and laboratory investigation,

- the fact that early life-stages of the POD species are difficult to obtain in viable condition from the field, which also implies a need for field and laboratory studies,
- the complexity of the San Francisco Estuary hydrologic system and geographic distribution of species, including the lack of a reference condition, meaning that laboratory and field studies will both be needed to increase the scope of inference, and
- the absolute need for a strategic, cost-effective, and time-sensitive framework of investigation.

3.2 RECOMMENDED TIER 1 APPROACH: FIELD AND LABORATORY INVESTIGATIONS AND SPECIAL STUDIES

A strategic suite of field and laboratory investigations, as well as high-impact "special studies" should be undertaken to discern the potential role of contaminants in the POD decline. The investigations must be highly integrated to maximize the likelihood of detecting contaminant effects. These recommendations are made from the perspective of biomarker implementation, since this was the focus of the Task Force, and we acknowledge that the broader perspective of the POD Contaminants Team and POD managers will differ.

FIELD INVESTIGATIONS

Objective: The primary objective of field investigations in the first year would be to assess the general health of juvenile and adult fish under realistic field exposure conditions. An additional goal would be to evaluate exposure to endocrine disrupting chemicals and planar aromatic compounds. Other toxic compounds may be of importance, but they are not easily studied with existing biomarker techniques. Responses relating to chemical exposure patterns would be measured, and sublethal effects on reproduction, growth, immune status, and organ condition would be quantified.

Recommended Biomarkers: We recommend vitellogenin/choriogenin as an indicator of exposure to feminizing (estrogenic or anti-androgenic) compounds and CYP1A and DNA adducts as indicators of exposure to planar aromatic hydrocarbons and note that DNA adducts are more likely to uncover positive results if applied to larger, longer-lived species such as striped bass.

Recommended Condition Indicators: We recommend a full suite of reproductive, growth, and histopathologic indicators with an archiving plan for some of the second phase histopathologic indicators and for RNA:DNA ratios as a measure of growth rates.

Integrating Chemical Analyses: We recommend that before field investigations begin, a chemistry team is formed, and a plan of analysis is formulated. Numerous decisions must be outlined in advance including: what media will be collected and why, how sampling will be conducted and where, and what the target analytes will be based on detailed understanding of inputs. These decisions will also directly inform the chemical

selections for some of the laboratory investigations below. The chemical analytical work should be integrated with the biological assessments in an interactive framework.

Interpretation: If the condition of adult and juvenile fish is relatively unimpaired over a period of two years, and biomarkers indicate no exposures of concern, it would lead to the conclusion that contaminants are not likely affecting the population--- at least in a manner that can be easily discerned using field collections of adult and juvenile fish. However, if after one year condition is poor, clues would be available as to what life-stages and types of effects should be the focus of subsequent investigations and whether exposures to endocrine disrupting and planar aromatic compounds are possibly involved.

LABORATORY INVESTIGATIONS

Objective: The two goals of laboratory investigations in the first year would be first, to assess effects of field-collected water samples on early life-stages which cannot be obtained from the field; and secondly, to conduct Toxicity Identification Evaluations (TIE) and on a limited basis, to screen individual chemical compounds and chemical mixtures to determine no effect-levels. Resident species toxicity tests would be used; and at the conclusion of the tests, tissues would be archived for subsequent biomarker analyses. To conduct these studies, an adequate supply of embryo and larval fish will be needed from hatcheries.

Selecting Toxicity Testing Endpoints: Biological responses measured in the resident species toxicity tests should include acute and chronic endpoints including: abnormal embryo or larval development, decreased growth (dry weight), hatching success, and survival. For some investigations, longer-term exposures, up to 28 days may be warranted.

