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Bivalve Biomarker Workshop: overview and discussion group summaries

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The Bivalve Biomarker Workshop, sponsored under a cooperative agreement between Marine Resources Research Institute (MRRI) and the National Oceanic and Atmospheric Administration (NOAA), was held in Charleston, SC at MRRI, March 19–21, 1998. The purposes of the workshop were to review the current state of knowledge on various biomarkers, discuss inferences from biomarker measurements to health and fitness parameters, evaluate the efficacy of biomarkers as monitoring tools, and identify critical research needs. A list of participants is provided at the end of this summary. The first day and a half were devoted to a series of presentations, most of which are summarized in the individual papers published in this volume. Then the participants divided into one of the three following discussion groups: Cellular Damage, Detoxification Responses and Stress Proteins, or Immune Function and Disease Responses. All members reconvened for a final plenary session to discuss working group summaries and other issues that were raised during the conference, and to develop recommendations for future efforts. To the extent possible, presenters as well as discussion groups were requested to address the following questions:

- Does the biomarker response indicate exposure to specific pollutants or different classes of pollutants; or does the biomarker function as a general indicator of anthropogenic stress?
- To what extent is the biomarker response affected by natural environmental variables (salinity, dissolved oxygen, temperature, etc.), or physiological variables such as reproductive cycle, age, or size? Can we correct or adjust for these sources of variation, and can we define normal ranges?
- Does the biomarker response enable us to distinguish between exposure and adverse effects?

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- Can we distinguish transient responses from sustained chronic responses that reflect irreversible damage?
- Are there quantifiable relationships between a biomarker response and contaminant concentrations of tissues and/or the environment?
- Is there evidence that the biomarker response is linked to adverse effects on fitness components such as growth, reproduction, or gamete viability?

Biomarkers of cellular damage

S. N. Luoma and R. T. DiGiulio (*Moderators*), M. Depledge, A. Ringwood, S. Steinert, S. Teh, R. Van Beneden, G. Winston.

It has long been recognized that the effects of pollutants on organisms are manifested as perturbations at different levels of functional complexity. Cellular responses to pollutant exposures are thought to be among the most sensitive and earliest detectable responses. In the mid-1980s, several authors (e.g. M. Moore, A. Viarengo) reviewed cellular disturbances such as lysosomal destabilization and lipid peroxidation in this context. Since then, a number of questions have been raised about appropriate uses of cellular biomarkers. Can we use them as early warning signals of pollutant impacts? Can we use them alone as a biological substitute for chemical information and as a signal of multiple chemical impacts? Is there a hierarchy of responses that we should expect to see as pollutant damage manifests itself, and can we employ that hierarchy in a diagnostic sense? Are cellular biomarkers just responses to exposure (exposure indicators) or do they truly indicate that the organism is stressed (effect indicator)? Are there pollutant-specific biomarkers? Do cellular changes similar to those caused by pollutants occur seasonally or in response to natural stressors such as salinity changes?

The fundamental questions about the use of cellular biomarkers have not changed greatly in the last decade. Superficially this might imply that substantial progress has not been made, but it is clear that our understanding of cellular biomarkers has matured. For example, basic mechanistic understanding of cellular biochemistry and the ramifications of the effects of lysosomal damage have contributed to the widespread application of lysosomal function as a tool for evaluating cellular responses to pollutants. Of all the cellular biomarkers, this may be the most effectively applied. On the other hand, it is now well established that neither this technique nor any other will provide the 'silver bullet' that can be applied anywhere to unequivocally demonstrate that pollutants are affecting organisms, populations, or communities. Only rarely (e.g. TBT and imposex) do we find simple responses that are unambiguously pollutant-specific or useful for determinations of cause and effect. While cellular processes are, indeed, quite sensitive to pollutant exposure, the hierarchical progression of responses with ascending levels of functional complexity has not been readily demonstrated. Populations stressed by pollutants may show signs of stress simultaneously at multiple levels of organization (e.g. G. Scott, this conference). It is possible that the cause of an effect observed at higher levels of organization can best be linked to pollutants (or better yet, explained) if cellular biomarkers are included in the study. We now appreciate that, analogous to diagnosis in human medicine, recognition of most pollutant effects will depend upon determination of suites of responses, rather than any magical pollutant-specific response. Some sensitive cellular responses, like lysosomal destabilization or cytochrome P450, might be used alone

as practical screening tools for evaluating whether pollutant effects might be present in an environment. However, biomarker tools may have limited use in isolation; but this is also true for other tools currently available for understanding pollutant effects. At present, cellular biomarkers are limited in regard to direct information concerning population-level effects. Because higher order effects are currently the focus of ecological risk assessments, it is critical to improve our understanding of how changes in biomarkers translate mechanically into implications for individuals and populations.

