

**EVALUATION OF TECHNIQUES FOR THE ASSESSMENT OF BIOMARKER
RESPONSES IN SELECTED MOLLUSCS AND CRUSTACEANS FOR *IN*
SITU ENVIRONMENTAL ASSESSMENT.**

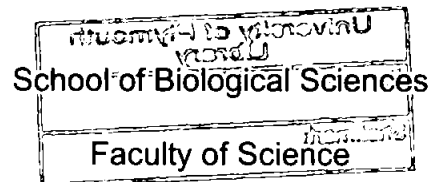
by

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ABSTRACT

There is a constant and increasing need for sensitive and relevant information regarding the effects of the chronic exposure of coastal and marine ecosystems to anthropogenically derived chemicals and stressors. If these environments are to be preserved and maintained then an increased availability of techniques to aid in the understanding of pollutant effect will be of significant advantage. This work examines the use of a suite of biomarkers of marine pollution exposure and effect to determine environmental quality and the effect of pollutants on marine organisms. The aim is to examine the sensitivity of a range of techniques and their applicability to field monitoring. The blue mussel *Mytilus edulis* and the shore crab, *Carcinus maenas*, were chosen as test organisms. A range of techniques, including lysosomal membrane tests, cardiac monitoring, histopathology, and embryonic bioassays have been utilised. The robustness of the neutral red test in mussels is demonstrated in a range of laboratory and field studies. Confounding factors, such as variability and operator bias, are discussed. Significant advances have been made in the application of the lysosomal neutral red test to crabs. Laboratory and field test data are presented demonstrating the application of this test. Significant new linkages are shown between the biomarkers under test. Field data are

presented showing links between subcellular membrane disruption, increased tissue abnormalities and the consequences of this on reproductive ability. Additional data are presented on the use of a freshwater mussel, *Anodonta cygnea*, as a sentinel animal. Links between cardiac activity and sub-cellular disruption are shown.

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1.0 INTRODUCTION

Assessment of the risk posed by the introduction of hazardous chemicals into the environment is an important task for regulators, industry, and governments. The marine, and particularly the coastal, environment is at risk from the anthropogenically derived effluent and waste that enter it. If damage to the integrity of the marine environment, through carefully focused management, is to be avoided then adequate information needs to be gained regarding the status of the ecosystems concerned.

However, the great complexity of natural ecosystems prevents most studies from being all encompassing in their approach. Furthermore, all studies tend to be constrained in the real world, by finite resources, such as time and money. However, the nature of environmental assessment requires information to be gained sooner rather than later, if it is to have any useful advantage in ameliorating, or preventing, harmful effects.

Thus, rather than studying an ecosystem in its entirety, it is theoretically simpler to examine only facets of it and use these to provide a diagnosis. A simple analogy of this is the observation of key biological symptoms in a patient, by a doctor, to enable a focused diagnosis of health condition to be obtained. In terms of environmental assessment, the 'symptoms' of pollutant induced stress are expressed in key organisms whose ecological niche and mode of existence make them ideally suited to reflecting prevalent environmental conditions. Certain specific characteristic responses can be used to determine if any affects observed are the results of pollutant toxicity or natural environmental fluctuation, and further, what pollutants are the likely culprits.

1.1 Background

1.1.1 Environmental Assessment

Environmental Assessment is the consideration of a local environment and the threat posed to it by activities which interact with it. The term is commonly used in the context of environmental legislation and refers to the appraisal of the likely effects of a proposed project, activity or policy on the environment. The inference being that negative effects will be identified, and managed, before they reach an unwanted or irreversible state. Environmental legislation is fundamentally based on the ability of available methods for environmental assessment to predict the effect of a process.

