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Appraisal of prospective bivalve immunomarkers

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Worldwide concern over threats to natural resources and public health has led to increased efforts to monitor and assess environmental conditions. This has stimulated the need for development and application of select biological and ecological measurements, or indicators, that are responsive to environmental stress. Measures of bivalve mollusc defence activities, such as haemocyte density, phagocytic activity, locomotion and production of cytotoxic molecules; and haemolymph constituents, such as agglutinins and lysozyme, have potential as indicators and appear to be responsive to xenobiotic chemical insults in the aquatic environment. However, basic research on the relevance of these measurements in inferring resistance to disease or enhanced survival is currently insufficient, reducing their value as potential biomarkers to address environmental objectives. In addition, variation in defence activities caused by seasonal temperature and reproductive cycling, salinity changes, nutritional status, diseases and parasites, and genetic stocks is high and may limit applicability of bivalve defence-related measurements as indicators. This review examines these sources of variability and their possible implications for interpreting changes in bivalve defence activity as an indicator of stress. Examples of contaminant-induced changes in bivalve defence functions are described.

Keywords: Indicators, bivalves, xenobiotic chemicals, defence activities, immunomodulation.

Introduction

The focus of environmental monitoring has evolved from measuring discrete sources of pollution, such as chemical emissions, toward a broader context that integrates and describes the effects of multiple, and sometimes unknown, stressors (Cairns *et al*. 1993, Munkittrick and McCarty 1995). Because the characteristics of an ecosystem are far too complex and numerous to wholly quantify, indicators are an efficient means to obtain useful and representative information about the condition of a resource or an ecosystem. A challenge for indicator research and development is to identify those measurable environmental characteristics that are essential to the integrity of a resource and responsive to stressors which threaten that integrity (Fisher 1998).

The use of bivalve molluscs in environmental monitoring has followed a similar evolution. Filter-feeding bivalves accumulate chemical contaminants and may acquire tissue concentrations of anthropogenic chemicals thousands of times higher than those in the water column. Chemical analysis of bivalve tissues can then be used to describe contamination profiles for different sites. This exposure monitoring approach was used in northern Europe and the United States in the 1960s and 1970s under the International Mussel Watch Program (National Academy of Sciences 1980), and continues as the National Mussel Watch Program conducted by the National Oceanographic and Atmospheric Administration.

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Bivalves (mussels, oysters and clams) are collected and analysed to characterize environmental levels of pollutants. Additional advantages to using bivalves as sentinels include their lack of mobility and their wide distribution throughout the coastal waters of the world. By 1980, it was recognized that monitoring bivalves for exposure was a chemical issue, but determining the significance of any environmental or ecological impact was a biological issue (Bayne *et al*. 1980):

'… if environmental conditions were severe enough to cause biological changes in mussels, they are likely also to have an effect on other organisms more sensitive to environmental stresses.'

This group proposed the measurement of physiological and biochemical responses of individual bivalves as indicators of the health of the larger population and community. Such measurements today are termed 'biomarkers' (McCarthy and Shugart, 1990, Huggett *et al*. 1992).

Since the immune system often reacts to contamination, 'immunomarkers' have become an important subject of biomarker research. Immune functions in vertebrates are susceptible to damage by several classes of xenobiotic chemicals (Koller 1979). In the aquatic environment, immunomodulation resulting from chemical exposure has been demonstrated in fish (Roszell and Anderson 1996a, b) and dolphins (Lahvis *et al*. 1995). Invertebrates do not exhibit the same memory and specificity of vertebrate immune systems, but their internal defence system offers a wide range of measurements that might be used to estimate defensive capacity. The potential for identification and use of immunomarkers based on the defensive responses of bivalve molluscs is examined in the following review.

Internal defences of bivalves: haemocytes and haemolymph

Measurements made on marine bivalve blood cells, or haemocytes, are attractive candidates for biomarker development. It is often noted that haemocytes are the first line of defence for bivalves, although other mechanisms, such as mucus production, have not been broadly investigated. Certainly haemocytes provide the first line of *internal* defence, largely due to their ability to phagocytose living microorganisms. They also serve in other defensive functions, such as inflammation, wound repair and encapsulation (Cheng 1979). These cellular activities are varied and complex, but each relies to some degree on the ability of the haemocyte to recognize foreign entities, locomote, secrete, adhere to substrates, bind and ingest particles, and kill and digest pathogens (Fisher 1986, Feng, 1988). These features, measured singly or in groups, all have potential as biomarkers. In addition to defence, haemocytes also play a role in bivalve nutrition and shell deposition (Feng 1988). Taken together, the complexity of haemocyte activities and their participation in multiple physiological functions supports the likelihood of finding measurable responses to chemical contamination that are a valid reflection of changes occurring in the organism.

Bivalve haemocytes also have a potential for contaminant exposure. These cells and the fluid medium in which they reside (haemolymph) are believed to be responsible for the transport of contaminants from the organ of entry (e.g. gill, mantle, digestive gland) to the kidneys or other tissues where detoxification or accumulation may occur (Ruddell and Rains 1975, George *et al*., 1978, George and

Pirie 1980, Pirie *et al*. 1984, Robinson and Ryan 1988). Further, haemocytes may be involved in metal detoxification and removal of certain contaminants (Takatsuki 1934, Stauber 1950, Tripp 1960, Ruddel and Rains 1975, Pirie *et al*. 1984, Seiler and Morse 1988). Because they exhibit transepithelial migration (diapedesis), haemocytes containing contaminants can move into the digestive tract or outward to the external surfaces of the oyster for elimination in the faeces or pseudo-faeces. Detoxification and elimination have been attributed primarily to granular haemocytes, and the proportion of this cell type is reported to be elevated in polluted environments (Ruddell and Rains 1975, Pickwell and Steinert 1984, Pirie *et al*. 1984, Seiler and Morse 1988).