While some of the simplest hopes for the use of cellular biomarkers may not be coming to fruition, it is interesting to evaluate the directions that the science is moving in response to the obvious need to learn on multiple fronts. The present workshop once again reiterated the importance of understanding basic cellular processes. While great emphasis still rests on the application of individual techniques, understanding the integrated response(s) of cellular processes to different types of stress is advancing. Effective diagnosis of the role of pollutants will probably advance accordingly.

Application of the basic knowledge to field studies is the ultimate goal. A variety of approaches to such applications were discussed. Monitoring programmes are beginning that employ a battery of biomarkers, and biomarker indices, that integrate the biochemical responses into a numerical index. Narbonne discussed uses of such an index to reduce variability in response and correlate overall cellular biochemistry with pollutant occurrence. Another approach has been to coordinate knowledge of pollutant exposures from specific environments with multiple biomarker responses and organismal and/or population biology (Scott, Casillas, Ringwood, Teh, Werner). The insights about the responses to pollutants of organisms and populations can be impressive; and it was clear that many of those insights would not have been attained without verification of pollutant-specific stress with cellular biomarkers. A. Ringwood used a suite of biomarker responses, organism-level studies, and analyses of contaminant concentrations to convincingly link reduced growth in juvenile oysters with glutathione depletion and lysosomal destabilization. G. Scott explained the changes in the oyster life cycle in response to urban runoff, agricultural runoff, and multiple contaminant exposures. E. Casillas showed impaired growth, altered population age structure and reproductive impairment in mussels, coincident with bioaccumulation of chemical contaminants. The association of these signs with subcellular biomarker responses was an important verification that organismic responses occurred concurrently with pollutant stress. The biomarkers also helped resolve the potential contribution of the different xenobiotics. Teh reported on a study that showed metal bioaccumulation and histopathological damage were statistically related to changes in condition and reproductive impairment in resident populations of a clam in San Francisco Bay. The cellular biomarker studies were essential in reducing the ambiguity in linking cause and effect in a complex setting.

Conclusions and recommendations

From the studies presented in this workshop it is clear that biomarker responses cannot substitute for understanding pollutant exposure and organism/population changes. However, biomarkers may be the only way to provide the crucial evidence that links pollutants to biological change. The separation of exposure biomarkers

from effects biomarkers is too simplistic. There is a continuum between the two. The great potential for biomarker applications will not be fully achieved without continued advances in understanding basic cellular and biochemical responses to pollutants. Undoubtedly new tools of increasing usefulness will emerge from that understanding. We must begin to understand variability of biomarker responses in nature, as well as simply trying to design studies that minimize variability. That means better understanding of the range and cycles of biomarker responses in the absence of pollutants as well as in the presence. Most importantly, we must understand the integrated responses of the organism at multiple functional levels as complex pollutant exposures are superimposed on natural stresses and cycles. Fifteen years ago this order sounded idealistic and impractical. In the intervening time, careful studies have demonstrated that many of these objectives can be achieved. Now a body of work is necessary to demonstrate where and how such applications are most useful.

Detoxification responses and stress proteins

R. Lee and M. Brouwer (*Moderators*), D. Epel, C. Minier, J. F. Narbonne, L. Peters, G. Roesijadi, A. Viarengo, I. Werner.

Changes in the concentrations of a variety of detoxification/stress proteins after exposure of bivalves to contaminants have been used in biomonitoring programmes. Examples presented at this workshop included metallothioneins (Ringwood, Roesijadi, Viarengo), multixenobiotic transporter proteins (Minier, Epel, Ringwood), cytochrome P-450 monoxygenase system (Peters, Narbonne), stress proteins (Ringwood, Werner), anti-oxidant enzymes (Winston), NADPH-ferrihaemoprotein reductase, catalase (Narbonne), diaphorase, gamma glutamyl transpeptidase (Casillas), glutathione S-transferase (Narbonne), and glutathione (Ringwood). Metallothioneins and cytochrome P-450 monoxygenase systems are examples of biomarkers that respond to specific pollutant classes. The other biomarkers discussed are more general indicators of anthropogenic stress.

An important factor that needs to be appreciated for all of these biomarkers is the normal range of concentrations at a fixed point in time. In addition, many of these biomarkers show marked seasonal changes, e.g. cytochrome P-450 systems (Peters and Livingstone) and multixenobiotic transporter proteins (Minier and Epel). When active reproductive periods cause such large variabilities in biomarkers, they should not be used during this period or scrutinized carefully. Natural environmental variables, such as temperature, can affect the level of the various detoxification/stress proteins. An important question is, how far outside the normal range do these biomarkers have to be before the animal is considered 'stressed'? For example, after many field and laboratory studies, Ringwood suggests that concentrations of glutathione below 400 nM g^{-1} are indicative of stress in the oyster, *Crassostrea virginica*. For other detoxification/stress proteins more work is needed to determine the normal range of concentrations. Depledge discussed the potential for using the variability in biomarker responses in bivalves as a tool for identifying pollutant effects.

