# Macroinvertebrate Biomarkers: Links to Toxicosis and Changes in Populations or Communities.

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## 1 Introduction

In Australia, through monitoring initiatives such as the National River Health Program, emphasis has been placed on measuring changes in macroinvertebrate communities or populations (Simpson and Norris, 2000). However, chemicals can cause changes at all levels of biological organisation (Table 1). It is now recognised that if the focus is only on community change, subtle or chronic biological effects that result in irreversible long-term changes could be occurring in apparently healthy ecosystems but would not be initially detected (Bunn, 1995, Maher *et al*., 1999). Another problem with measuring changes only at the population level is that little inference can be made with regard to the cause of the population decline.

Although the population, community and ecosystem are the important levels at which to monitor toxic effects, toxic effects are also manifested at the molecular-subcellular level by impaired biological function (Table 1).

The present paper reviews the general use of biochemical measurements that can be used as individual biomarkers of impaired biological function in invertebrates. The emphasis is on biomarkers that can be potentially associated with changes at population and/or community levels. The lack of knowledge on the linkages between biomarker variations and macroinvertebrate population and community response is highlighted.

## 2 Concept of Biomarkers

Biological markers have been defined as ‘xenobiotically-induced variations in cellular or biochemical components or processes, structures or functions that are measurable in a biological system or sample’ (NRC, 1987). They are classified as biomarkers of exposure to a toxicant, biomarkers of effects of exposure or biomarkers of susceptibility to the effects of exposure (Peakall and Shugart, 1993). According to this definition, biomarkers adequately describe effects induced by various environmental stresses at any level of biological organisation, from the cellular to the ecosystem level. In practice, the term biomarker is more commonly used in a more restrictive sense, namely biochemical sublethal changes resulting from individual exposure to xenobiotics. From the ecological point of view, environmental changes at community and ecosystem levels are mostly assessed by the use of bioindicators rather than biomarkers. In this context, a bioindicator is a species of a group of species for which the abundance reflects the quality of the environment. Distinction should therefore be made between biomarkers and bioindicators to prevent misunderstanding of the nature and effects of environmental stress. Here we define biomarkers as a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect. Table 2 gives a summary of the major biomarkers used to assess impairment of biological function in organisms. Most of these are not applicable to invertebrates because insufficient biological material can be obtained for analysis or the different physiology of invertebrates.

Most studies on biomarkers are carried out in organisms exposed to selected stress under controlled laboratory conditions. These cause and effect relationships should ideally be applicable to the natural environment. The variation of the

biomarkers at molecular, cellular or organism level should ideally provide information on the stress syndrome of the animal and on its repercussions at the population level (McCarthy and Shugart, 1990). However, this is not often the case because a number of biotic and abiotic factors can influence the extrapolation of individual biomarkers to the field monitoring of contaminant effects at population and community levels. (Lagadic *et al*, 1994).

## 2.1 Linking the responses of individuals to populations and community effects.

Biomarkers have been identified that indicate that organisms have been exposed to stress (general biomarkers; Mayer *et al.,* 1992) and in some cases, even specific stressors (metallothionein responses following metal exposure (Benson *et al.,* 1990), acetylcholinesterase inhibition following exposure to organophosphorus pesticides (Mayer *et al.,* 1992). Whilst signalling that an exposure has taken place, such biomarkers contribute little to the prediction of the direct consequences for the organism or population in question. For this to be possible, a particular biomarker response should be related to a degree of impairment of growth, reproductive output or metabolic function which directly affects the survival of the organism and which can be attributed to exposure to a known amount of the specific toxicant (Depledge and Fossi, 1994).

Correlations of population and community level changes with variations of selected biomarkers in field-collected aquatic invertebrates are poorly documented (Lagadic *et al.*, 1994). Some biomarkers can be proposed to evaluate the degree of exposure of individuals in order to predict population level consequences. However, the effects of contaminant exposure are difficult to evaluate because there is often a long latent period between exposure and the expression of an adverse effect. Moreover, biological variability among individuals and environmental factors can exert confounding influences. Also, the relationships between biomarker responses and population or community structures and functions is not straightforward because of the compensatory mechanisms which regulate population dynamics in natural systems.

Neuroactive compounds have been the dominant insecticides for the last 50 years and the exposure to neurotoxicants could be assessed through the measurement of biomarkers related to their target activity or detoxification (Casida and Quistad, 1998). Cholinergic effects of some pollutants (for example, organophosphorus pesticides) have sometimes been associated with behavioural changes in invertebrates (Scherer and McNicol, 1986) but the direct correlation between individual acetylcholinesterase activity and population or community level responses remains to be established. Stream-dwelling invertebrate populations constitute a valuable model for establishing such a correlation.

The biomarker approach should not be used as a replacement for conventional assessment techniques, but used as a supplementary approach for interpreting links between sub-lethal biochemical and cell changes (biomarkers) and adverse effects on populations and communities. Biomarkers that measure toxic interactions at the molecular level (eg. the inhibition of brain acetylcholinesterase activity by organophosphorus pesticides) provide predictions of a toxic effect upon individuals. The remaining research challenge for using biomarker measurements in individuals is

to predict effects at the population level from effects measured upon individuals collected in the field.

## 2.2 Consequences at population and community levels

From the ecotoxicological point of view, resistance to pesticides in invertebrates is the only population-level biomarker that can be directly correlated with enzyme biomarkers such as mixed-function oxidase (MFO) activity, glutathione S-transferases (GST) activity, acetylcholinesterase (AChE) activity or other esterases (Lagadic *et al*., 1994; Gunning *et al.,* 1997). The development of resistance depends on genetic variability already present in a population or arising during the period of the selection.