Archiving for Biomarkers: Tissue samples from the bioassays which are not used in chemical analysis should be archived for potential biomarker analyses. This would serve two purposes. First, for field samples that contain chemical mixtures, biomarker responses would provide clues as to what chemical compounds are bioavailable and/or producing detrimental effects. For chronic effects, bioassay-directed fractionation techniques (TIE) are often infeasible; therefore, biomarkers can be used to confirm exposure to selected compounds including acetylcholinesterase for organophosphates and carbamates, CYP1A and DNA adducts for a variety of aromatic hydrocarbons, and metallothionein for metals. While these analyses would in no way produce a complete list of chemicals of concern, they would serve to narrow down possibilities. A second application of biomarkers would be to use tissues from the chemical exposures to analyze for response mechanisms that may be important in the field and to validate new methodologies that might also be applied in the field or follow up laboratory research. This is, perhaps, the most important application. For example, pyrethroid insecticides are known to damage DNA, and genotoxic effects have been recorded in a native fish species in the San Joaquin River (Whitehead et al., 2004). If similar effects are

confirmed in POD species, then it might trigger a special study to perfect one or more genotoxicity tests for application in the second year.

It is important to note that the use of some of the aforementioned biomarkers at this stage is advised, their use could be considered complementary or optional. For example, for metals and some pesticides, for which few biomarker options exist, a reasonable approach may be to initially use long term toxicity tests involving early life-stages. Such an approach may provide effective assessment of the magnitude of chemical effects in wild fish. If effect levels observed in the laboratory are not within an order of magnitude of field exposure levels, then more detailed biomarker investigation may help shed light on the discrepancies observed between laboratory and field studies.

Prioritizing field sampling: Samples from areas that represent worst case exposure conditions (along with appropriate reference-site controls for biological biomarkers) should be emphasized in the first year. Samples should be collected as close to the source of contamination as possible, where dilution is minimal and be collected during periods of greatest concern. Examples might include water from dead end sloughs adjacent to agricultural fields, with collections occurring immediately after a first flush event. Other examples might include samples collected adjacent to a wastewater treatment plant, marina, aquatic herbicide application, or dredging operation. Water, sediment, extracts from SPMD, and other samples could be returned to the laboratory. Decisions regarding sampling protocols would be outlined in a detailed study plan.

Prioritizing Chemicals and Mixtures: We advise that a chemistry panel and the POD contaminants work team prepare a prioritized list of chemicals and chemical mixtures for testing and analysis and that some are screened to establish no-effect levels for the POD species. However, we emphasize that TIE approaches should be emphasized whenever feasible as the most effective method of discerning chemicals of concern.

Integrating Chemical Analyses: The analytical team would assist in the planning of exposures, field sampling, and validation of chemical exposure levels during testing. Analyses of several contaminants of concern may not be routine, and targeted research and close collaboration with biologists will be a key to success.

Interpretation: If worst-case exposure conditions produce no effects on embryos and larvae in the laboratory, then the sampling program has either failed to produce worst case conditions or it would appear unlikely that contaminants are causing serious harm to these life-stages. It would be important to undertake a revision of sampling procedures and strategy in the second year to confirm. For example, onsite bioassay trailers, SPMD techniques, and integrated water samplers may all be considered and sampling frequency and locales adjusted. In addition, if long term bioassays of key contaminants and contaminant mixtures in the system produce few or no effects, within an order of magnitude of levels observed in the field, then it would imply that these are unlikely to produce significant stress on the population.

RECOMMENDED SPECIAL STUDIES

This task force believes that special investigative studies are also needed to explore potential effects of contaminants. We urge the POD management to consider three particular types of investigations that drew our attention and have relevance to the topic of biomarkers.

First, it is possible that the combination of studies described above would be inadequate to reveal neurological damage in fish associated with pesticide exposure. This has been shown to be an important issue in salmon (Scholz et al., 2000; Sandahl et al., 2005). In this context, acetylcholinesterase inhibition is a useful biomarker, and it can be combined with other neurophysiological and behavioral observations to perform cumulative ecological risk assessments for increasing levels of contaminant exposure.