Haemocytes are easily accessible in relatively large numbers in haemolymph fluid drawn from adductor muscle sinuses of bivalves and can be held *in vitro* for several hours to examine their living properties (Tripp 1963, Tripp and Kent 1967). Measurements can also be made on the biochemical properties of the haemolymph serum, or plasma (Chu 1988). Some components of the plasma, such as lysozyme and agglutinins, are secreted by the haemocytes (Cheng *et al*. 1975) and may be important in defence. It is generally believed that contaminant exposure leads to suppression of defence activities and thereby reduces the ability of bivalves to defend against invading parasites and pathogens (Winstead and Couch 1988, Anderson 1994, Chu and Hale 1994, Anderson *et al*. 1996, Fisher *et al*. 1999). However, some studies have shown that relatively low concentrations of certain chemical contaminants can create a hormesis-like effect, activating or enhancing haemocyte defence activities (Fisher *et al*. 1989, *in preparation*, Anderson *et al*. 1997). Enhanced activity may permit bivalves to survive in a polluted environment.

Biomarker criteria

An indicator is a sign or signal that relays a complex message, potentially from numerous sources, in a simplified and useful manner. A biomarker may indicate exposure to environmental stressors, or their effects. As exposure indicators, biomarkers may 'diagnose' specific stressors or point to multiple, synergistic stresses. Biomarkers of effect are valued for their potential to predict future effects at higher levels of biological organization; that is, suborganismal effects may sufficiently precede population effects to allow implementation of resource management alternatives (Munkittrick and McCarty 1995).

There have been several criteria described for environmental indicators (Messer 1990, OECD 1993). Recently, the US Environmental Protection Agency summarized criteria for indicators to be used in the Environmental Monitoring and Assessment Program (EMAP) (Fisher 1998). This guidance was presented in four phases:

Phase 1—Conceptual Relevance: Is the indicator relevant to ecological function and to an identified assessment question?

Phase 2—Feasibility of Implementation: Are the methods for sampling and measuring the environmental variables technically feasible, appropriate, and efficient for use in a monitoring programme?

Phase 3—Response Variability: Are human errors of measurement and natural variability over time and space sufficiently understood and documented?

Phase 4—Interpretation and Utility: Will the indicator convey information on ecological condition that is meaningful to environmental decision-making?

Although not all environmental indicators are developed for programmes like EMAP, any successful indicator will be responsive to these criteria. Whether or not a measurement is a 'simple and useful' indicator of environmental condition is ultimately decided by programme managers who must weigh a variety of factors, including programme objectives, scale of application, cost, etc. At present, we feel the greatest challenges for the development of bivalve immunomarkers are (Phase 1) linkage of defence measurements to real effects in the population or community, and (Phase 3) the ability to discriminate meaningful differences among sites (because of high natural within-site variability between individuals and the relatively weak signal elicited from anthropogenic exposures).

In this review, we examine the issues of conceptual relevance and response variability for prospective immunomarkers of bivalve molluscs, with emphasis on the eastern oyster, *Crassostrea virginica*. There are several advantages to developing biomarkers with this species. Eastern oysters occur along the Atlantic coast and Gulf of Mexico in North America and are highly valued as a fisheries resource. They inhabit primarily estuarine environments, and as such are often exposed to pollution in terrestrial run-off. Because of major diseases that have afflicted eastern oysters, a relatively expansive literature is available on defence capabilities and activities. In the Gulf of Mexico, where populations of mussels are rare, the NOAA Status and Trends Program (National Mussel Watch) monitors eastern oysters for accumulated concentrations of anthropogenic chemicals. As with all bivalve molluscs, oysters are sessile organisms that bioaccumulate both inorganic and organic contaminants by filter feeding (National Academy of Sciences 1980). As such, bivalves may act as environmental sentinels for contaminant levels and for sub-lethal biological effects resulting from pollutant exposure. Although some issues presented here may be unique to eastern oysters, we feel they are generally representative of issues that must be considered for all bivalves that are candidates for biomarker studies.

Conceptual relevance

A successful indicator must be relevant to the objective of the monitoring programme. The objective is the first, and one of the most important, determinations in development of a biomarker or other environmental indicator—why is the indicator needed? As obvious as this may seem, many indicator research projects lack this simple direction. Such projects are often driven by basic, exploratory questions that address biological and ecological mechanisms, and are only secondarily considered to have some potential as an environmental indicator. A more effective approach to biomarker research will concentrate on achieving a justifiable objective that addresses an important societal value. In ecological risk assessment, the *assessment endpoint* must be defensibly linked to society's concern for ecological condition (US EPA 1996).

There are at least three societal values that could be addressed through implementation of bivalve immunomarkers. The most obvious, since bivalve fisheries and bivalve aquaculture exist throughout the world, is the status of an important economic resource. For example, immunomarkers may be useful for predicting oyster disease impacts and, consequently, the future strength of the fishery. The second is the prediction of public health risks from ingesting contaminated bivalve products; decreased defences may reflect a decreased capacity

to rid oyster tissues of human pathogens such as *Vibrio vulnificus*, *V. parahaemolyticus* or *V. cholerae* (Murphree and Tamplin 1991, Harris-Young *et al*. 1993, 1995). A third potential objective is assessment of environmental condition, as described above for the International Mussel Watch Program, with the embedded premise that the 'health' of an ecosystem will be reflected by the health of its inhabitants (Bayne *et al*. 1980). In all cases, it must be conceptually established that changes in the measured endpoint will correspond with concomitant or future changes in the bivalve resource (case 1), risk to public health (case 2) or condition of the ecosystem (case 3). If the endpoint cannot be linked to the objective, even conceptually, then there will be little benefit from further research and development. Conversely, those measurements that are most closely linked to the objective are the best candidates for further research.