Biochemical techniques offer the possibility of rapidly detecting the initial stages of resistance in a population and the mechanism(s) of resistance involved (Table 3). Amongst the various mechanisms so far identified, enhanced detoxication by pesticide-metabolising enzymes and decreased target site sensitivity are the most efficient and the best documented (Terriere, 1984, Oppenoorth, 1985; Brown and Brogdon, 1987; Soderlund and Bloomquist 1989; Gunning *et al*., 1997,1998).

The cotton bollworm, *Helicoverpa armigera* (Hubner) is a serious pest for cotton and other summer crops in Australia and insecticides are widely used for its control on cotton. Biochemical studies have shown that both pyrethroid, carbamate and organophosphorus resistances are directly related to biochemical resistance mechanisms (Gunning *et al*., 1996, 1998). Pyrethroid resistance in Australian *H. armigera* is largely attributable to an increased production of esterase enzymes that are not present in susceptible *H. armigera,* which apparently detoxify pyrethroids by sequestration and hydrolysis (Gunning *et al*., 1996). Recent studies have identified an insensitive acetylcholinesterase (AChE) as the resistance mechanism causing resistance to methyl parathion and profenofos in *H. armigera* (Gunning *et al*., 1998). Fortunately for the Australian cotton industry, *H. armigera* remain susceptible to chlorpyrifos. In field selection for chlorpyrifos resistance in the mosquito *Culex pipiens*, resistance was initially monofactorial and associated strictly with a highly active detoxifying esterase, but later evolved to include at least two more factors, an altered acetylcholinesterase and a microsomal monoxygenase (Raymond *et al*., 1986).

Non-target macroinvertebrate species should also be studied to determine if reduced acetylcholinesterase activity or an insensitive acetylcholinesterase, or increased esterase activity is developing in riverine macroinvertebrates adjacent to the cotton growing regions.

## 2.3 Use of biomarkers in ecological risk assessment

Risk assessment requires hazard identification and estimates of the likely exposure to a hazard. Biomarkers are important in indicating exposure and for determining the likely effects if exposure continues. All ecological risk assessments assume that for each community or ecosystem, there is a range of conditions associated with normal, sustainable functioning (analogous to homeostasis in an individual organisms) and that there is a quantifiable risk that the community or ecosystem will depart from the normal range of conditions when pollutant chemicals

are added. With regard to the predictive component, this necessitates knowledge of how populations comprising the ecosystem will respond to a given pollutant load.

Protocols for the use of biomarkers in ecological risk assessment have been formulated (Fossi and Leonzio 1993, Halbrook *et al*., 1993). The approaches focus on establishing the risks to key components in the ecosystem, the inference being that if these components are adversely affected, the ecosystem structure and/or function (ecological integrity) will be at risk.

McCarty and Munkittrick (1996) and Holdway (1996) have highlighted the inappropriate use of biomarkers in ecological risk assessments that cannot be correlated to population or community effects. Mayer *et al*., (1992) and Wolfe (1996) proposed specific evaluation criteria against which biomarkers could be selected for meeting monitoring and field assessment objectives.

The criteria are reformulated and summarised below:

(1) The biomarker must be causally related to population or ecological effects.
(2) The biomarker should respond on a dose-dependent manner to the toxicant.
(3) The biomarker should be field validated.
(4) The specificity of the biomarker response and its independence from non-contaminant sources of variability (eg. season, sex and temperature) should be established.
(5) The biomarker should be sensitive and have broad applicability on large scales of time and space.
(6) The biomarker should be a measure of a response that is representative of an important biological process.
(7) The biomarker should be relatively easy to measure at low cost.

## 2.4 Field use of biomarkers

Choice of the appropriate biomarker for monitoring toxicant exposure and effects in the field requires consideration of a variety of factors (Mayer *et al*., 1992, Peakall and Shugart, 1993). Sex, reproductive status, age and diet (composition and amount) are among the factors that can influence the biomarker response of many invertebrate animals. There is evidence that multiple forms of some enzyme biomarkers exist in a variety of insects and aquatic invertebrates (Terriere, 1984; Clark, 1989; Habig and Di Giulio, 1991). Each form of the enzyme may differ in its sensitivity to the toxicant under study. These enzyme biomarkers can also vary in form, location and activity within a species.

The concentration of biochemical biomarkers in a given tissue can also vary in response to characteristics of the organism such as development stage and age within a particular development stage. This is of particular concern with the application of biomarkers to invertebrate species in riverine studies where the immature nymphs and larvae are typically at various stages of development. Thus it is critical to use well-defined biological material for prediction of toxicity.

Many biomarker responses are transient. An exposure to a pollutant may elicit a response that lasts for a matter of hours. However, some biomarker responses persist for weeks or months with continued exposure of the organisms. Species-specific differences in the rate of recovery from poisoning from a particular toxicant have been demonstrated (Benke and Murphy, 1974). Hence it is vital to establish in advance what duration of response can be expected. This is important if biomarkers in invertebrates are used to measure the environmental impact of agrochemicals.

The effects of environmental (abiotic) factors, for example temperature, must be accounted for in the design, analysis, and interpretation of the biomarker study, if they potentially exert an effect. Following sampling, attention should be paid to the effect of time elapsed from collection to storage and to the effects of storage conditions on the biomarkers. Collection and storage of samples should be consistent across all sampling periods and sites and should be appropriate to the chosen analyses.

