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BIOCHEMICAL AND PHYSIOLOGICAL INDICATORS OF CONTAMINANT STRESS IN AQUATIC ORGANISMS OF LARGE RIVER SYSTEMS

by

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ABSTRACT

Examination of the biochemical and physiological responses of organisms to xenobiotic chemicals has been an area of scientific investigation for many years. Historically, these techniques have been used in the development and screening of natural and synthetic pharmaceuticals, biocides, and other biologically active chemicals and in elucidating their modes of action. However, only recently have these techniques been applied as indicators of the nature and extent of chemical pollution of aquatic ecosystems. Today, research on the effects of chemical contaminants at the molecular level of biological organization is one of the most rapidly developing and intensively studied areas in the field of environmental toxicology. In this paper, we discuss the relationship of biochemical and physiologic responses to effects at higher levels of biological organization, examine general categories and provide specific examples of techniques that are presently being applied to aquatic systems, and provide recommendations for implementation of biochemical and physiological techniques in aquatic contaminant assessment programs.

INTRODUCTION

Water, soil, and sediments serve as the ultimate sinks for most chemicals produced and used by man. General sources of anthropogenic chemical contaminants are from industrial, agricultural, and urban uses. These uses result in both point and nonpoint source pollution of our environment. Large rivers are of particular concern because they receive and integrate pollutants from man's activities and land use practices over large geographical regions. Large rivers are the direct recipients of municipal and industrial effluents and additionally receive contaminant loadings from tributaries and agricultural practices in their watersheds.

These multiple sources of contaminant input result in complex mixtures of contaminants being present in the water and sediments of large river systems. Unfortunately, most of our knowledge and understanding of the effects of chemical contaminants in aquatic organisms is based upon the effects of single compounds tested in the laboratory. Much less is known about the effects of complex environmental mixtures. Field studies can document the correlation of population-level effects with contaminant exposure, but the complexity of environmental variables and multiple contaminant exposure makes it difficult to establish cause and effect relationships with specific chemical pollutants. Environmental scientists are presently focusing more effort on

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expanding our understanding of the effects of pollutants at the molecular level to help discriminate among the various environmental stressors affecting aquatic populations and communities.

Management practices for aquatic resources and regulatory practices regarding contaminant discharge are primarily directed at the population level. However, biochemical and physiological measures of contaminant stress have received increased attention in recent years due to their rapid response, sensitivity, and potential to provide a further level of discrimination in the environmental risk assessment process. In this paper, we discuss the relationship between contaminant effects at the biochemical level and those at higher levels of biological organization, examine general categories and specific examples of techniques that can be applied to aquatic systems, and provide recommendations for integration of biochemical and physiological measures of contaminant stress with other environmental risk assessment procedures.

RELATIONSHIP OF BIOCHEMICAL AND PHYSIOLOGICAL STRESS TO EFFECTS AT HIGHER LEVELS OF BIOLOGICAL ORGANIZATION

A basic premise in the field of toxicology is that to elicit a toxic response, a chemical must first reach and interact with its biomolecular site of action. This response on a molecular level may then be followed by a tissue response, an organ response, a whole animal response, and finally a population or community response, but it must occur first at the biomolecular level.

Each level possesses an inherent resiliency, or assimilative capacity, that allows it to maintain homeostasis. To provide a continuum of response, the magnitude of effect at each level of organization must be great enough to exceed the assimilative capacity of the next higher level. Ultimately, for a contaminant to elicit a response at the population or community level, it must exceed the capacity of all lower levels to absorb or mitigate the insult.

These relationships and interdependencies provide both promise and challenge to the use of biochemical and physiological responses as indicators of aquatic community health. First, it is apparent that biochemical changes must be the first and most sensitive biological responses that occur following contaminant exposure. Thus, bioindicators may be useful as early warning indicators of contaminant problems. Alternatively, because the continuum may be broken at subsequent levels of biological organization, effects at the biochemical level may not be ecologically significant. Thus, the challenge facing the environmental scientist is to develop an understanding of the magnitude of effect at the biochemical level that is required to elicit an ecologically significant response at higher levels of organization.

Toxicants interact with organisms on the molecular level in a variety of ways that can challenge the maintenance of homeostasis. Metals can bind to various proteins disrupting membrane integrity, ion transport, and cellular metabolism. For example, mercury can bind to sulfhydryl groups on structural proteins (Luckey and Venugopal 1977), thereby disrupting membrane integrity. Copper can interfere with ion transport by affecting gill ATPase activity, reducing the ability of coho salmon (*Oncorhynchus kisutch*) to adapt to seawater (Lorz and McPherson 1976). Lead can deactivate δ-aminolevulinic acid dehydratase (ALAD), disrupting red blood cell metabolism.