Defence against disease, at least for eastern oysters, can be assuredly linked to the health and survival of oyster populations. The vast populations of eastern oysters that once inhabited the Chesapeake Bay have been decimated by two disease agents, *Perkinsus marinus* or 'dermo' (Andrews 1988, Burreson and Ragone Calvo 1996) and *Haplosporidium nelsoni* or 'MSX' (Andrews 1979, Haskin and Andrews 1988). Infection by *P. marinus* can cause a multitude of sublethal effects as infections progress, including reduced growth (Menzel and Hopkins 1955), biochemical changes (Soniat and Koenig 1982), and impaired reproductive capacity (Dittman 1993, Choi *et al*. 1994). Prior to causing death of its oyster host, *H. nelsoni* disease results in impaired feeding (Newell 1985), compromised condition index, reduced reproductive effort (Barber *et al*. 1988a, Ford and Figueras 1988), depressed haemolymph protein (Ford 1986), reduced glycogen (Barber *et al*. 1988b) and altered enzyme and free amino acid levels (Feng *et al*. 1970, Douglass and Haskin 1976).

Although more difficult to measure than direct effects on oysters, the impact of these diseases on functions performed by bivalves in estuarine ecosystems has been severe. In particular, the decreased ability of oysters to filter matter from the water column has greatly affected the ecology of estuaries. Newell (1988) estimated that the pre-1870 Chesapeake Bay oyster population could filter the entire water column of the Bay within 3–6 days, and that the current population—devastated by disease and heavy harvesting—requires 325 days. Increased water turbidity in the Chesapeake Bay, linked primarily to industrial and demographic growth, probably has been exacerbated by the loss of oysters. Furthermore, oyster beds provide epibenthic habitats for other organisms such as polychaete worms, barnacles and mussels (Dauer *et al*. 1982), and this habitat plays a key role in maintaining food web associations (Newell 1988).

An indicator endpoint should also be recognized as a critical factor in the system under study. This is a particularly important concern in biomarker research, since it must be demonstrated that characteristics measured at the suborganismal level are transferred as real effects to higher levels of organization. Many seemingly aberrant physiological characteristics, especially in bivalves, may simply be short-term or acclimation responses to changing environmental conditions with no ultimate effect on survival, growth or reproduction (Bayne *et al*. 1980, Munkittrick and McCarty 1995) and certainly no effect on the population and community. In this criterion, our prospective haemocyte and haemolymph biomarkers are lacking. Whereas quantifiable immune functions are essential in humans, or at least positively associated with bactericidal capability (Gifford and Malawista 1972,

Horan *et al*. 1982), such associations have yet to be confirmed between defence measurements and immunocompetence in oysters. Defence mechanisms are undoubtedly a factor in oyster interactions with *P. marinus* and *H. nelsoni*, but there is no evidence to date that any measured haemocyte activity or haemolymph constituent is associated with greater resistance to these parasites. This is an important unknown in our current concept. When a defence mechanism or resistance factor is identified that has some measurable impact on an important disease, then it will become a valid candidate for biomarker development.

There is a similar concern for the public health objective. The concept that defence activities of oysters can modulate the level of bacteria in their tissues is plausible, but currently there is only indirect evidence that supports this connection. Certain bacterial strains that are highly pathogenic to humans are retained in oyster tissues while other species may be depurated (Murphree and Tamplin 1991). In one example, haemocytic binding, phagocytosis and degradation of an avirulent translucent strain of *Vibrio vulnificus* exceeded that of the virulent opaque strain, suggesting that oyster haemocytes may be involved in depuration of *V. vulnificus* (Harris-Young *et al*. 1993, 1995). However, further study is required to connect oyster defence activities to depuration of potential human pathogens. From a different perspective, it is possible that certain defence measurements can be considered surrogates for the functional activities of all cell types in the oyster; thus, if haemocyte activities were compromised, then the overall health of the oyster would be compromised and the likelihood of acquiring oyster or human pathogens, increased. These suppositions have not yet been adequately explored.

Recent studies have begun to address these concerns over the relevancy and importance of the measurement to the objective. A haemocyte microbicidal assay has been developed to determine the killing ability of bivalve haemocytes (Volety *et al*. 1999). The assay can be applied to a variety of microorganisms, including *P. marinus* or human bacterial pathogens associated with oysters. The assay integrates all the cellular functions that have been measured previously (e.g. haemocyte locomotion, chemotaxis, particle binding and chemiluminescence) into a single functional measurement that is clearly linked to the objectives of characterizing oyster health and the ability to defend against microorganisms. The idea to measure microbicidal activity is not new (Tripp and Kent 1967, Hartland and Timoney 1979, Harris-Young *et al*. 1995) and the technique is well established for other phyla (Graham *et al*. 1988, Roszell and Anderson 1996a), but it has never been adapted to bivalve haemocytes or developed as a potential biomarker for environmental monitoring. Such a measurement would provide an important step toward linking defence activities with the ultimate purpose of an indicator.

Response variability

Perhaps the greatest challenge for development of useful bivalve immunomarkers is the ability to discriminate significant differences attributable to contaminant exposure over the high background of natural variability. By most accounts, variability of physiological characteristics in eastern oysters is driven by their seasonal reproductive cycle and the changing salinity and temperature conditions of their estuarine habitat. Bivalves are poikilothermic and osmoconforming, so the haemocytes are subjected to temperature and salinity

fluctuations that occur in their ambient environment which create variability in haemocyte defence activities (Fisher 1988). Furthermore, haemocytes are involved in a variety of physiological functions that may not be related to defence, such as intracellular digestion (Feng *et al*. 1977, Morton, 1983), shell growth and repair (Bubel *et al*. 1977, Watanabe, 1983), and repair of damaged tissue (Tripp 1963). These functions may drive their responses as much as, or more than, anthropogenic contaminants. Less understood, but undoubtedly important, is the variability created by differences in nutrition, diseases and parasites, and genetic stock. Consequently, even oysters collected from the same site at the same time of year can show wide variation among individual (coefficients of variation of 50–100%) for potential immunomarker measurements such as haemocyte number, rate of locomotion, and nitroblue tetrazolium reduction (Fisher *et al*. 1996b). Some of these issues are discussed below.