### 2.5 Biomarker selection

Biomarkers can be used to measure a wide range of responses to chemicals at the biochemical, cellular or tissue levels. Responses may also be measured at the physiological (eg. scope for growth), behavioural (eg decreased foraging behaviour in bees) or population level (eg, changes in gene frequency in a population that confers resistance to biocides). These have been reviewed extensively (McCarthy and Shugart, 1990; Benson and Di Giulio, 1992; Mayer *et al*, 1992; Lagadic *et al*., 1994; Holdway *et al*., 1995). Some biomarkers (eg metallothionein) do not appear to have a direct relationship to a mechanism of toxicity. In this case, the use of the biomarker may not give a reliable prediction of toxic effects upon organisms and is, therefore, only likely to indicate exposure to chemicals. In using such biomarkers of exposure, it may not be possible to predict effects at the population level from biomarker changes measured upon a sample of individuals.

The primary concern of the present review is with the measurement of biomarkers in macroinvertebrates under field conditions, where effects of environmental chemicals at different levels of biological organisation can be examined. At present the selection of biomarkers of effects that may be applied in many species is limited by lack of knowledge of basic mechanisms of action. The reliability of biomarkers also may depend on knowledge of the mechanisms involved in the particular response. However, for insecticides there is a growing understanding of their mechanistic basis which can assist in the identification of suitable biomarkers that can be linked to population effects (Table 4). The development of increased numbers of suitable biomarkers is dependent largely on fundamental research into the underlying mechanisms of action. In a site where the identity of contaminants is unknown, a range of general biomarkers of exposure (eg. scope for growth, metallothionein) could be evaluated at various levels of organisation to determine if a hazard existed. Once selected, it is important in the application of biomarkers to field studies to establish how environmental and biotic factors will modify the biomarker responses to pollutants relative to those seen in laboratory conditions where these factors are controlled.

## 3. General Biomarkers of Contaminant Exposure

## 3.1 Rate of growth and *in situ* growth Rate

The rate of growth is a fundamental measure of physiological fitness/performance and provides one of the most sensitive measures of stress in organisms. However, growth in taxa such as bivalves is difficult to quantify and interpret, especially in relation to environmental pollution, due to a lack of coupling between shell and other growth components (ie. somatic and gonadal) and the difficulty in separating 'nutrition effects' from 'toxicant effects' (Widdows and Donkin, 1991).

Increased emphasis has been placed on the development and use of *in situ* testing techniques to assess toxicity of contaminated aquatic environments. Of particular interest is the desire to incorporate this type of information into ecological risk assessment frameworks.

By enclosing the test organisms in mesh containers and placing them in the field, direct exposure to the changing aquatic and sediment environment is possible. The use of *in situ* testing reduces many uncertainties and artefacts associated with laboratory bioassays, such as collection and storage of samples. *In situ* testing also takes into account intermittent changes and pollution events.

Successful *in situ* testing has been achieved with the chironomid *Chironomus tentans* based on survival and growth data (Sibley *et al*., 1999). In a laboratory study (Colville *et al.,* 2000) established culture conditions that supported the constant growth for up to 30 days, nymphs of the native Australian mayfly species *Atalophlebia* sp. (length 4 to 7 mm), with a growth rate of 0.208 to 0.228 mm/day. The application of an *in situ* testing procedure using mayfly nymphs with growth and survival as endpoints would provide ecologically realistic assessments of pollutants.

## 3.2 Scope for growth

Many of the problems associated with measuring growth can be overcome by determining the energy available for growth and reproduction (termed the "scope for growth") based on the physiological analysis of the energy budget.

Scope for growth (SfG) represents the difference between the energy gained from the food and the energy lost by respiration and excretion. Scope for growth values can be positive, indicating that energy is available for growth and reproduction, zero when energy inputs balance energy losses or negative when the animal must use its reserves for essential metabolism. Scope for growth is often considered as a good physiological indicator of pollutant-induced stress (Donkin and Widdows, 1986). Most of the research on SfG use in environmental impact assessment has been conducted in marine bivalves and more recently in freshwater crustaceans (Maltby *et al*., 1990a, 1990b).

Field and microcosm studies have confirmed that the long-term consequences to growth and survival of individuals and the population can be predicted from measured effects on energy balance observed at the individual level (Widdows and Donkin, 1991). A decline in SfG in the freshwater amphipod *Gammarus* sp. due to increased stress was associated with a decline in feeding rates. This led to decreased

offspring weight and increased numbers of abortions with important consequences for the long-term viability of affected populations (Maltby and Naylor, 1990; Maltby *et al*., 1990a).

The main disadvantage of scope for growth measurements for freshwater studies is the lack of test organisms for which feeding rate can be quantified.

## 4 Biomarkers of Organic Chemical Exposure

### 4.1 Mixed-function oxidases (MFO)

Cytochrome P450 (or simply P450) refers to a family of enzymes that transform the structure of organic chemicals (for review, see Nebert *et al.,* 1981). More than 100 P450 genes or proteins from procaryotes and eucaryotes have been sequenced, and organised into 27 gene families (Nebert *et al*., 1991). The reactions catalysed by these proteins are of a type generally referred to as mixed-function oxidase (MFO) or monooxygenase reactions which function through the haemoprotein cytochrome P450. The toxicity of organic chemicals such as pesticides can be drastically altered by structural transformation. By affecting chemical structures, cytochrome P450 enzymes may render a given compound non-toxic or, by contrast, drastically increase its toxicity. Many organophosphorus pesticides require bioactivation of sulfur analogues to more potent oxygen analogues prior to exerting their anticholinesterase effects. This desulfuration reaction is mediated by microsomal mixed function oxidase (MFO) enzyme systems. Pyrethroid resistance in the cotton ballworm, *Helicoverpa armigera* has been correlated to the activity of two enzymes namely monooxygenase and an esterase (Barden *et al*., 1992).