Organic contaminants also interact with organisms on the molecular level by disrupting membrane integrity and function, displacing endogenous substrates of enzymes, or by diminishing energy reserves through increased demands upon detoxification mechanisms. For

example, pentachlorophenol uncouples oxidative phosphorylation by interacting with the mitochondrial membrane (Moreland 1980). Organophosphates and carbamates can displace acetylcholine and inhibit its hydrolytic cleavage by acetylcholinesterase (AChe), thereby affecting nerve transmission (O'Brien 1976). Widdows and Donkin (1991) report a variety of ways that toxicants can diminish the energy reserves of an organism to the detriment of survival.

To cause effects at higher levels of biological organization, the effects at the molecular level mentioned above must be of sufficient severity to challenge the organism's ability to maintain homeostasis. Organisms invite a variety of mechanisms for dealing with toxicants. In some cases, they may be able to detect and avoid the toxicant (Folmar 1976). They may sequester the toxicant (e.g., through induction of metal-binding proteins, such as metallothioneins), or they may metabolically alter the compound to a less toxic form that may be more readily eliminated (e.g., oxidative metabolism of pyrethroid insecticides). Alternatively, they may simply repair the cellular damage caused by the toxicant. Each of these mechanisms incurs some physiologic cost on the part of the individual organism (Calow 1991). When the organism's ability to absorb or mitigate the toxic insult is exceeded, stress will be applied to the higher levels of organization such as the population or community.

At present, a wide range of biochemical and physiological parameters are known to be responsive to chemical exposure. However, our understanding of how these responses are quantitatively linked to effects at the aquatic population and community levels is much more limited. Relatively few of the measurable biochemical and physiological responses are specific to certain chemical classes (e.g., AChe inhibition by organophosphates and carbamates; ALAD inhibition by lead). The majority of responses are sensitive to a wide variety of chemical compounds and other environmental stressors. Thus, with a few exceptions, the majority of biochemical and physiological measures are more useful as *general indicators* of organism health rather than *specific indicators* of specific chemical exposure. It is important to recognize the range of sensitivity of the indicators we use in aquatic contaminant risk assessment, because our goal is to establish cause and effect between contaminant exposure and responses at the population and community level. Responses that are general indicators of organism health must be used in concert with other measures of chemical exposure, such as chemical residue analysis. Responses that are more specific indicators of individual chemical exposure must be calibrated to quantify the continuum of response from the biochemical to the population and community level.

Within the broad range of measurable biochemical and physiological parameters, a further distinction can be made between *regulated parameters* and *regulatory mechanisms*. Regulated parameters are usually maintained within relatively narrow ranges to maintain physiological function. Contaminant exposure that displaces these parameters beyond the tolerated range will usually result in acute injury or death (Zachariassen et al. 1991). However, the regulatory mechanisms that govern these parameters may fluctuate much more widely before significant injury or death occurs in an attempt to maintain the optimal range, and may be more responsive and sensitive indicators of contaminant insult (Zachariassen et al. 1991). For example, the level of ATP present in an organism is a highly regulated parameter. ATP levels can be maintained at the expense of other endogenous high-energy phosphate compounds. Aunaas et al. (1991) demonstrated that sublethal exposure of the blue mussel (*Mytilus edulis*) to contaminants generally resulted in larger fluctuations of components of the phosphate pool other than ATP. Thus, the regulatory mechanism (the phosphate pool) was more responsive than the regulated parameter (ATP).

The range of sensitivity, level of variability, and relationship to responses at higher levels of biological organization are all important parameters for consideration in selecting biochemical and physiological indicators for application in aquatic contaminant risk assessment programs.

CATEGORIES AND EXAMPLES OF TECHNIQUES

Biochemical and physiological indicators of contaminant stress can be categorized in a variety of ways--general versus specific sensitivity to compounds, regulatory versus regulated parameters, indicators of exposure versus indicators of effect, or by category of biochemical and physiological function. In this section, we will identify general categories of biochemical and physiological function and discuss representative examples of indicator techniques for each category that are presently being used or show promise for further development.

OSMOREGULATION

A variety of inorganic and organic environmental pollutants affect osmoregulation in fishes. For this reason, many osmoregulatory stress indicators are more useful as indicators of general organism health rather than diagnostic tools for identification of specific pollutants.