Temperature and seasonal reproductive cycle

Temperature affects the cellular and biochemical processes of oysters, including defence-related activities. Temperature effects were recognized early in the study of bivalve haemocytes when Feng (1965) reported a linear relationship between holding temperature and both heart rate and leucocyte counts. It was also found that clearance by oysters of chicken red blood cells after intracardial injection was slower at 6 °C compared with 15–19 °C (Feng and Feng 1974). Subsequently, temperature has been noted to affect haemocyte density and morphology (Chu and La Peyre 1993) as well as activities such as aggregation, spreading, locomotion, and particle binding (Fisher 1988, Fisher and Tamplin 1988). It should be noted that haemocyte activities are not always positively associated with temperature. For example, the rate of haemocyte locomotion of estuarine oysters from the Chesapeake Bay was lower after holding at 25 vs 15 °C, a possible consequence of summertime high temperature stress (Fisher 1988). The source of oysters may also be a factor; haemocytes from oceanic oysters responded more to short-term temperature change than estuarine oysters (Fisher 1988).

The rate of response to temperature change by haemocytes is generally unknown and probably dependent on the characteristic being measured. However, day-to-day fluctuations in temperature may be sufficient to create variations that obscure detection of contaminant effects. Considering this possibility, sampling techniques that alter the temperature of the oysters may also alter defence characteristics. This is certainly a possibility when sampling at multiple and/or distant sites requires chilling and re-warming oysters after overnight cold storage (Fisher *et al*. 1996a,b). In such cases, consistent procedures will preserve the validity of comparisons among samples, even if the measurements may not reflect *in situ* responses.

Longer term, or seasonal, effects of temperature have also been documented and are inextricably tied to physiological changes associated with the bivalve reproductive cycle. Haemocyte density of mussels *Mytilus edulis* collected at Venice Lagoon, Italy (Pipe *et al* 1995) was lower in summer, a period of spawning, compared with winter and spring. The spring onset of spawning in oysters from Apalachicola Bay, FL (May), was accompanied by reduced density of circulating haemocytes, a reduced haemocyte ability to produce the cytotoxic superoxide anion (O_2^-) and an increase in haemolymph concentration of lysozome, all of which

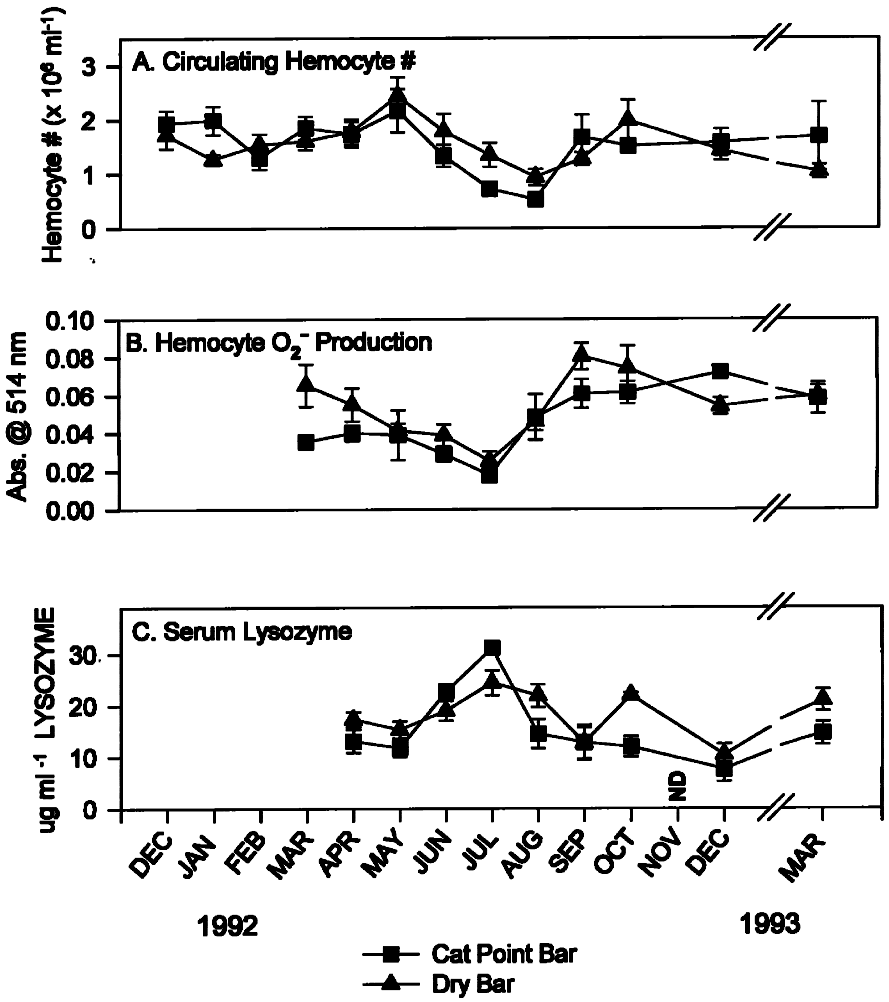

Figure 1. Defence responses of oysters collected from two sites, Cat Point Bar and Dry Bar, in Apalachicola Bay, FL during the period from December 1991 through March 1993. Bars indicate standard errors. 'ND' indicates no data for that date. (A), Circulating haemocyte number, (B) haemocyte superoxide (O_2^-) production, as measured by NBT reduction assay, (C) serum lysozyme concentration, (data from Fisher *et al*. 1996b).

returned to previous levels as spawning ceased in autumn (figure 1) (Fisher *et al*. 1996a, b). In spite of potentially decreased haemocyte density and O_2^- production during the spring and summer, preliminary data indicate that the ability of Gulf of Mexico oyster haemocytes to kill *Vibrio parahaemolyticus* (*in vitro*) increases during this time of year (Volety, personal communication). In another apparent contradiction, lysozyme in haemolymph plasma was lower during the summer in New Jersey oysters (Feng *et al*. 1970) and Chesapeake Bay oysters (Chu and La Peyre 1989), but higher in Florida oysters (Fisher *et al*. 1966b). These reports imply that seasonal effects of temperature on haemocytes and haemolymph constituents are dependent on the characteristic being measured and the geographic source of the oysters.