The amounts of some types of P450 can be induced (increased) in response to an organism's exposure to many types of chemical. As a result, the rate of chemical transformation catalysed by these enzymes is altered. P450 induction can also serve as a highly sensitive indicator of an organism's toxic burden, or the extent to which it has been exposed to chemical inducers in the environment.

The properties of MFO systems in marine invertebrates have been detailed in a number of recent reviews (James, 1989; Livingstone, 1993). Molluscan and crustacean microsomal enzymes catalyse transformation of a diverse suite of xenobiotic substrates including aromatic hydrocarbons, but the rates of these processes in invertebrates are substantially lower than those in most fish. Thus, at present, there seems to be little potential for using monooxygenase activity or P450 levels in molluscs or crustaceans to assess their exposure to pollutants such as aromatic and chlorinated hydrocarbons. Induction has been suggested to occur after weeks of treatment, but there is no convincing evidence that hydrocarbons can rapidly induce P450 forms in molluscs or crustaceans (Stegeman *et al*., 1992).

In natural populations, the combined influences of biological and environmental factors are known to cause background and seasonal variations in P450 content and activity. The content of P450 and induction of monooxygenase activities in fish can be suppressed by estradiol (Forlin *et al*., 1984) during reproduction in some species. Similarly, low temperature can cause attenuation of the induction response (Stegeman *et al*., 1992). Environmental factors (eg. temperature and photoperiod) and biotic

factors, have to be taken into account in the use of P450 induction as a biomarker for environmental contamination.

### 4.2 Glutathione S-transferases

The glutathione S-transferases (GST) represent an important family of enzymes named for their role as catalysts for the conjugation of various electrophilic compounds (eg. epoxides of PAH) with the tripeptide glutathione (Buhler and Williams 1988; Clark, 1989). These enzymes exist in multiple forms and are known to be involved in insect resistance to organophosphorus insecticides (Terriere, 1984). The glutathione S-transferases of larval insects are induced up to 18-fold by plant chemicals (Terriere, 1984). Attempts to detect chemically induced levels of GST in fish collected from the field have yielded conflicting results (Stegeman *et al*., 1992). Few studies have been carried out in freshwater invertebrates, most of them using GST as biomarker of individual contamination (Blat *et al*., 1988; Boryslawskyj *et al*., 1988).

The level of biotransformation activity in aquatic invertebrates and its responsiveness to organic xenobiotics is usually very low when compared with vertebrates and their biological significance in these organisms is not yet fully understood. Therefore, in spite of the undoubted responsiveness of the detoxication enzymes to some pollutants, more research is needed on the characterisation of these enzymes in aquatic invertebrates before valid interpretations of such data in terms of biomarker response that can be linked to population changes.

## 5 Biomarkers of Metal Exposure

### 5.1 Metallothioneins

Metallothioneins (MT) comprise the major metal-binding proteins in animals and occur in plants and prokaryotes as well. Toxicological effects of heavy metals can be countered by detoxification mechanisms. Two of the major detoxification pathways in aquatic invertebrates involve the storage of metal into metal-rich granules and the binding of metal to metallothioneins (Brown, 1982; Roesijadi, 1992). The formation of metal-rich granules is important for the long-term storage of essential and non-essential metals, while binding to metallothionein and metallothionein-like proteins is the major mechanism for regulating internal metal concentrations on short time scales (Brown, 1982; Roesijadi, 1992). Although there is a dynamic equilibrium in binding of metal among cytosolic ligands, once metal accumulation exceeds metallothionein production, the detoxification system can become saturated and excess metal may bind to sensitive enzymes and exert toxic effects. This has been referred to as spill-over (Brown *et al*., 1977). For example, reductions in the growth rate of fish and zooplankton coincided with metallothionein saturation with mercury (Brown and Parsons, 1978). Reductions in oxygen consumption by oysters occurred when metallothionein became saturated with cadmium (Engel and Fowler, 1979), This spill-over effect, however, is not always found in metal-exposed organisms. For instance, growth reductions in crab larvae were more closely related to the binding of copper to metallothionein and low molecular weight proteins than to the binding to high molecular weight proteins (Sanders *et al*., 1983; Sanders and Jenkins, 1984).

The ability of relatively low exposure to non-essential trace metal contaminants (eg. cadmium) to induce metallothionein has generated interest in the use of metallothionein as a biomarker for metal pollution. Also, while the function of metallothionein in the metabolism of non-nutrient metals, such as cadmium, is not fully understood, it appears that metallothionein often plays a protective function by sequestering these metals and inhibiting interactions with sensitive cellular components, such as enzymes. Cellular toxicities may ensue after the metal-binding capacity of metallothionein has been exceeded. Therefore, it has been proposed that metallothionein measures may provide considerably more information about potential health hazards of metals in exposed animals than tissue metal residues alone.