Plasma ion concentrations are responsive to heavy metal exposure. Sublethal exposure to copper causes transient decreases in plasma chloride concentrations in brown bullheads (*Ictalurus nebulosus*) and brook trout (*Salvelinus fontinalis*); however, the levels return to normal after about 3 weeks even during continuous exposure, indicating the presence of a compensatory mechanism for maintenance of homeostasis (Christensen et al. 1972, McKim et al, 1970). Zinc and cadmium also affect plasma ion concentrations (Lewis and Lewis 1971, McCarty and Houston 1976).

Gill ATPase activity in fishes is also sensitive to chemical exposure. This measure of osmoregulatory ability is affected by a wide range of environmental pollutants including copper (Lorz and McPherson 1976), PCBs (Davis et al. 1972), and a variety of chlorinated pesticides (Davis et al. 1972). Gill ATPase activity has direct ecological relevance in that it is intimately linked to the migratory success of anadromous fish species (Epstein et al. 1967, Zaugg and Wagner 1973).

Histological and histochemical examination of gill tissue has also been used successfully to demonstrate the effects of osmoregulatory stressors. As with ATPase activity, a wide variety of environmental contaminants can cause histological lesions in fish gill. Aluminum (Jagoe et al. 1987), copper (Baker 1969), mercury (Wobeser 1975), sodium arsenite (Gilderhaus 1966), and zinc (Skidmore and Tovell 1972) are some of the metals that cause gill lesions in fishes. A wide variety of organic compounds also cause histological lesions in fish gill. Endrin (Eller 1971), heptachlor (Andrews et al. 1966), methoxychlor (Lakota et al 1978), mirex (Van Valin et al. 1968), naphthalene (DiMichele and Taylor 1978), and phenol (Mitrovic et al. 1968) are only a few of the organic contaminants that cause gill lesions.

METAL SEQUESTRATION AND REGULATION

Metallothioneins are a class of metal-binding proteins found in a wide variety of organisms (Roesijadi 1992) that are inducible after exposure to Cd, Cu, Hg, and Zn (Noel-Lambot et al. 1978). Metallothioneins are thought to reduce the toxicity of heavy metal exposure by sequestration (Hamilton and Mehrle 1986), with toxicity occurring only after its binding capacity is

exceeded (Brown and parsons 1978). Hepatic metallothionein levels in feral fishes from lakes contaminated with Cu, Zn and Cd are closely correlated to metals exposure (Roch et al. 1982). The inducible nature of metallothioneins suggests that they represent a regulatory mechanism, and as such provide a useful indicator of exposure to certain metals. Other metal-binding proteins such as ferritin (Fe) and copper-chelatin (Cu) have been less thoroughly studied in aquatic organisms.

OXIDATIVE METABOLISM

Oxidative metabolism serves three important functions in eukaryotic organisms that provide useful biochemical indicators of stress. First, oxidative metabolism plays a central role in catabolic energy production. The resultant storage currency is adenylate, and its utility as a biochemical indicator of contaminant stress is discussed in the next section.

Secondly, oxidative metabolism is associated with the reduction of oxygen radicals and the maintenance of oxidative homeostasis within the cell. This critical function also lends itself for use as a biochemical indicator of stress in aquatic organisms. Oxygen radicals are formed as intermediates of cytosolic enzymes, electron transfer systems, and by activated cells of the immune system. Inducible prophylactic enzymes such as superoxide dismutase, catalase, and glutathione peroxidase serve a vital role in protecting the cell from oxidative stress and are useful indicators of contaminant stress in aquatic organisms (DiGiulio et al. 1989). Additionally, the pools (and relative ratios) of reducing equivalents (e.g., glutathione, α-tocopherol, and ascorbate) that are required to maintain oxidative homeostasis are useful indicators of contaminant stress. These antioxidant defense mechanisms of the cell are sensitive to compounds that block or inhibit electron flow and, in particular, those that can undergo cyclic reactions (redox cycling) to produce oxygen radicals. These important classes of environmental contaminants include paraquat, diquat, and various amines.