Differing effects of seasonal temperature change on oyster populations of

different geographic origin should not be unexpected; oyster gametogenic cycles are unique for different latitudes (Loosanoff 1965, Kennedy and Krantz 1982), and threshold temperatures for gametogenesis are thought to be genetically determined (Loosanoff 1969, Barber *et al*. 1991). Accordingly, any influence that reproductive cycling (or associated seasonal temperature changes) may have on haemocytes and their activities must be modulated by the geographic and genetic source of the oysters.

The seasonal cycle of temperature and/or gametogenesis may even affect the predominant types of haemocytes present and, in so doing, skew haemocyte activity measurements. For example; a haemogram shift from granular to agranular cell types of Chesapeake Bay oysters between January and May was observed by McCormick-Ray and Howard (1991). Geographic differences may create the same circumstance; Apalachicola Bay oyster haemocytes were morphologically different (generally smaller, with fewer granulocytes) from Chesapeake Bay oyster haemocytes when sampled at the same time of year (March 1992), and were also less active in particle binding, superoxide anion production, and mobility (Oliver and Fisher 1995). Cell composition is an important concern because, for most marine bivalves, granular cells are more active in phagocytosis (Foley and Cheng 1975, Renwrantz *et al*. 1979), production of lysosomal enzymes (Cheng and Downs 1988), production of cytotoxic oxyradical species (Bachère *et al*. 1991) and detoxification and elimination of trace metals (Ruddell and Rains 1975, Pickwell and Steinert 1984, Pirie *et al*., 1984, Seiler and Morse 1988). Estimates of immunocompetence may be easily misinterpreted if seasonal or geographic differences influence haemocyte composition.

Finally, changes in the reproductive cycle influence the accumulation of chemical contaminants in bivalves. Weight-specific concentrations of lipophilic organic compounds such as polychlorinated biphenyls tend to increase with fat accumulation during gametogenesis, and contaminants may be shed during active spawning (Hummel *et al*. 1989, 1990). Gonadal growth and accompanying weight gain can also dilute the weight-specific concentrations of some metals just prior to spawning (Boyden 1977, Phelps *et al*. 1985). Exceptions exist, however, as in the case of manganese which is enriched in the gonads of some bivalves during gametogenesis (Galtsoff 1964, Páez-Osuna *et al*. 1995). Again, oyster genetics and geography should be expected to influence the seasonal variations in contaminant accumulations.

It is obvious that temperature and seasonal variability must be taken into account in the development of bivalve immunomarkers, that different measurements may respond differently to temperature and season, and that oysters from different geographic regions may exhibit disparate seasonal variations. A partial resolution to this problem is to select an index period during which the bivalve physiology is relatively stable. Bayne *et al*. (1980) suggested a winter index period for bivalves due to reproductive stability and relatively high contaminant concentrations. Although a reasonable alternative, this approach may limit the potential application and versatility of an indicator.

Salinity

Several mechanisms exist for organisms to survive salinity changes in estuarine habitats, but not without affecting cellular and biochemical processes. An explicit

effect of salinity on eastern oyster haemocytes has been demonstrated *in vitro*. Fisher and Newell (1986) found that haemocyte mobility decreased after an acute increase in salinity, and hypothesized that haemocytes are unable to move until they volume regulate, i.e. build up sufficient osmotic effectors in the cells to prevent cell shrinkage. In declining salinity, the cells merely need to release osmotic effectors to prevent swelling, so there is little effect on other haemocyte activities. The effect of acute salinity change on haemocyte spreading was found to magnify during the hot summer months in Chesapeake Bay (Fisher *et al*. 1989), indicating possible interactive effects of other factors such as temperature or spawning. In fact, salinity and temperature appear to have many interactive effects on oyster haemocytes (Fisher 1988, Fisher and Tamplin 1988).

Haemocyte response to hyper-osmotic change is immediate—presumably the cells will not spread until they have reached osmotic equilibrium. Longer-term effects may also occur. In one study, it was shown that acclimation of low-salinity oysters to high salinity for 1 month eliminated any hindrance to cell spreading at high salinity (Fisher and Newell 1986). However, the rate of locomotion of haemocytes was still significantly retarded, implying that previous environmental conditions, at least 1 month preceding, still influenced haemocyte activities.

Salinity can also influence bioavailability, uptake and relative toxicity of many contaminants. For example, metal concentrations in Chesapeake Bay oysters were shown to be inversely correlated with salinity (Phelps *et al*. 1985). This may be partly due to the effects of salinity on metal speciation (Stumm and Morgan 1981), which in turn influences toxicity (Engel and Fowler 1979, McLusky *et al*. 1986). Comparisons of physiological responses are best made among bivalves from similar salinities to avoid error introduced from salinity effects on toxicant bioavailability.