It is now clear that, at least in mammals, insects, and crustaceans, metallothionein is induced under many other conditions besides metal exposure. Both glucocorticoid hormones (progesterone, glucagon) and peptide hormones (interleukin I and interferon) have been found to induce metallothionein synthesis. As a consequence levels of metallothionein, and the amounts of copper and zinc bound to it, are affected by processes such as growth, reproduction, tissue regeneration, and, in the case of insects and crustaceans, moulting. It has been demonstrated that factors, such as temperature and/or nutritional status may have profound effects on the copper/ zinc ratios bound to metallothionein in marine invertebrates (Stegeman *et al*., 1992).

Measurement of nonessential trace metals such as mercury, silver, and cadmium will be less likely to be confounded by these sources of variability. Analysis of the metal composition of metallothionein confers a degree of chemical specificity with regard to the probable inductive agent. Tissue concentrations of non-essential metals (Ag, Cd, Hg) in invertebrates reflect environmental concentrations, whereas, copper and zinc are regulated (Stegeman *et al*., 1992).

In conclusion, the literature suggests that it would be feasible to use metallothionein as a biomarker for metal exposure or metal-induced stress, but restricted to non-essential trace metals such as mercury, silver and cadmium. Metallothionein induction by non-essential metals and the attendant metal-binding appear to be a cellular defence mechanism against cell injury and metal toxicity occurs after this capacity is exceeded. If applied to aquatic macroinvertebrates, then the effects of these contaminants could have a predictive value for effects at the population or community level. The main disadvantage of using metallothionein as a biomarker is that the procedures for quantifying metallothionein and its bound metals are elaborate and probably do not offer much advantage over measuring metal concentrations in invertebrates. If a threshold metallothionein concentration over which impaired biological function occurs eg reproduction or toxicity could be established, metallothionein concentration could be used to predict the likelihood of population changes

## 6. Biomarkers of Pesticide Exposure

### 6.1 Acetylcholinesterase

Many of the commonly used insecticides are toxic because they inhibit the animal nervous system enzyme, acetylcholinesterase (AChE). Since this reaction is

substantially irreversible in response to many pesticides, percentage inhibition of acetylcholinesterase activity can be used as an indicator of exposure of an organism to organophosphorus pesticides for a considerable period after the toxicant itself is metabolised or eliminated from the organism's body. The monitoring of acetylcholinesterase activity in the brains of fish and birds in the field has become a technique commonly used for diagnosing the exposure to cholinergic poisons (Greig-Smith, 1991; Zinkl *et al*., 1991). The successful use of acetylcholinesterase inhibition to identify exposure of vertebrates to organophosphorus and carbamate insecticides prompts the question as to whether measurement of acetylcholinesterase activity in aquatic invertebrates could similarly reflect their exposure to cholinergic pesticides quantitatively and enable them to be used as biomonitors. There are several reasons why invertebrates could be attractive substitutes for vertebrates. Firstly, there is evidence that certain aquatic invertebrates are more sensitive to organophosphorus insecticides than vertebrates (Giesy *et al*., 1999). Secondly, acetylcholinesterase activity has been measured in a number of aquatic invertebrates species (Day and Scott, 1990; Kozlovskaya *et al*., 1993; Diamantino *et al*., 2000). Thirdly, many aquatic invertebrates are important components of aquatic food chains, hence, a significant reduction in populations of invertebrates can be linked to important ecological consequences.

Although aquatic invertebrates have been used routinely as biological monitoring agents, the use of acetylcholinesterase inhibition as a diagnostic tool or as an indicator of pollution has been largely neglected. Coppage and Matthews (1974) reported that pink shrimp had about 75% inhibition of acetylcholinesterase activity when exposed to a lethal dose of organophosphorus insecticides. Repeated exposures of the freshwater shrimp, *Paratya australiensis*, to chlorpyrifos and profenofos at sublethal concentrations caused increased depression in acetylcholinesterase activity (Abdullah *et al*., 1993; 1994). Percentage inhibition of acetylcholinesterase activity in nymph heads of stoneflies was linearly correlated with concentration in nymphs poisoned by fenitrothion (Flannagan *et al.*, 1978). Day and Scott (1990) and Ibrahim *et al*., (1998) demonstrated similar results for stoneflies and chironomids, respectively, exposed to several organophosphorus compounds. However, the results from study of Day and Scott (1990) indicated that the choice of the aquatic invertebrate species, and a knowledge of their sensitivities to organophosphorus insecticides, would be important in any hazard assessment program which uses changes in acetylcholinesterase activities as an indication of sublethal toxicity.

### 6.1.1 Multiple and insensitive acetylcholinesterase

Although vertebrate acetylcholinesterases are primary membrane-bound enzymes, there is evidence to indicate that insect acetylcholinesterase exists in hydrophilic (buffer soluble) and amphiphilic (membrane-bound) states. This has been reported for Coleoptera, Lepidoptera, Diptera, Hymenoptera, Plecoptera, Ephemeroptera and other aquatic taxa (Krysan and Kruckberg, 1970; Belzunces *et al*., 1988; Day and Scott, 1990), with the proportions of both states varying from species to species. A major concern is that multiple forms of acetylcholinesterase might obscure the correlation between symptoms and inhibition of acetylcholinesterase activity. Among terrestrial insects, in particular the housefly, the degree of inhibition needed to produce observable symptoms range from a low value of 17% to a high of 98%

(Edwards and Fisher, 1991). This disparity initially compromised the use of acetylcholinesterase inhibition as a predictive tool until it was realised recently that those studies in which very high levels of acetylcholinesterase inhibition were needed to produce an effect might have been inaccurate. There is now substantial evidence to indicate that the inhibition of thoracic acetylcholinesterase is more important in producing symptoms of intoxication, than acetylcholinesterase derived from other sites (Edwards and Fisher, 1991).