The determination of environmental quality can be based on all, or some of, the following:

- Risk Assessment
- Direct Toxicity Assessment (DTA)
- Chemical and Biological Monitoring

Risk assessment of chemicals, before their introduction into the environment is commonly assessed using toxicity testing. In aquatic toxicity tests groups of selected organisms are exposed to test materials (water or sediment samples) under defined conditions to determine potential adverse effects (Anderson *et al*, 2004).

Toxicity testing can be divided into:

Acute Toxicity Testing: The concentration of a substance (compound or drug) required to kill a predetermined proportion of test organisms within a relatively short period of time.

Chronic Toxicity Testing: This is used to determine the effects of a sub-lethal (i.e. one which does not kill) concentration of a substance applied on part, or all, of an organisms life cycle.

(Forbes and Forbes, 1994)

The results of toxicity tests are normally expressed as an effect on a standard test species to a given test substance for a set time period. Thus toxicity results will often be expressed as, for example, a 48 hour LC₅₀ i.e. the concentration which kills 50% of the test organisms in 48 hours (LC standing for lethal concentration). When the endpoint is a sub-lethal effect, for example larval deformity, the expression effect concentration (EC) is used.

Single species toxicity testing includes the use of 72 hour algal testing (*Skeletonema costatum*), 48 hour *Acartia tonsa* testing (crustacean), 96 hour *Schophthalmous maximus* (fish) and 10 day *Corophium volutator* testing (crustacean). Other tests include 15 min Microtox testing (utilizing the inhibition of bioluminescence), 48 hour *Tisbe battagliaia* testing, and 24 hour oyster embryo testing. Water or sediment can be tested.

The advantages of toxicity testing include:

- Demonstrable biological effect
- Uniform testing procedures
- Simplified logistics
- Data can be compiled, linked with results from other tests e.g. for use in structure-activity relationship research
- Establishes a definite baseline
- Variability, to a certain extent, is controlled

Toxicity testing has the advantage that it provides sufficient data to determine threshold or no-effect levels (Landis and Yu, 2003). Toxicity test data principally determines lethal concentrations of a chemical following uptake and enables

the identification of no observable effect concentrations (NOEC) (Fossi and Leonzio, 1993). NOECs are the highest concentration of a toxicant to which organisms are exposed to and which causes no observable adverse response.

The use of such testing is world wide and detailed procedures exist to support its use. All toxicity tests are based, in some way, on the relationship between the dose (or concentration) of a toxicant and a biological response (Forbes and Forbes, 1994). This dose-response relationship is fundamental to all toxicity testing, and is generally measured as the dose that elicits a particular effect (usually death) over a fixed time interval.

The results of toxicity tests are directly utilized in decision making and legislation formulation. Toxicity tests are an extremely important area in terms of public health, as they provide the underlying basis for decisions on the safe levels of chemicals in, for example, food and hygiene products. They also provide the basis for how much of a given chemical can be allowed to enter the environment, before it has the potential to cause harm.

Current toxicological test procedures focus on ranking the relative toxicities of chemicals that might, or have, gained entry into the natural environment (Fossi and Leonzio, 1993). NOECs are established with a small number of test species in controlled laboratory conditions (Fossi and Leonzio, 1993). The critical level at which a chemical is observed to have an effect is then the focus of legislation, as targets are created to ensure that environmental concentrations do not exceed this concentration. Many environmental assessments of the marine environment are undertaken using information derived from physical characteristics of compounds and laboratory-based toxicity tests (Depledge *et al.*, 1993; Rashid *et al.*, 1991).