Nutrition

Bivalve digestive tubules undergo morphological changes associated with feeding status. Intertidal bivalves cease feeding when tides recede and tubule columnar epithelium thins to a squamous morphology with an accompanying increase in lumen size (Morton 1970). Digestive tubules of subtidal bivalves that can feed continuously tend to maintain columnar epithelium with narrow lumens unless subjected to stress (Robinson and Langton 1980). Tubule atrophy in several bivalve species has been associated with stress from xenobiotic exposure (Couch 1984, Low 1988) but also result from insufficient nutrition (Winstead 1995) or an intertidal lifestyle (Winstead 1998). Although digestive tubules may seem more closely linked to food digestion than to defence, haemocytes also migrate into the gut to phagocytose and digest food (Stauber 1950, Tripp 1957, Feng *et al*. 1977) and may be equally influenced by the nutritional status of the bivalve, so even short periods of poor or high food availability could confound interpretation of haemocyte measurements. Digestive tubule morphological changes can occur in only 2–3 days (Winstead 1995). Following a 7-day period of starvation, circulating haemocytes of clams (*Ruditapes philippinarum*) were significantly reduced (Oubella *et al*. 1993). These authors hypothesized a (reversible) mobilization of haemocytes from haemolymph into soft tissues to compensate for the lack of nutrients. Regardless of the mechanism, effects of altered nutritional status on circulating haemocyte numbers could interact with or overshadow effects from chemical exposure.

Anoxia

Oysters typically inhabit estuarine environments and are therefore potentially subjected to periods of low oxygen, especially in the summer. Haemocyte viability and phagocytic ability appear to be insensitive to oxygen partial pressure as low as 9 mm Hg (Alvarez 1989, Alvarez *et al*. 1992). However, circulating haemocyte numbers in oysters living in a near anaerobic environment was one order of magnitude lower than that of oysters living in aerobic conditions (Alvarez *et al*. 1992).

Diseases and parasites

Infection of oysters by parasites may also influence defence activities and could interfere with accurate interpretation of defence-related measurements. Anderson *et al*. (1995) discovered that oysters with heavy *Perkinsus marinus* infections had greater numbers of circulating haemocytes and greater levels of zymosan-induced chemiluminescence. Both of these measurements are candidate indicators of xenobiotic exposure and may, in fact, respond to certain chemicals. However, interpretation of field results must take into account the potential influence of *P. marinus* and other parasites. An increased number of circulating haemocytes probably represents a generalized (inflammatory) response to the presence of infectious agents. Although increased haemocytes are present in heavily infected oysters, no resistance to *P. marinus* is conferred since haemocytes can phagocytose, but are unable to kill enough parasites to thwart infection progression (La Peyre *et al*. 1995, Volety and Chu 1995).

Since *P marinus* is virtually ubiquitous in both Gulf of Mexico and Atlantic coast oysters, some researchers are attempting to use *P. marinus*-free oysters for various types of experiments (Chu and Volety 1997, Bushek and Erskine 1998, Fisher *et al*. 1999). Such oysters must either originate in (rare and decreasing) areas that are unimpacted by *P. marinus*, or be carefully reared in filtered seawater that is free of the parasite. Experimental approaches using *P. marinus*-free oysters are attractive for bioindicator development since effects of xenobiotic chemical exposure can be tested without interference from pre-existing *P. marinus* infections. Even where *P. marinus*-free oysters can be employed in this manner, comprehensive parasite evaluation should always be done where possible to eliminate confounding factors potentially attributable to other parasites.

Metazoan parasites may also cause elevation of defence-related measurements. During a survey of polluted and reference sites in Tampa Bay, Florida, gonadal infestation of metacercariae of a previously unidentified trematode was observed in histological sections of oysters (Winstead *et al*. 1998). Oyster haemocytes from this relatively unpolluted site exhibited heightened haemocyte activities that were comparable to those from polluted sites (Fisher *et al*. in preparation). These activities included high haemocyte mobility, high particle binding ability, high levels of NBT reduction (indicative of O_2^- production) and high concentrations of plasma lysozyme.

The presence of bacteria may also alter serum and haemocyte characteristics. Increased haemocyte numbers were seen in two bivalve species injected with *Vibrio alginolyticus* (Suresh and Mohandas 1990a) and a third species exhibited increased haemocyte number and serum peptidase when exposed to *Vibrio* P1, the agent of Brown Ring Disease (Oubella *et al*. 1994). Based on these results, it seems plausible

that different concentrations or genera of bacteria could activate or enhance the activities of haemocytes.

Geographic origin of oysters is equally relevant to this discussion of the influence of parasites and disease on haemocyte activities. Cheng *et al*. (1993, 1995) described regional differences in oyster haemocyte agglutination responses to various carbohydrates. He believed that significantly higher lathyrose-positive haemocytes in oysters from Malpeque Bay, Canada, and Galveston Bay, TX, where *Haplosporidium nelsoni* has never been observed, indicated that lectin expression on haemocyte surfaces may be partly determined by local parasites. If so, then defence responses may be geographically specific, influenced as much by local parasites as by contaminants.

Genetic stocks

Several examples have been given regarding the importance of genetics and geographic origin of bivalves used for biomarker studies. Definition of normal ranges for unimpacted populations is needed to effectively apply indicators, and these may differ among geographically separate locations. Estuarine oysters in Chesapeake Bay were unable to locomote as quickly as oceanic oysters, even after 1 month in elevated salinities (Fisher and Newell 1986). Chesapeake Bay oyster haemocytes varied in both density and activity from Apalachicola Bay haemocytes in both spring and autumn (Oliver and Fisher 1995). Differential resistance to *Perkinsus marinus* has been demonstrated between geographically distinct oyster stocks (Bushek and Allen 1996), differences that could be related to a variety of defence responses. Furthermore, localized oyster populations may become more tolerant of local contaminants through physiological responses like induction of metallothioneins (Roesijadi and Fellingham 1987, Viarengo *et al*. 1987), or through genetic selection.