The third, and probably the most thoroughly studied, function of oxidative metabolism is xenobiotic metabolism. Xenobiotic metabolism associated with the cytochrome P450 monooxygenases (MO) has been reviewed for use in biomonitoring by Payne et al. (1987), who concluded that induction of MO could be a sensitive biochemical indicator of exposure in many aquatic organisms. Induction of MO activity is caused by a wide variety of chemical contaminants (e.g., PAHs, PCBs, PCDDs, and PCDFs). The MO system plays a central role in the detoxification of these and many other hydrophobic compounds largely by facilitating their elimination. Additionally, MOs are highly conserved phylogenetically. All of these characteristics enhance the potential utility of MO activity as a biochemical indicator of contaminant stress. However, its use in feral organisms has been limited because of the confounding factors of age, sex, reproductive status, diet, disease, and general health conditions that can all greatly affect the MO response to xenobiotics (Neal 1980). A thorough understanding of the effects caused by these variables is lacking in most species of fish and wildlife. Even if these relationships were understood in aquatic species, it is often difficult to obtain such information on feral organisms. Thus, MO activity in this context offers a more qualitative than quantitative measure of contaminant exposure. However, successful use of MO activity as a quantitative bioindicator of contaminant stress in the field has been accomplished using caged fish studies (Lindstrom-Seppa and Oikari 1990) and by using developing embryos (Hoffman et al. 1987). In both of these cases, the organismal and environmental factors affecting MO activity could be controlled or monitored during the period of exposure.

Another alternative to limiting the confounding factors that influence MO activity is the use of *in vitro* systems where the conditions are tightly controlled. In these assays, the environmental contaminant mixture is brought to the laboratory to expose the system, as opposed to taking the system (organisms) to the environment for exposure. An example of such an *in vitro* model system for MO activity is the H4IIE hepatoma cell bioassay (Tillitt et al. 1991). In this bioassay, environmental contaminants are extracted from the species of concern and serial dilutions are used to dose the H4IIE cells. After a period of incubation, ethoxyresorufin-o-deethylase (EROD) activity is measured in the cells. The MO response of the H4IIE cells is highly correlated to the effects observed in the whole organism (Safe 1987). Therefore, the MO response *in vitro* can integrate the interactions of complex mixtures of environmental contaminants at the cellular/biochemical level and serve as a quantitative measure of both exposure and effect in the whole organism from which the contaminants were extracted. Recent studies have demonstrated the ability of the H4IIE bioassay to be predictive of contaminant responses at higher levels of biological organization, such as egg mortality rates in PCB-exposed populations of fish-eating birds (Tillitt et al. 1992).

MAINTENANCE OF ENERGY STATUS

Adenylate energy charge is a term developed by Atkinson (1968) to describe the dynamic equilibrium between the various components of the cellular energy system--ATP, ADP, and AMP. It is calculated as (ATP + 0.5 X ADP)/(ATP + ADP + AMP) and its value reflects the energy status of the organism. Adenylate energy charge is sensitive to a variety of environmental factors such as food availability and temperature (Moal et al. 1991), season (Giesy and Dickson 1981), salinity (Ivanovici 1980), and pollutants (Verschraegen et al. 1985). Because ATP levels are regulated within relatively narrow limits in many organisms, Aunaas et al. (1991) have proposed the use of a phosphorus index--which reflects the status of a larger phosphorus pool--as a more sensitive indicator of energy status. The broad spectrum of environmental stressors to which energy status is sensitive indicates that this biochemical parameter may be most useful as a general indicator of organism health, rather than a specific indicator of contaminant exposure.

REPRODUCTION

Another category of biochemical and physiological function that is sensitive to contaminant exposure is reproduction. The complexity of the reproductive process in aquatic organisms and the confounding influence of behavior, nutritional status, seasonality, and other variables makes it difficult to ascertain the effect of contaminants on the reproductive success of natural populations. However, a number of biochemical reproductive parameters have been investigated in the laboratory in terms of their sensitivity to contaminant exposure. Vitellogenin is the major yolk protein in salmonids and many other species. It is produced in the liver and transported to the ovaries in the blood. Blood levels of vitellogenin are elevated during ovary maturation. Vitellogenin levels in brook trout and rainbow trout are affected by low pH exposure (Tam et al. 1987, Parker and McKeown 1987). Reproductive endocrine function in fishes has also been studied to a limited extent in terms of the effects of contaminants on steroid hormone levels. Thomas (1990) demonstrated an increase in plasma hormone levels in Atlantic croaker (*Micropogonias undulatus*) after exposure to lead, benzo(a)pyrene, or Arochlor 1254; vitellogenin levels were similarly affected by cadmium and PCBs.

NEUROTRANSMISSION

One of the more specific biochemical indicators of contaminant exposure is measurement of the neurotransmitter acetylcholinesterase (ACHe). Organophosphates and carbamates are specific inhibitors of ACHe activity (O'Brien 1976), although other physiological factors can influence activity levels. It should be noted that measurement of brain activity levels represents primarily true acetylcholinesterase whereas plasma levels generally reflect both acetylcholinesterase and butyryl(pseudo)cholinesterase (O'Brien 1976). Depressed ACHe activity is associated with a variety of behavioral responses in fishes (Symons 1973) and it has been measured in feral fishes from polluted waters (Williams and Sova 1966). However, brain activity levels resulting in death of fishes can be quite variable (Weiss 1961).