Tiered risk assessment describes a common method of phased or stepwise toxicity testing. Such schemes have been recommended for a number of years and have been developed in many countries e.g. much of Europe, including the UK and the USA (Smrcek and Zeeman, 1998). The EU, and the OECD, have both advocated the use of a tiered approach. The pathway through such a scheme can be initiated by the likely high volume production of a chemical, or the suspicion that a chemical may be toxic (Smrcek and Zeeman, 1998). Specific triggers initiate further testing. Tiered risk assessment tests can follow the following progression:

Tier 1. Single species lethality

Tier 2. Expanded acute tests, using a wider a range of organisms

Tier 3. Chronic and bioconcentration tests

Tier 4. Field and mesocosm tests

(King, 1998)

While these tests are low in cost and well established they do not address the sub-lethal effects of contaminants nor the environmental context in which pollutant exposure takes place (Peakall, 1992). The practice of conducting tests in a stable physical environment, such as laboratory aquaria, which demands little or no response from physiological processes prevents the accurate determination of the potential impact that contaminants may have on natural populations of animals (Cairns, 1986; Bamber and Depledge, 1997).

Forbes and Forbes (1994) believe that there is considerable room for improvement in current ecotoxicological testing. The fundamental issues which underpin this view are that:

- Dose response relationships are frequently misunderstood or the initial experimentation performed poorly

- Acute toxicity tests, limited to a single species, are widely used – even though there is widespread doubt on whether this can predict chronic effects in any meaningful way
- Lack of standardized protocols

(Forbes and Forbes, 1994)

At best, standard toxicity data gives a measure of toxicity under closely defined sets of operating conditions (Walker *et al.*, 2000).

Direct Toxicity Assessment (DTA) and chemical/biological monitoring satisfy, to a certain extent, the shortcomings of risk assessment by aiming to detect chemicals which slip through the more theoretical approach of tiered toxicity testing (Kenny *et al.*, 2002). DTA is a process by which whole effluent toxicity tests are used to help assess and control complex effluents discharged to the marine environment. DTA is used in conjunction with substance specific tests to control effluent discharge. DTA has been recently introduced, in the UK, in parallel with the introduction of the Water Framework Directive. The Water Framework Directive requires the combination of biological classification and chemical monitoring in coastal environments (Kenny *et al.*, 2004). DTA, whilst addressing the problem of mixtures of chemicals, still relies on tests which provide little information on sub-lethal effects. Substance specific measures are based largely on toxicological data such as Environmental Quality Standards (EQSs) which set maximum values for specific substances. EQSs, whilst quickly providing specific information for chemicals which have been characterized, may fail to identify substances that may account for observed toxicity. They do not address any of the issues of complex mixtures or bioavailability, they require large quantities of data and dubious extrapolation procedures (Kenny *et al.*, 2002).

There is still a reliance – at least in Europe – to use chemical analysis as the most significant tool for environmental monitoring (Tarazona, 1998). The reasons for this can be broadly attributed to:

- Monitoring tends to be in response to a specific concern (e.g. PCBs, DDT, Lindane)
- It has frequently been assumed that the chemical responsible for a biological effect of concern will be easily identifiable
- Chemical analysis is perceived as more instrumental, reproducible and sensitive than biological methods

(After Tarazona, 1998).

Most chemical monitoring is focused on priority lists of chemicals and operates within the framework provided by OSPAR. Beyond this monitoring focus tends to be largely based on analytical ability (Kenny *et al.*, 2002). The use of chemical monitoring, in the UK, can be argued to have undergone only a small advance since the early use of chemical monitoring in the '70s and '80s (Kenny *et al.*, 2002). Chemical monitoring could be extended to include more chemicals but the cost, the number of differing analytical techniques required and the sheer complexity of the potential interaction of chemicals with the environment (the mixture of organic and synthetic material for example) makes this, at present, an unrealistic proposition. Chemical monitoring, critically, says nothing about the potential effects of a chemical's presence in the environment, partly as a result of resolution issues (being unable to detect other chemicals with which it may interact), the difficulty of assessing bioavailability and the inability to set a 'safe' limit with any degree of certainty (Kenny *et al.*, 2002).