Chemical exposure/biomarker response linkages

The sources of variability discussed here do not preclude development and application of bivalve immunomarkers; however, variability must be incorporated or minimized in experimental and monitoring designs. Indicator development is an iterative process that includes both field surveys and controlled laboratory exposures. Putative relationships between contaminant exposure in the field and measured effects must be verified by conducting controlled exposures in the laboratory to confirm causal associations. Conversely, effects generated in the laboratory must be tested in field situations where there is less control over bioaccumulation and physiological status, and where interaction with multiple chemicals and biotic factors can occur. Many investigations have shown contaminant effects on bivalve defence measurements in laboratory experiments, but relatively few have examined these in field settings. Results from studies on several bivalve species are presented here, but it should be noted that each species may respond differently.

In vitro exposures of haemocytes have shown toxicant-induced effects on several defence processes including chemiluminescence or CL (Larson *et al*. 1989, Fisher *et al*. 1990, Anderson *et al*. 1992, 1994). and locomotion (Fisher *et al*. 1990). *In vitro* testing may be useful to screen chemicals for their relative toxicity, however

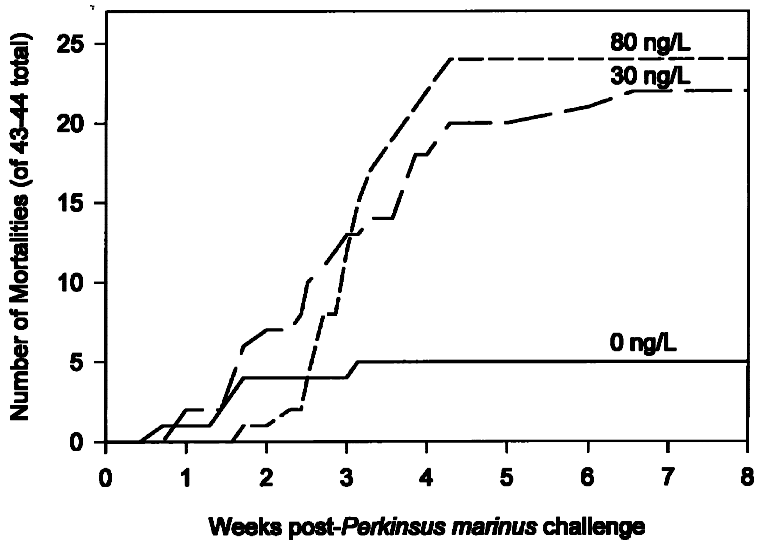

Figure 2. Number of mortalities of oysters that were exposed to 0, 30 or 80 ng l^{-1} tributyltin (TBT) for 9 weeks, then challenged with *Perkinsus marinus* parasites for an additional 8 weeks in the absence of TBT (data from Fisher *et al*. 1999).

translating *in vitro* effects to real situations requires verification using intact animals. For example, Fisher *et al*. (1990) showed a dose-dependent suppression of eastern oyster haemocyte CL caused by *in vitro* exposure to 4–400 ppb tributyltin (TBT), while 0.4 ppb TBT caused a slight hormetic effect (increase) in haemocyte CL compared with unexposed controls. Anderson *et al*. (1997) also reported inhibitory effects of *in vitro* TBT exposure (≥ 80 ppb) on oyster haemocyte CL. However, investigation of *in vivo* effects of TBT (100 pptr for a maximal exposure duration of 21 weeks) on defence responses and disease susceptibility (Anderson *et al*. 1996) showed no clear effect of TBT on haemocyte CL. Despite the lack of clear effects of *in vivo* TBT exposure on individual elements of the oyster defence system, both Anderson *et al*. (1996) and Fisher *et al*. (1999) showed that TBT exposure did exacerbate the *P. marinus* disease process and associated mortalities (figure 2). Similarly, *in vitro* exposure of *C. virginica* haemocytes to cadmium caused dose-dependent reduction in haemocyte CL, which was significant at concentrations from 10 to 50 ppm in the absence of oyster serum (Anderson *et al*. 1992). *In vivo* exposure of oysters to cadmium (0.25 ppm $CdCl_2$) for 14 days did not produce interpretable differences in haemocyte CL, due in part to high individual variability and possibly to insufficient accumulation of Cd in haemocytes (Oliver 1993).

Considerable data obtained from controlled exposures have demonstrated that oyster defence activities do respond to anthropogenic chemicals such as heavy metals. Cheng (1988a, b) measured significantly lower percent hyalinocytes in oysters exposed to 1 ppm Cu^{2+} ($CuSO_4$) and significantly higher percent hyalinocytes in oysters exposed to 1 ppm Cd ($CdCl_2$). Phagocytic activity was also affected by these exposures regimes; Cu^{2+} had an inhibitory effect while Cd had a stimulatory effect on haemocyte phagocytosis of *Escherichia coli* bacteria. Mussels (*Mytilus edulis*) exposed to Cd for 7 days followed by 7 days exposure to the bacterium *Vibrio tubiashi* had higher densities of haemocytes than non-Cd exposed groups (Pipe and Coles 1995). The same study showed that *Vibrio*-challenged

mussels not exposed to contaminants had elevated haemocytic NBT reduction activity compared with those pre-exposed to 0.2 ppm Cu for 7 weeks. Coles *et al*. (1995) measured a significant increase in circulating haemocyte numbers in mussels *Mytilus edulis* resulting from exposure to 400 ppb Cd for 7 days. Exposure to 40 ppb Cd suppressed haemocyte release of degradative enzymes during phagocytosis. Mussels (*Mytilus edulis*) exposed to copper also had increased granular blood cells by factors of 3–4 over unexposed controls (Pickwell and Steinert 1984). Clams (*Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis*) exposed to copper (1.0 ppm and 0.15 ppm for the two species, respectively) had elevated haemolymph levels of acid phosphatase compared with unexposed controls after 24–96 h, presumably released by haemocytes via destabilization of lysosomal membranes (Suresh and Mohandas 1990b). Haemocytes of freshwater zebra mussels (*Dreissena polymorpha*) exposed to lead and zinc contained enlarged and/or more numerous lysosomes compared with controls (Giamberini and Pihan 1997).