INTERACTIONS WITH GENETIC MATERIAL

The genotoxic effects of contaminants are receiving increased interest in the area of environmental contaminant assessment. A variety of techniques are becoming available for use in aquatic systems. Detection of the presence of DNA adducts using ^{32}p-postlabeling is one method of assessing exposure to contaminants that are capable of interacting with genetic material. Varanasi et al. (1989) used this method to correlate DNA adducts in livers of English sole (*Parophrys vetulus*) and winter flounder (*Pseudopleuronectes americanus* from Pugent Sound, with exposure to PAHs in sediments. Another method that shows promise is the use of the DNA alkaline unwinding assay. Toxic chemicals that interact with genetic material can cause strand breaks in DNA. The rate of unwinding of the DNA double helix when placed in an alkaline solution is a measure of DNA integrity that has been shown to be related to exposure of fish to genotoxic chemicals (Shugart 1988).

IMMUNOLOGY

A wide range of environmental pollutants affect the immune system of fishes (Zeeman and Brindley 1981). Immunosuppression can result in increased susceptibility of fishes to disease (Sinderman 1979). Contaminant-reduced immunocompetence of fishes has been a subject of study in both feral fishes from polluted environments (Warinner et al. 1988), and laboratory studies with single compounds (Walczak et al. 1987) and complex mixtures (Secombes et al. 1991). Various approaches to assessment of immune status have been used. These include differential white blood cell counts (Gardner and Yevich 1970), serum protein levels and ratios (Smith et al. 1976), agglutination titer (Roales and Perlmutter 1977), phagocytosis (Secombes et al. 1991), and disease susceptibility (Hansen et al. 1971). As with many other biochemical and physiological indicators, measures of immunocompetence are more useful as indicators of general organism health rather than a diagnostic indicator of specific contaminant exposure.

RECOMMENDATIONS FOR IMPLEMENTATION

In this brief overview, we have had the opportunity to focus on only a minute fraction of the biochemical and physiological response of aquatic organisms that have been measured in relation to contaminant exposure. Of the literally hundreds of measurable biochemical and physiological responses of organisms, most have been studied at least to some extent in relation to exposure to one chemical or another. The wide variety of species of aquatic organisms and the wide variety of chemicals that pose a threat as environmental pollutants provides an extremely extensive and complex matrix of possible combinations of exposure and response for study.

Relatively few biochemical and physiological responses of aquatic organisms are specific to individual chemicals (e.g., ALAD); several others are sensitive to general chemical classes (e.g., acetylcholinesterase activity; EROD activity). These *specific* indicators may be very useful in environmental assessments where sources and types of contaminants are unknown, by directing evaluation efforts toward particular chemicals. The sensitivity of these tests may also make them useful for documenting the fringes of environmental disturbance or areas of subtle contaminant exposure when the types of contaminants present in a system are known. Their use, coupled with supportive analytical chemistry, can provide important information for establishing cause and effect relationships and interpreting observed effects at higher levels of biological organization.

The majority of biochemical and physiological responses of aquatic organisms are sensitive to broad ranges of chemical contaminants and other environmental stressors. For this reason, these techniques can provide sensitive measures of organism health, but are not diagnostic for specific chemical exposure. These *general* indicators are most effectively applied in environmental contaminant assessments in concert with other measures of contaminant effects and exposure. The use of general biochemical indicators should be coupled with chemical analysis, on-site and *in-situ* toxicity assays, infaunal surveys, and other classical contaminant assessment procedures to identify the nature and extent of the contaminant problem. As with specific indicators of contaminant exposure, these general indicators of organism health may provide additional sensitivity in identifying the fringes of environmental disturbance and areas of subtle contaminant exposure. However, supporting information for establishing cause and effect is extremely important when general indicator techniques are applied in environmental contaminant assessments.

In summary, measurement of the biochemical and physiological responses of aquatic organisms to toxic chemicals has provided extensive insight into the modes of action of environmental contaminants. However, their use in the identification and monitoring of the nature and extent of aquatic pollution is a relatively new application. Their rapid response and sensitivity provides potential for adding a further level of discrimination in the environmental risk assessment process, particularly in marginally impacted areas. Proper application of these techniques requires a thorough understanding of their specificity in regard to the types of environmental stressors to which they are sensitive, and a quantitative understanding of the linkages between effects at various levels of biological organization. For many biochemical and physiological indicators, these are topics of current and future work.

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