The major advantage of utilizing biological methods, such as bioassays or biomarkers, is that they offer the potential to supplement and improve on the weaknesses of chemical analyses. However, the difficulty with biological tests

is that, in most cases, they cannot identify the pollutant responsible. Many assays have been significantly simplified, with implications for cost and applicability. *In Situ* biological methods provide an overall check on ecosystem health by diagnosing causes and providing early warning of environmental problems (Kenny *et al.*, 2004). The *Ex Situ* testing of waters and sediments by means of bioassays, utilizing whole organism responses, indicate that pollution has occurred but are not diagnostic of particular pollutants (Kenny *et al.*, 2004). It is generally recognized that there is no single method of monitoring, it must ultimately be a combination of physiochemical measurements and biological measurements (Widdows and Donkin, 1992). The UK has now realised the importance of integrating biological and chemical monitoring (Kenny *et al.*, 2004).

1.1.2 Pollutants in the Marine Environment

Approximately 70% of the human population lives within 60km of the coastal ocean, and this percentage is increasing (Gray, 1991). The dependency of humans on the worlds oceans is increasing, with a corresponding increase in anthropogenic stress imposed on marine ecosystems. Pollutants can enter the marine environment via a number of different pathways, including:

- Riverine input
- Diffuse runoff from land
- Direct discharges
- Ship discharges/dumping
- Atmospheric deposition
- (Kennish, 1996)

The areas most at risk, therefore, are the coastal environments which receive the highest concentration of pollutant input. Contaminants of the marine

environment can be divided into three main groups, non halogenated hydrocarbons, halogenated hydrocarbons, and metals.

Halogenated hydrocarbons

These are some of the most persistent, ubiquitous and toxic pollutants in estuarine and marine ecosystems (Kennish, 1996). They contain halogens such as chlorine, fluorine, bromine and iodine. Their use's include a role in plastics manufacturing, pesticides, the cleaning of electronic components and propellants. The higher weight halogenated hydrocarbons are of particular concern as they are extremely persistent and accumulate preferentially in lipid rich tissue (Kennish, 1996). The lower weight members of this class are not of such concern, as they do not accumulate – to such an extent – in biota (Kennish, 1996). It is the specific chemical properties of this class of chemical that makes them such a risk to marine environment. This includes:

- Chemical stability
- Great mobility in the marine environment
- Hydrophobicity
- Resistance to degradation
- Persistence in the environment
- Affinity for living systems
- Bioaccumulative capacity
- Generally high toxicity

A classic example of such a chemical is DDT (dichloro-diphenyl-trichlorethane); a pesticide which was found to be extremely persistent and mobile in the environment. Its use was largely banned in 1972 when it was found to accumulate and effect other species.

Non-halogenated hydrocarbons

Extremely common, non-halogenated hydrocarbons, are also known as Poly Aromatic Hydrocarbons (PAHs). They are of particular environmental concern due to their widespread introduction into the environment and their potential carcinogenicity, mutagenicity and teratogenicity to aquatic organisms and humans (Kennish, 1996). High levels of PAHs are commonly found in coastal areas and are formed, for example, by the incomplete combustion of carbon containing fuels. Examples of PAHs include anthracene, benzopyrene, naphthalene and pyrene. PAHs are characterized by:

- High hydrophobicity
- Persistence
- High toxicity (lower weight PAHs)
- Susceptible to transformation processes e.g. photooxidation, chemical oxidation, and biotic metabolism
- Rapid sorption to particulate matter and accumulation in sediments
- The body burden of PAHs in a marine organisms is principally determined by:
 - Concentration of PAHs in the environment
 - Degree of bioavailability of PAHs
 - Capacity of organisms to metabolise them
 - (Kennish, 1996)

Metals

Among the most intensely studied contaminants of marine environments are heavy metals (Kennish, 1996). They may be divided into two categories:

- Transition metals e.g. cobalt, copper, iron
- Metalloids e.g. arsenic, cadmium, lead, mercury

Transitional metals are essential for metabolism, but are toxic at higher concentrations whereas metalloids are generally not required for metabolic function and are toxic at low concentrations (Kennish, 1996). Toxicity of a given metal can vary between organism species and its accumulation is dependent on a large range of factors. Trace metals can occur in a variety of forms during transit to the sea:

- In solution, as inorganic ion and inorganic/organic complexes
- Absorbed onto surfaces
- In solid organic particles
- As coatings on detrital particles
- In lattice positions of detrital crystalline material
- Precipitated as pure phases

(Kennish, 1996)

Most metals tend to be associated with bottom sediments and are not common in dissolved form (Kennish, 1996). The distribution of metals is controlled by:

- Resuspension during storms
- Precipitation/interaction with particle surfaces
- Activity of organisms
- Mobilisation of metals to interstitial waters
- Transformation of metals

(Kennish, 1996)

Contaminant Behaviour

The behaviour of a contaminant in the environment is highly complex and dependent on a multitude of factors. Generally, its movement, availability and

toxicity is determined by which class it is, the magnitude of its presence, interaction with environment factors (such as salinity, temperature and dissolved oxygen levels) and the condition (e.g. life stage, health, metabolic rate) of the organisms it comes into contact with (Lawrence and Hemingway, 2003).

Biotic and abiotic variables influence the kinetics of a pollutant in the environment, and must be considered when examining biomonitoring data (Philips, 1980). Observations in toxicity studies have shown that the physiochemical properties of the test water such as the presence of cations, pH, temperature, salinity and the presence of other inorganic/organic constituents can change the chemical speciation of the contaminant (Erickson *et al.*, 1994). The affects of salinity, temperature on bioavailability as well as the relative proportions of a pollutant taken up from food, seawater or sediments are important factors in assessing the ecotoxicological significance of particular pollutant loads (Depledge and Rainbow, 1990).

The initial fate of a organic compound in the environment is based on three factors:

- Prevailing environmental conditions at the point of discharge
- Physiochemical properties of the compound
- Patterns of use (e.g. location and timing of introduction)

(Pontolillo and Eganhouse, 2001)

The transition from the freshwater to the marine environment can have an immediate effect on the marine distribution of organic compounds (Knezovich, 1994). Body burden of PAHs differs widely among estuarine and marine organisms due to variable concentrations of PAH in coastal environments,

differing degrees of bioavailability and variable capacities of the organisms to metabolize them (Kennish, 1996). For example, PAHs bound to sediment have a limited bioavailability, which reduces toxic potential (Kennish, 1996). The most important physicochemical properties relating to the environmental behaviour of organic compounds are aqueous solubility and octanol-water partition coefficient (Pontolillo and Eganhouse, 2001; Knezovich, 1994). In the case of marine mussels, solubility limits the quantity of contaminant available for uptake (Widdows and Donkin, 1992). Reduced solubility reflects increasing hydrophobicity and an increasing tendency to partition into organism tissue (Widdows and Donkin, 1992). Chemicals vary considerably in their solubility in water, their ability to bind to soil like materials and in their propensity to be absorbed into biological fats (Kenny *et al.*, 2002).

The octanol-water partition coefficient (commonly written as K_{ow}) is a parameter used to determine the fate of chemicals in the environment. It is the ratio of the concentration of a chemical in octanol and in water at equilibrium, and at a specified temperature. Octanol, an organic solvent, is used as a surrogate for natural organic material. K_{ow} has been correlated to water solubility therefore the water solubility of a substance can be used to estimate its K_{ow} value. K_{ow} can also be correlated with soil/sediment sorption coefficient and bioconcentration. The use of K_{ow} values is widespread and are routinely used in risk assessment and the formulation of environmental legislation. However, the reliability of K_{ow} values currently being used has recently been questioned by Pontolillo and Eganhouse (2001) based on unknown levels of accuracy in the prevailing literature, unvalidated computational methods and a general lack of satisfactory data quality. This is particularly significant as K_{ow} values are widely used to estimate or predict other properties, such as bioconcentration and

bioaccumulation factors, as well as indices of biodegradability (Pontolillo and Eganhouse, 2001).