Effects of organic chemicals have also been examined via *in vivo* exposures. Sami *et al*. (1993) exposed oysters to sediment from a polycyclic aromatic hydrocarbon (PAH)-contaminated environment. Haemocytes from exposed oysters showed progressively lower Concanavalin A agglutination sites with increased exposure time. Haemocytes of *Mercenaria mercenaria* exposed to phenol concentrations above 10 ppb had reduced phagocytosis of yeast (Fries and Tripp 1980). Above 100 ppb, cell lysis was observed in 25–30% of cells examined. Coles *et al*. (1994) showed that 7-day exposure to 200 and 400 ppb fluoranthene caused increased numbers of circulating haemocytes in mussels *Mytilus edulis*, as well as stimulation of reactive oxygen metabolite production. The release of a chymotrypsin-like enzyme during phagocytosis was reduced to only 25% or less in all fluoranthene treated mussels compared with controls. Reduced lysosomal integrity, as measured by a neutral red retention assay, was observed in haemocytes and isolated digestive cells of mussels exposed to 100 ppm fluoranthene for 1 and 7 days (Lowe *et al*. 1995). Grundy *et al*. (1996) also tested the effects of *in vivo* exposure to a mixture of PAHs on phagocytic activity and lysosomal integrity of *Mytilus edulis* haemocytes. After 2 and 4 weeks exposure to the mixture of anthracene, fluoranthene and phenanthrene, phagocytosis of zymosan particles and retention of neutral red were significantly inhibited.

Chemical effects on defence activities have been demonstrated in field studies where indigenous oysters were collected from natural sites in the field and assessed for both defence functions and contaminant burdens. This approach allows potential relationships to be identified, but often produces results that are difficult to interpret in light of the sources of variability in oyster physiology already discussed, as well as inevitable exposure to complex mixtures of chemicals in the environment. Pipe *et al*. (1995) sampled mussels from a polluted area, Venice Lagoon, and a reference site in the Adriatic Sea. The highest levels of NBT reduction at two of three sampling times by mussel (*Mytilus galloprovincialis*) haemocytes were from sites with lowest levels of organic contaminants in the digestive gland. Seasonal changes were also observed, illustrating the complexity of interpreting such data. *Crassostrea virginica* collected from a PAH-contaminated site had a higher percentage of small haemocytes and an accompanying decline in large haemocytes as compared with the reference site (Sami *et al*. 1992). The changes were shown to be both inducible and reversible by moving oysters between

sites, demonstrating the potential of haemocyte morphological changes and relative composition as possible indicators of pollution. Fisher *et al.* (1998) studied eastern oysters collected from 16 sites in Tampa Bay, FL, that varied in pollutant type and quantity. Oysters with high tissue levels of metals, particularly Cu, Sn and Zn, had the highest average haemocyte density, percent and rate of locomotion, and NBT reduction activity. Reference sites had relatively low contamination by metals and organic chemicals; and haemocyte density and activities were low. Although no attempt was made to empirically measure food availability at the sites, it was observed that oysters from some highly contaminated (and eutrophied) sites were relatively large, had excellent shell growth and good tissue structure under histological examination. Nutritional status could have played a role in the differences observed, since oysters at reference sites had poor shell growth and higher [wet: dry] weight ratio.

Conclusion and outlook

Variation in bivalve defence measurements among individuals tends to be high and may be further compounded by seasonal and salinity fluctuations, parasite burdens, anoxia, genetic stock, and numerous other factors. Understanding these sources of variation will enhance the potential for these measurements to provide meaningful information about the status of bivalve populations and their habitats. At this stage relationships between exposure to specific contaminants and immune effects cannot be generalized. Nonetheless, several promising associations have emerged, e.g. metals exposure with increased haemocyte density and percentage granulocytes, and both inorganic and organic exposure with haemocyte lysosomal destabilization.

Indicator development will require further experimentation in laboratory and field settings. It should be noted that bivalves collected from sites impacted by pollution may be selected based on their ability to survive there. Only those able to tolerate the adverse conditions would be sampled in field studies, and any deviations from relatively unimpacted or reference sites observed may represent adaptive mechanisms rather than responses to pollutant stress *per se*. Oysters are able to survive and even thrive in very polluted environments, perhaps by virtue of the plasticity of their estuarine-adapted physiology. An alternative to studying natural populations is deployment of genetically-similar bivalves at natural sites along pollution gradients; this approach offers the advantage of reduced intra-individual variability and circumvents the potential problem of selecting only hardy survivors at impacted sites. Disadvantages of this approach include the potential influence of the previous environment and the loss of relevance to local resources (bivalve stocks).

Although certain changes in bivalve defence activities have been associated with xenobiotic exposure in both laboratory and field experiments, the dilemma in terms of relevance persists. How are immunological changes to be interpreted? Since a functional immune response is needed to fight parasitic diseases, instances of immunosuppression may be the most quickly recognized as deleterious. However, evidence points toward elevations in circulating haemocyte numbers as well as certain haemocyte activities in response to some stressors. Haemocyte recruitment in response to pollutant stress could divert energy away from other vital physiological processes. Better definition of 'normal' ranges is essential to expand

application of defence measurements as indicators. In addition, progress must continue in basic research on bivalve defences and disease processes in order to accurately interpret changes in immune function as indicators of oyster population health, and to identify the most relevant defence systems to apply as indicators. This will require measurement of defence characteristics in bivalves to be made repeatedly from the same population and location, in conjunction with certain environmental measurements that are known to affect these characteristics.

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