Thus, studies, which used whole body of cephalic homogenates, tended to conclude that high levels of acetylcholinesterase inhibition were required to produce an effect, because they were testing a relatively non-critical form of the enzyme. It is now evident in some insects that lethal acetylcholinesterase inhibition is a localised rather than a generalised phenomenon. The average level of acetylcholinesterase inhibition in an insect may not be high. However, if certain critical forms of the enzymes are inhibited (eg thoracic acetylcholinesterase), a considerable effect will be seen (Tripathi and O'Brien, 1973). The honey bee, *Apis mellifera*, is the only terrestial insect in which head acetylcholinesterase activity has been correlated with a biological effect and used for diagnostic purposes (Bendahou *et al*., 1999).

To be used as a biomarker, candidate species must be screened carefully for the anatomical location of multiple forms of acetylcholinesterase activity and the relative sensitivity of each molecular form to cholinergic inhibitors must be determined. The predictive value of the diagnosis will very much depend on how carefully these distinctions are made.

### 6.1.2 Variations in endogenous acetylcholinesterase activity

As well as acetylcholinesterase activity varying in its form and location, as discussed in the previous section, the amount of acetylcholinesterase activity in a given tissue can also vary in response to characteristics of the organism such as the developmental stage and age within a stadium. Casida (1955) measured different cholinesterase activity in the eggs, larvae, pupae and adult heads of the housefly and other insects, with the highest specific activity in the heads of adults. Quantifiable levels of acetylcholinesterase activity first appeared at stage V of grass shrimp embryonic development and increased as development progressed (Lund *et al*., 2000).

It is also important in any study to determine if the enzyme activity being studied is exclusively acetycholinesterase. Cholinesterases are typically subdivided into two major classes, the true or acetycholinesterases (AChEs) and butytylcholinesterases (ChEs), sometimes referred to as pseudocholinesterases. These cholinesterase enzymes can be distinguished by differences in their catalytic properties towards various substrates and determining the effect of iso-OMPA and BW284C51, specific inhibitors of butytylcholinesterase and acetylcholinesterase activity, respectively (Eto, 1974; Belzunces *et al*., 1988).

### 6.1.3 Characteristics of the chemical which affect acetylcholinesterase

The two major classes of cholinergic insecticides, organophosphorus compounds and carbamates differ fundamentally in their acetylcholinesterase

inhibitory properties. Organophosphorus compounds are considered to be functionally irreversible inhibitors of acetylcholinesterase since the time necessary to liberate the enzyme from inhibition may be in excess of the time required for synthesis of new acetylcholinesterase. Carbamates, on the other hand, can have a fairly rapid decarbamylation step so that substantial recovery of the enzyme, can occur in a finite period of time. Recovery from non-lethal carbamate poisoning in vertebrates can be complete within 24-48 hours (Habig and Di Giulio, 1991). Invertebrates also recover quickly from carbamate intoxication. On the other hand, the inhibitory effects of organophosphorus compounds may often be long lasting, suggesting recovery in such cases results primarily from new enzyme synthesis. For example, recovery of fish brain acetylcholinesterase activity requires one to four weeks following short-term sublethal exposure to organophosphorus pesticides (Habig and Di Giulio 1991; Zinkl *et al.*, 1991). Recovery from the effects of organophosphorus compounds is both chemical and species specific (Zinkl *et al*., 1991). Abdullah *et al*., (1994) reported that acetylcholinesterase activity of the Australian freshwater shrimp, *Paratya australiensis*, required seven days for recovery from inhibition by sublethal concentrations of profenofos. This difference between the effects of these two types of pesticides must be accounted for in making diagnoses of field samples.

### 6.1.4 Environmental parameters which affect acetylcholinesterase

A number of environmental variables have been suggested as factors that could interfere with the accurate interpretation of acetylcholinesterase activity data in invertebrates. Among these, temperature emerges as the single most important factor controlling levels of acetylcholinesterase activity and inhibition. There is abundant evidence that endogenous acetylcholinesterase activity increases as a function of temperature (Zinkl *et al*., 1991; Edwards and Fisher, 1991). However, using control samples, which have had similar environmental temperatures, can eliminate this source of variability.

Animals collection in the field must be properly stored between collection and analysis in order to ensure that any acetylcholinesterase activity reduction is due to pesticide inhibition rather than protein degradation. Immediate freezing of specimens on dry ice at the time of collection is a conservative standard procedure that will halt postmortem changes in tissue or enzyme-inhibitor complexes. When freezing is not possible or if specimens are to be processed for acetylcholinesterase assay on the day of collection, chilling of brain or head segments at $4^{o}$ C is recommended for 2-14 days, without significant loss of acetylcholinesterase activity (Ludke *et al*., 1975). Storage of specimens for more than several weeks is often necessary pending procurement of appropriate controls. Although freezing depresses brain acetylcholinesterase activity by up to 25% during the first 12-24 h of storage, it appears to proportionally affect inhibited and uninhibited brain acetylcholinesterase activity alike; stabilisation of activity occurs after this initial depression (Ludke *et al.*, 1975; Zinkl *et al.*, 1991).