Not only do environmental factors, such as salinity, affect the compound itself but they will also have an affect on the organisms present in the environment. Salinity induced changes on an organisms physiology may alter the availability of a contaminant by effecting changes at the site of uptake or in its metabolic capabilities (Knezovich, 1994). Both physical and geochemical processes control the distribution and transport of metals in the environment, for example, sediment sorbed metals are often resuspended during storms (Kennish, 1996). Metals which enter the marine environment usually do so as part of a freshwater discharge, sorbed onto the surfaces of dissolved ligands and particulate matter, which reduces their availability to marine organisms (Knezovich, 1994). However, the high dilution ratios that accompany such discharges favor metal desorption as does the increased presence of soluble anions which compete for the sorption of metals to form soluble complexes (Knezovich, 1994). Such factors will therefore influence the overall bioconcentration of trace metals within an ecosystem (Knezovich, 1994).

The geochemical nature of sediment will affect binding e.g. sediment with a low organic carbon content might bind less contaminant chemical than one with a high organic carbon content. Salinity will affect the flocculation processes that will, in turn, affect the binding of chemicals to sediment or organic carbon compounds suspended or dissolved in the water column (Kenny *et al.*, 2002).

The accumulation of metals in biota occurs via several pathways, including:

- Ingestion of food and suspended particulate material containing sorbed materials

- The uptake of metals directly from bottom sediments or interstitial waters
- The removal of metals from solution

(Kennish, 1996)

Numerous factors affect the uptake of metals including dissolved metal concentration, temperature, salinity, presence or absence of chelating agents and other metals, and other intrinsic variables such as organism surface permeability, nutritional stage, molt cycle stage, size and through put of water (Kennish, 1996; Ansari *et al.* 2004). The largest factor affecting metal bioavailability is the route metals take from sediment to biota i.e. as soluble or suspended form (Ansari *et al.*, 2004). Depledge and Rainbow (1990) emphasized that the systemic mechanisms of handling metals and an organisms physiological condition can determine the significance of the body burden of specific metal.

The most significant factors affecting metal toxicity are:

- Chemical speciation (e.g. may be free ions, organometallic molecules and be transported in dissolved or particulate phase)
- Presence of other metals or toxicants (this can cause antagonistic or synergistic effects)
- Environmental conditions (e.g. temperature, pH, salinity, dissolved oxygen condition the physiological activity and metabolism of marine organisms)
- Condition of the organisms (e.g. health, age, sex and life stage)
- Adaptation of the organism to the absorption of metals

(Ansari *et al.*, 2004)

Speciation refers to the various physical and chemical forms in which an element may exist in the marine system (Ansari *et al.*, 2004). Various metals,

upon dissolution in seawater, may give a number of different chemical species at different pHs which directly affects their resultant toxicity (Ansari *et al.*, 2004). In marine environments metals occur in dissolved form (e.g. as free ions or complexed ions) or in the solid state (as colloids, sorbed onto particle surfaces or in mineral matrices) (Kennish, 1997).

The bioavailability of metals in estuarine waters may depend on:

- Mobilisation of metals and their speciation
- Transformation (e.g. methylation)
- Level of binding to sediment components
- Competition between sediment metals
- The influence of bioturbation, salinity, redox and pH

(Bryan and Langston, 1992)

1.2 Biomarkers

Pollutant induced changes at the population, community, ecosystem, and human health level of biological organisation are the ultimate concern, but they are too complex and too far removed from the initial causative events to be of much use for the early detection and prediction of the consequences of environmental stress (Moore, 2002). Furthermore, the use of toxicity testing and chemical measurement ignores the issues of, for example, mixture effects and does little to address the complexity of the pollutant-organism-environment triumvirate.