Secondly, effects of multiple stressors could initially be examined by focusing on the interaction between salinity stress and contaminants. We noted that biomarkers of ion regulation and of multixenobiotic response (MXR) mechanisms might be invoked to determine whether any of the POD species are more likely to be highly susceptible to a combination of contaminant and salinity stress. Of course, food stress, disease, and other factors could be incorporated into a more complex experimental design if resources are available.

Third, toxicogenomic techniques using fish are becoming more common and are increasing our understanding of mechanisms of actions of chemicals, as well as showing promise in providing new biomarkers. However, understanding gene expression in fish exposed to multiple stressors in the field is problematic. It is reasonable to consider this area in the context of a long term investment. Specifically, the development and application of microarrays specific for POD species and initial studies in the laboratory could be useful. Ultimately, toxicogenomic approaches may be able to shed some light on the nature of chemical and environmental stressors. A limited investment might be a hedge on the future.

3.3 GENERAL APPROACH FOR TIER 2 INVESTIGATIONS

Investigations conducted in Tier 1 will provide information regarding three lines of evidence: the classes of chemicals that pose the greatest risk, the types of effects or mechanisms of effect that predominate, and/or which life-stages seem to be most critical for further study. Such findings would then trigger a second phase of study design that would be more focused and definitive. For example, a decision to focus on early life-stages would lead to the need for more resources for laboratory exposures and perhaps *in situ* transplant studies or onsite exposure facilities. Whereas, discovery of histopathologic abnormalities in juveniles and adults might argue for a more intensive use of immunologic and DNA damage biomarkers to determine whether contaminant exposure is likely the cause. While we attempted to formulate a detailed decision tree that would aid planning for Tier 2, we ultimately resolved that this was more

appropriately an aspect of the formal study design. However, the need for flexible planning and expertise should be considered from the outset.

3.4 FURTHER TECHNICAL CONSIDERATIONS

Several technical considerations must be outlined when a detailed study plan is drafted. First and foremost, sampling design and plans for statistical analysis of field data must be carefully considered. With integrated studies, several measurements are made on the same individual fish, and this permits use of several multivariate techniques (Chevre et al., 2003; Galloway et al., 2003) and an iterative multivariate approach suggested by Bennett and coworkers (unpublished, PEEIR website, http://www.bml.ucdavis.edu/peeir/). These techniques can be powerful in ascribing relative risk associated with multiple environmental stressors. Other significant issues include but are not limited to:

- plans for chemical analysis based on a knowledge of the landscape, priority analytes, and extensive experience with multiple environmental media,

- plans for collecting environmental samples for return to the laboratory, also considering any unique resources to create cost-effective sampling (e.g. mobile laboratories or any strategic approach that addresses spatial and temporal dimensions of sampling),

- needs for ancillary data collection such as environmental variables that document temperature, salinity, flows, suspended solids,

- preparation of a detailed archiving plan in consultation with each laboratory involved and given a realistic assessment of capabilities and storage capacity,

- budgeting for other support such as data management and archiving, internet conferencing, administrative and contracting support as well as funding for annual planning retreats, and

- possible utility of several additional screening tests such as Ames assays, to reveal genotoxicity, as well as cellular tests, to reveal endocrine disruptors. These were beyond the scope of this committee, but we recommend that they be considered in the future.

3.5 RESOURCE AND IMPLEMENTATION ISSUES

Integrated assessments of effect of contaminants on fish population status have been undertaken (e.g. http://www.bml.ucdavis.edu/peeir/) but, none provide an ideal precedent for planning the POD investigations. What is essential, however, is an understanding that integrated work requires a total commitment to the integration. This means that adequate funding must be available to the individual laboratories,

hatcheries, sampling teams, and for a scientific director and external steering committee. When this type of scientific consortium is formed, it also generally serves as a magnet that attracts outside involvement and resources, such as collaboration with EPA or NOAA laboratories. Finally, a targeted RFA should be considered to add new laboratories to the team, with different resources, to maintain the intellectual vigor of the undertaking.