### 6.1.5 Chemical reactivation of inhibited acetylcholinesterase

Chemical reactivation is a means of confirming whether low acetylcholinesterase activity is due to pesticide inhibition. Reactivation of acetylcholinesterase activity is produced by addition of aqueous pyridine-2-aldoxime

methiodide (2-PAM), which will produce a substantial recovery of activity in cases where there has been inhibition by organophosphorus pesticides (Martin *et al*., 1981). Measurement of spontaneous reactivation can also help to identify the action of a carbamate compound (Martin *et al.,* 1981). Results obtained by the application of this technique might be biased by the pesticides involved, for it is known that dimethyl-substituted organophosphates are less prone to reactivation by 2-PAM than are diethyl-substituted compounds (Martin *et al.*, 1981).

## 6.2 Cellulase/carbohydrase

The physiology of digestion in aquatic macroinvertebrates has received little attention, and the few studies suggest that the digestive processes of various functional groups are adapted to the locally available food supply, primarily terrestrial leaves (Martin *et al*., 1980; Kesler, 1982). Aquatic insects, including shredders, show a positive selection for “conditioned” leaves, that is, leaves in the early stages of decay due to the presence of colonised aquatic hyphomycete fungi and bacteria (Barlocker and Kendrick, 1975). The fungi species on leaves have been shown to secrete three enzymes, collectively known as cellulase, that degrades cellulose to glucose (Martin, 1983). The digestive systems of most macroinvertebrate shredders are designed to decompose dietary polysaccharides and oligosaccharides. Thus their digestive systems contain either endogenous or ingested cellulose and cellobiase (α-glucosidase) activity (Kesler, 1982; Martin, 1983; Barlocher and Porter, 1986) and β-glucosidases such as amylase or β-amylose. Amylase catalyses the digestion of starch, the nutrient reservoir in plants (Bernard and Lagadic, 1993), whereas, β-amylose catalyses the digestion of β-1,4- glucans in the fungal tissue associated with plant debris (Martin et al., 1980). Caddisflies and mayflies feed very little as adults, thus essentially all the nutrients required for pupation and reproduction must be accumulated by the larva. Some species preferentially ingest lipid-coated detritus during the last larval instar (Cargill *et al*., 1985). Pyrethroids at sub-lethal concentrations have been shown to decrease gut amylase activity in larvae of the beetle *Tribolium castaneum* larvae (Saleem and Shakoori, 1985; 1986; 1987). The organophosphorus insecticide, fenitrothion, has also been reported to inhibit the gut carbohydrase activity of earthworms (Patnaik and Dash, 1993). Decreased feeding will result in decreased growth and reproduction, which can severely affect the survival of some populations. Therefore, the measurement of decreased cellulase, β-glucosidase or α-glucosidase activity in shredders such as the mayfly nymphs of *Jappa kutera* and *Atalophlebia* sp., which are dominant macroinvertebrate species in the Namoi River, NSW, has potential as a biomarker of pesticide exposure.

## 6.3 Carboxyesterase

The primary target for pyrethroids and DDT analogues in insects is the voltage-dependent sodium channel of nerve-cell membranes (Narashashi, 1992) where the insecticides prolong the opening time of the channels (Bloomquist and Miller, 1986). An important mechanism that confers resistance to pyrethroids and DDT, known as knockdown resistance or *kdr*, is due to single mutations, has been reported in many insect species (Soderlund and Bloomquist, 1989; Gunning *et al,* 1991). However, induction of detoxifying enzymes in insects is another important mechanism for resistance to these insecticides (Terriere, 1984). After the introduction of a pest management strategy that placed restrictions on the agricultural use of pyrethroids

against the Australian cotton bollworm, *Helicoverpa armigera*, resistance due to *kdr* nerve insensitivity rapidly declined to virtually undetectable levels in field populations (Gunning *et al.,* 1996). However, pyrethroid resistance in *H. armigera* persists and is largely attributable to a massive overproduction of esterase enzymes, which detoxify pyrethroids by sequestration and hydrolysis (Gunning *et al.,* 1996). The increased esterase activity of up to 50-fold is likely to be the result of gene amplification (Devonshire and Field, 1991). Quantitative changes in esterase enzyme titres were correlated to resistance factors determined by laboratory bioassay from eggs collected in the field (Gunning *et al.,* 1996).

Carboxyesterase activity is present in a variety of tissues in vertebrate and invertebrate species (Leinweber, 1987). In insects, juvenile hormone increases carboxyesterase activity in the haemolymph up to 8-fold and appears to regulate its own destruction near the end of the last larval instar (Sparks and Hammock, 1979). Other studies have demonstrated that carboxyesterases also protect against the toxicity of organophosphorus pesticides (Maxwell, 1992). Whether carboxyesterases also protect against the toxicity of organophosphorus pesticides in field populations of aquatic invertebrates in agricultural catchments needs to be investigated.

As in the case of acetylcholinesterase, there are multiple forms of carboxyesterase activity in most species (Terriere, 1984). Therefore it cannot be assumed that measuring total activity with a selective surrogate substrate will provide useful information regarding resistance in which an insecticide is detoxified. Naphthyl acetate is a general substrate for a variety of hydrolases, which is commonly used for determining carboxyesterase activity. Other than juvenile hormone or the commonly used test substrates 1-napthyl acetate and *p*-nitrophenyl-acetate, there has been no careful study of suitable substrates, although various butyrate thioesters have been reported to eliminate background activity in insects (Brown and Brogdon, 1987). Esterase inhibitor/ synergist compounds can provide information on the mechanism of resistance (Brown and Brogdon, 1987).