Thus, there has been a focus on 'early warning' responses to pollution which could enable identification of problems before irreversible damage occurs and address some of the problems with current monitoring approaches. The wealth of available information appears to demonstrate an impact, of pollution, on a variety of organisms (including mussels, oysters, crabs, and fish) at a sub-lethal

level (Lawrence and Hemingway, 2003). This bias, towards sub-lethal methods, has largely arisen from a need to detect and assess the impact of pollution on environmental quality at a more sensitive level of understanding than that gained from using lethality (Forbes and Forbes, 1994). The measurement of sub-lethal effects represents an opportunity to identify the presence and action of pollutants before damage reaches an irreversible level.

Biological measurements of pollutant effect have come to be collectively known as 'biomarkers' (McCarthy and Shugart, 1990; Huggett *et al.*, 1992; Depledge *et al.*, 1993; Livingstone, 1993; Forbes and Forbes, 1994; Schlenk, 1996; Walker *et al.*, 1996). Peakall and Walker (1994) defined biomarkers as:

“a biological response to a chemical or chemicals that gives a measure of exposure, and sometimes, also of toxic effect.”

In the broadest definition the word 'Biomarker' can be used to describe any test which reflects the interaction between a biological system and an environmental agent (Kenny *et al.*, 2002). Biomarkers can be used to measure exposure, effect or susceptibility (Kenny *et al.*, 2002). There are, however, numerous definitions of biomarkers which all, in some way, relate to measuring a change in biological effects, from molecular to behavioural, at or below the individual level, which demonstrate a change from the normal functioning of the organism (English Nature, 2004).

Biomarkers have proved useful tools in establishing evidence of damage to the health of sentinel organisms (Depledge *et al.*, 1993). Human and veterinary medicine also makes use of biomarkers of health change in a similar way (Moore and Simpson, 1992). There is an increasing trend to focus on

biochemical measures as biomarkers (Handy *et al.*, 2003) but the total number of putative biomarkers is, after at least 15 years of recognizable biomarker research, large.

Biomarkers can be classified based on the organization level of biological hierarchy that they examine:

- Receptor Binding Biomarkers e.g. TCDD binding to Ah receptor
- Biochemical Response e.g. Induction of MFOs
- Physiological Alterations e.g. Imposex
- Individual Affect e.g. Changes in scope for growth

(Walker *et al.*, 2000)

Common biomarkers are, for example, the presence of pathological tissue, elevated levels of certain proteins, or reduced membrane viability. Membrane damage is significant, given their purpose in acting as a selectively permeable barrier between the cell, or cell organelle, and the external environment.

Biomarkers are generally specific changes that can potentially be linked to stress and in particular pollutant exposure. However, one of the challenges for ecotoxicologists has been to distinguish between general biomarkers, which respond to most types of environmental stress, and specific biomarkers, which respond only to a particular class of contaminants (Livingstone *et al.*, 2000). Furthermore, all biomarkers can be classified as responding to exposure and effect or in some cases both. Biomarkers of exposure indicate a general stress response whereas a biomarker of effect indicates that an adverse reaction has occurred (Elliott, *et al.*, 2003). The measurements of alterations in functional responses of organisms have been considered at different levels of complexity. These range from relatively specific responses at the molecular and cellular levels, to more general (non-specific) responses to the sum of the

environmental stimuli at the level of whole-animal physiological status (Bayne *et al.*, 1979, 1985; Livingstone, 1985; Moore, 1985; Widdows, 1985; Moore *et al.*, 1987).

Biomarkers which can be considered for use today include: DNA strand breakage as an indicator of genotoxicity (Shugart, 1990), the CYP1A protein as an indicator of organic pollution (Gibson and Skett, 1994), multidrug resistance as a measure of xenobiotic expulsion (Kurelec, 1992), histopathology (Hugget *et al.*, 1992), cardiac monitoring (Depledge and Andersen, 1990) and lysosomal membrane integrity (Allen and Moore, In Press). Recent studies of biomarker use have been described by Peakall and Shugart (1993), Fossi and Leonzio (1994), Livingstone *et al.* (2000), and Lawrence and Hemingway (2003). A review of selected biomarkers is shown in table 1.