Given that resources are not infinite, the committee made an effort to highlight some guidelines that would define a minimum effort. We concluded that investigations might focus on two fish species that would be selected based on policy considerations and factors such as available expertise and hatchery capacity. Investigations would be conducted year round but, for some species, more intensive sampling should be conducted in the late winter and early spring, when contaminant levels may be highest in the system. The minimum investment in time would be three to four years after funding and would include six months for planning and method development, two to three years of field and laboratory investigations, and six months for synthesis. Inadequate funding or lack of integration might result in any of a number of problems including: flawed project design, sampling difficulties, slow turnaround of laboratory samples, problems at the hatcheries, poor linkage to contaminant exposure, and limited possibilities for statistical analysis.

4. CONCLUSIONS REGARDING STRATEGIC FRAMEWORK

The central questions that we addressed were “What is the most strategic use of biomarkers to determine whether contaminants are causing significant stress in POD populations, and how should they be integrated into the larger perspective of other toxicological methods and multiple stressors?” Our simple answer to these questions is that most biomarkers are not meaningful in the context of the POD unless they are thoughtfully implemented in an integrated program of study. The promise of biomarkers is that they can help indicate exposures to specific classes of chemicals and that they may increase the understanding of subtle chronic effects that are not obvious in controlled laboratory studies, or with other techniques such as condition indicators. Thus, many biomarkers can be valuable if used in a weight-of-evidence approach to assess whether poor fish condition is related to contaminant exposure. After extensive deliberation and analysis, the committee documented several key factors unique to the POD that ultimately drive the strategy that we have summarized. Each of the major sections of the report include summary key conclusions in bulleted lists that document the most important items that shaped our suggestions below.

The group concurred that a minimum effort would be to select two of the POD species, largely based on policy relevance, and to undertake an integrated contaminants study with year round sampling but extra effort during the late winter and early spring months, when contaminant exposures are likely to be greatest. We recommend an integrated assessment using field-collected juvenile and adult fish as well as laboratory exposures of embryos and larvae to field water samples and putative chemical compounds (that are hopefully selected based on TIE results). The embryos and larvae

would be obtained from hatchery stock, and this implies adequate support for the hatcheries. For field studies, one would measure a small suite of biomarkers (vitellogenin/choriogenin, CYP1A, and DNA adducts) in combination with a larger initial suite of fish condition indicators (reproduction, growth, histopathology, disease incidence). A sophisticated archiving program would be used to save tissues for additional analyses that might be relevant after a first tier of data comes in. Analytical chemical analyses would be needed in concert, and analyses should be conducted in an iterative research mode. A parallel program of screening contaminant sources using resident species toxicity tests in the laboratory is needed and would provide direct feedback for a more definitive field study the next year. Specimens from laboratory studies would be archived for biomarker analyses. Subsequent laboratory-based biomarker studies would elucidate responses that may be of relevance in the field and hence serve as validation for biomarker techniques that could be implemented in Tier 2. Three types of special investigative studies are also recommended, including an examination of salinity on contaminant stress, a more detailed analysis of pesticide effects, and, tentatively, a toxicogenomics pilot study.

This integrated effort would require a designated lead person, an external scientific steering committee, adequate funds, and a variety of types of capacity building. The lead person should also be delegated to attract interest of EPA research laboratories and other entities that might provide assistance.

The issues surrounding the POD are unique. We conclude that it will be difficult to confirm with 100% certainty the exact role of contaminants in the status of the POD populations. However, we agree unanimously that an integrated use of “Best Available Technologies” within the fields of toxicology, environmental chemistry, and ecology, can lead to a better understanding of the contributions of chemical hazards and increase our understanding of the temporal and spatial aspects of any detrimental effects. The payback for undertaking the work in an integrated framework is significant, and this also represents the pathway to true multiple stressor investigations. An answer to the simple question “Is it plausible that contaminants pose a significant stress to POD populations?” is indeed attainable, provided there is commitment to all components of an integrated study design.

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