Some biomarkers are most useful as an indicator of exposure, e.g. cytochrome P-450 systems and metallothioneins. Dose–response relationships have been demonstrated for a few of these biomarkers, including metallothioneins (Ringwood, Roesijadi), glutathione (Ringwood), multi-xenobiotic transporter proteins

(Minier), and stress proteins (Werner). The linkage between a biomarker's response and an adverse effect, such as effects on growth and reproduction, was demonstrated in a few cases (Casillas, Ringwood, Werner).

Conclusions and recommendations

The detoxification/stress proteins that were evaluated in this workshop may not be the best biomarkers, but they are the ones that are available at this time and many, if not most, are under development. It seems likely that useful and new detoxification/stress proteins will appear from ongoing studies and advances in molecular tools. Establishment of the normal ranges for selected biomarkers should have high priority. In addition, the workshop group recommended a multi-marker approach with the type of contaminants at a particular site as one of the driving factors for selecting the biomarkers to be used at a site. Caged/transplanted sentinel species may offer some advantages for some studies by reducing the effects of endemic tolerance and the variability associated with different ages and exposure history. In some sites, it may be useful to conduct both multi-marker and multi-species assessment work. For example, infaunal as well as epifaunal bivalves may provide valuable insights, particularly since sediments are the primary sinks for contaminants. However, just because a species is infaunal does not necessarily mean it would serve as a better indicator species. For example, it has been observed that clams (infaunal bivalves) may have much lower metal bioaccumulation rates and reduced MT expression compared to oysters (epifaunal bivalves). More field testing with laboratory verification should be carried out with all of the biomarkers reported in this workshop. Greater emphasis should be placed on relating the biomarker responses to effects, particularly at the population and community level. All of these biomarkers should be useful for assessing contaminants from both point sources and non-point sources. Since changes in the responses can typically be measured after even a short exposure period, they may also be particularly useful for monitoring the effectiveness of remediation efforts. It is hoped that a multi-marker and multi-species approach, along with linkages to ecological effects, will provide useful diagnostic and predictive capabilities in risk assessment work at contaminated sites.

Immune function and disease responses

E. C. Peters and G. I. Scott (*Moderators*), R. Anderson, L. Burnett, F-L. Chu, L. Oliver, K. Paynter.

Immune systems provide the means by which organisms are protected from the harmful effects of pathogens such as viruses, bacteria, fungi, protozoa, and multicellular parasites that are capable of invading and producing disease. In bivalves this defence is provided by the circulating haemocytes, some fixed phagocytic cells, and secreted products of cells, e.g. the humoral immunity factors. This workgroup identified the following as the most critical issues pertaining to immune system responses and exposure to pollutants. Genetic variations, both interspecific and intraspecific, of the immune responses have been noted that might enable one group or some individuals to have an advantage over others in resisting invasion by pathogens and reducing its susceptibility to disease. Age, gender, reproductive state, nutritional status, and natural environmental variables can affect

the immune system and the type of immune response that an organism is capable of mounting. Work to date suggests that the indicators can probably be corrected or adjusted to control for these natural variables (Oliver and Fisher), and normal ranges for values of these indicators in different groups of organisms can be derived, but the number of individuals that must be examined under a variety of conditions to develop this framework needs to be defined. Indicators of immune system function have shown dose-dependent correlations with exposure to chemical contaminants in both field and laboratory studies, but these studies are very limited in scope, e.g. number and classes of chemicals tested, species tested, indicators evaluated. Indicators of immune system function do not provide specific information about exposure to individual pollutants or other anthropogenic stressors (Anderson). Indicators of immune system function could serve as biomarkers of effect because chemically mediated reduction of immuno-competency has been linked to adverse effects on host resistance to infectious diseases (e.g. increased MSX and *Perkinsus marinus* infection rates in oysters from polluted habitats, Chu).

This workgroup discussed the advantages and disadvantages associated with a variety of parameters that have been examined to qualitatively and quantitatively provide information on the functioning of the immune system in bivalves. However, for many of the immune system biomarkers, different techniques have been used by different laboratories, in different field or laboratory situations, and with different bivalve species. For example, the immune system of the quahog *Mercenaria* spp. is quite sensitive to certain chemical contaminants, but few pathogens, especially bacteria, affect quahogs or other bivalves in epizootic proportions. Mussels and oysters have been studied more in relation to contaminant exposure effects, and the oyster pathogen *P. marinus* is now relatively well understood, but oyster immune responses are easily overwhelmed making it difficult to identify contaminant effects on the immune system when this pathogen is present (Paynter, Burnett). Thus, although data have been accumulating, the primary issues remaining are the comparability of measurements and how to interpret results. The workgroup narrowed down the list of immune system parameters to the most significant indicators and divided them into two groups or tiers based on their characteristics:

Tier I—Morphometric Indicators: haemocyte number and differential cell count

Tier II—Functional Indicators: killing index, phagocytic index, NBT reduction, serum lysozyme, chemiluminescence, agglutinens, MTT reduction, and neutral red uptake and retention.