## 7. Conclusions

The measurement of *in situ* growth and scope for growth of macroinvertebrates provide an early warning system that macroinvertebrates are stressed but provide little evidence of the cause of the stress. The use of *in situ* growth measurements requires the effects of transplanting and cages on macroinvertebrate growth and survival to be established for each species. The main disadvantage of applying the scope for growth measurements for freshwater studies is the lack of test organisms for which feeding rates can be quantified. The slow induction of monoxygenase activity and P450 in response to contaminants in invertebrates precludes the use of mixed functional oxidases as a measure of stress. Two biochemical mechanisms that confer high resistance to organophosphorus and pyrethroid insecticides are increased detoxification by the over-production of esterases and insensitive acetylcholinesterases. Measurement of these enzyme activities in aquatic macroinvertebrates could be used as a biomarker of susceptibility to sub-lethal toxicity (decreased acetylcholinesterase activity) or as a biomarker of resistance (insensitive acetylcholinesterase or increase carboxyesterase activity) that can be linked to changes in population densities. Other enzymes such as ✂-glucosidase or 🖇-glucosidase activity, which are involved in the digestion of food, have potential as

biomarkers that could be linked to the survival of aquatic macroinvertebrate populations. Metallothioneins can be used as a biomarker to indicate exposure of macroinvertebrates to some metals but not common contaminants such as copper and zinc. Metallothionein as a biomarker of exposure probably does not offer much advantage over detailed chemical studies of the metal concentrations in macroinvertebrates. If a threshold metallothionein concentration over which impaired biological function occurs eg reproduction or toxicity could be established, metallothionein concentration could be used to predict the likelihood of population changes.

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**Table 1.** Levels of biological organisation and measurements.

| Level | Measurement | Effects |
|---|---|---|
| Community | Structure/ function | Loss of diversity |
| Population | Mortality/ genetic pool/ disease/abur recovery rates | Population decline |
| Organism | Metabolism /fitness/fecundity/ bel lifespan/ scope for growth (SfG)/susce | Reproductive failure |
| Cellular | Metabolism/ immunosuppı fertilisation/tumors | Loss of homeostasis and in mortality |
| Sub-cellular | Neurone function/stability of lysc organelles/necrosis/genotoxicity | Loss of homeostasis and in mortality |
| Molecular | Enzyme activity/ adenylate energy (AEC)/ oncogenesis/ RNA:DNA metallothionein | Loss of homeostasis and in mortality |

**Table 2.** Summary of major biomarkers presently used to assess impaired biological function. Adapted from Peakall (1992), McCarthy and Shugart (1990), Benson and Di Giulio (1992).

| Biomarker | Tissue | Use |
|---|---|---|
| Vitellogenin | Liver | Indicator of exposure to estrogenic contar |
| Mixed function oxidases | Liver | Indicator of exposure to organic chemica as PAHs and PCBs |
| Glutathione S-transferases | Liver | Indicator of exposure to pesticide metalloids |
| Cellulase/carbohydrase | Stomach | Indicator of exposure to pesticides |
| Acetylcholinesterase | Brain | Indicator of exposure to organophosph carbamate pesticides |
| Carboxylesterase | Various | Indicator of exposure to pyrethroi carbamate pesticides |
| DNA strand breakage, formation, chromatid exchange | Various | Indicator of exposure to alkylating or a agents |
| Aminolevulinic acid dehydratase | Blood | Indicator of exposure to lead |
| Metallothionein | Various | Indicator of exposure to metals |
| Retinoids | Liver | Indicator of exposure to dioxin and furans |
| Porphyrins | Liver | Indicator of exposure to chlorinated a hydrocarbons |
| ATP | Blood | Indicator of exposure to stress |
| Scope for growth (energy balanc | Whole animal | Indicator of non specific contaminant exp |
| Stress proteins | Various | Indicator of cells experiencing stress |
| Glutathione | Liver | Indicator of oxidative stress |
| Histopathological indices | Various | Indicator of non specific contaminant exp |

**Table 3.** Resistance mechanisms to insecticides. Adapted from Casida and Quistad (1998).

| **Insensitive target** | **Chemical class affected** |
|---|---|
| Voltage-dependent $Na^+$ channel | DDT, pyrethroids |
| GABA-gated chloride channel | Cyclodienes |
| Acetycholinesterase | Organophosphorus, carbamates |
| **Enhanced detoxification** | |
| Cytochrome P450 | Chlorinated hydrocarbons, pyre organophosphorus, carbamates |
| Carboxyesterases and other esterases | Pyrethroids, organophosphorus, carbam |
| Glutathione S-transferase | Chlorinated hydrocarbons, organophosp |

**Table 4.** Insecticide mode of action.

| **Nerve Target** | **Chemical Class** | **Pesticide Example** |
|---|---|---|
| Voltage-dependent $Na^+$ channel | DDT and pyrethroids | Deltamethrin |
| ☎-aminobutyric acid (GABA) | Cyclodienes | Dieldrin, endosulfan |
| Nicotinic acetylcholine receptor | Nicotinoids | Imidacloprid, spinosad |
| Acetylcholinesterase | Organophosphorus compounds | Chloropyrifos |
| **Other Targets** | | |
| Mitochondria | Respiratory inhibitors/ uncouplers | Chlorfenapyr |
| Insect growth hormone receptors | Juvenile hormone and ecdysone agonists | Methoprene, tebufenozide |