The Morphometric Indicators provide information about the ability of a bivalve to develop an inflammatory response when challenged by a pathogen in terms of numbers and types of circulating haemocytes present and can be used to quickly screen animals. Increased haemocytes are also indicative that the bivalve is diseased and that an inflammatory response is present, but will not provide definitive evidence that a pathogen is present (e.g. inflammatory responses have been found in bivalves exposed to toxicants in the absence of pathogens and parasites and in cases of neoplasia). The Functional Indicators provide information about the ability of a bivalve to neutralize or kill an invading pathogen. Dysfunction in these indicators is more useful in determining why a bivalve is diseased, but they require more time and expertise to perform.

Both field and laboratory approaches should be used for studying the effects of

xenobiotics on immune system function. Laboratory assays, exposures to bivalves or their cells and tissues (*in vitro* and *in vivo*), can be controlled to examine the role of other environmental factors in the immune response, such as water temperature and salinity, and rapid screening procedures can be used to identify immunotoxicant chemicals for further study. Field-collected bivalves will reflect long-term cumulative exposure to altered water quality, chemical contaminants, and pathogens so correlations of immunosuppression to specific pollutants may be difficult. Likewise, distinguishing pollutant effects from the effects of natural variables may be problematic. They can be used, however, for spatial and temporal studies to distinguish the relative effects of exposures occurring at different sites or the effects occurring during specific time periods. Finally, use of field-deployed bivalves is increasing because disease-free stocks can be used or they can be bred for certain traits. The place and period of deployment also can be easily changed to address spatial and temporal questions of exposure to xenobiotics and pathogens. The use of suitably large sample sizes will increase the power to discriminate effects on the immune system. The workgroup advocated using all approaches to improve our interpretation of the condition of the immune system and the impacts of chemical exposure across studies.

Conclusions and recommendations

A team of experts needs to complete a 'state of knowledge' report to provide guidance on further research needed to understand the bivalve immune system and the appropriateness of specific biomarkers of immune system function. At least some techniques for measuring immune system function in bivalves should be standardized with respect to procedures and materials, and quality control standards or reference materials should be developed to improve assays and permit interlaboratory comparisons. 'Normal' ranges need to be determined for different parameters for a variety of bivalve species at a range of reference sites. A supply of 'disease-free' oysters should be developed for use in transplantation studies and in the development of standardized techniques. Work must continue to distinguish transient reversible responses from chronic responses that reflect irreversible damage.

The ability of the immune system to effectively challenge invading micro-organisms can be measured by certain indicators. Additional studies are also needed on the potential suppressive effects of specific chemical contaminants on the immune system that make an organism more susceptible to infection by pathogens and disease. Likewise, the effects of pathogens on an organism and its immune system can affect how an organism reacts to a chemical contaminant; and pathogens, as well as the host, can be affected by environmental exposure to contaminants. Thus, the Workgroup on Immune Response Biomarkers noted that much remains to be done in the development of biomarkers of immune responses, but this promising field warrants further investigation.

Final commentary

The summaries of the three working groups include most of the general conclusions. The most prevalent findings that were commonly reiterated were as follows. Most biomarkers function as general indicators of stress but specific suites of responses or a multi-marker approach should enable scientists to focus on putative causes. Many individuals presented a bio-medical model analogous to the

approach used in human health, i.e. that we should be able to use suites of diagnostic and prognostic tools. Within this context, human health diagnostics is successful because of the well-defined 'normal ranges'. A strong consensus of the participants was the need to conduct the research and establish an information management system that would facilitate defining normal ranges for many of the responses. A number of studies have successfully documented seasonal changes that would need to be incorporated into a normal range approach. When extrapolating from individuals to populations, the data should be presented in such a way that skewness and variability can be derived. Variability in data as well as central tendencies in the data (i.e. means and medians) were regarded as important information for interpreting responses. Although relating alterations in cellular responses to fitness is desirable and empowering, establishing those relationships could be difficult in some cases. Increased collaborative research, more field studies, and developing a good understanding of normal ranges, as well as increases in mechanistic studies, will continue to facilitate the advancement of this field and the utility of cellular biomarkers as monitoring tools. Merging these sensitive tools with advances in biotechnology should increase their application. It is time to develop strategies for incorporating cellular biomarkers into risk assessment and environmental management.

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