The optimum biomarker measurements should be complementary, at the various levels of biological organisation, and where possible they should have ecological significance in terms of an adverse effect on growth, reproduction or survival of the individual and the population (Moore *et al.*, 1987). The rationale for this approach has been described by Bayne *et al.*, 1979, 1982, 1985; Livingstone, 1985; Moore, 1985; and Moore *et al.*, 1987. The key point is that the results of tests of biota for biologically, pollution mediated, change must be capable of being linked to ecologically relevant end points (Moore, 2002). Furthermore, these data must then be capable of being usefully applied in some form, for example ecosystem ranking in terms of condition, to enable justification for more study or action (Depledge *et al.*, 1993; Moore and Simpson, 1992; Moore, 2002).

Biomarkers offer the following advantages:

- Indicate the presence of biologically available contaminants
- May identify contaminants overlooked, or undetected by other methods
- Persist after a transient exposure, in some cases after the chemical responsible has degraded
- In many instances, biomarkers are easy tests to perform
- Give a measure of mixture effects
- Can demonstrate the effectiveness of remedial action

(English Nature, 2004; Handy *et al.*, 2003)

Biomarker	Type /Chemical Class	Current Status	Advantages	Limitations	Method
Increases in Cytochrome P450 MFO enzyme activity (Livingstone <i>et al.</i> , 2000)	Biochemical Organic, lipophilic compounds e.g. PCBs	Recommended by ICES/OSPAR. In use in academic and regulatory environment	Sensitive biomarker of organochlorine and PAH exposure. Inexpensive potentially non-destructive.	Destructive. Response not well characterized. Response may be inhibited by certain chemicals.	Fluorimetric and electrophoresis assays.
Increases in Glutathione S-transferase activity (Romeo <i>et al.</i> , 2002)	Biochemical Organic, lipophilic compounds	In use in academic sectors.	Inexpensive and validated method for invertebrates.	Destructive. Different isozymes.	Spectrophotometric assay.
Antioxidant Enzymes e.g. superoxide dismutase (Manduzio <i>et al.</i> , 2004)	Biochemical All	Recommended by ICES. In use in academic sectors.	Can be used for monitoring responses throughout an ecosystem. Inexpensive	Destructive. Many confounding factors.	Spectrophotometric assay.
Delta-aminolevulinic acid dehydratase inhibition (ALAD)	Biochemical Metals, organic compounds	In use in the academic sector as a general indicator of ecosystem health.	Specific for lead, absence of strong confounding factors. Inexpensive	Enzyme is unstable.	Atomic absorption spectroscopy.
Metallothionein Induction (Viarengo <i>et al.</i> , 1997)	Biochemical Molecular Metals	In use in the regulatory sector, standard assay for determining lead exposure in human	Sensitive marker of metal exposure, highly conserved. Inexpensive	Destructive. May be induced by other factors.	Spectrophotometric assay.
Inhibition of cholinesterase activity (Moulton <i>et al.</i> , 1996)	Biochemical Organophosphate pesticides, metals and detergents.	In use in academic and regulatory sectors. Recommended for use by ICES.	Inexpensive, non-destructive biomarker of organophosphate exposure.	Natural variation and transient.	Spectrophotometric assay.
Increase in DNA adduct formation (Harvey <i>et al.</i> , 1998)	Molecular PAHs organic compounds	In use in both academic and regulatory sectors.	Can be widely used in all organisms, Inexpensive.	Stability of adducts unknown, and background variation needs to be defined.	Post labeling techniques.
Increase in DNA strand breakage (Livingstone <i>et al.</i> , 2000)	Cellular Organic compounds, radiation.	In use in academic sector. Suggested as promising by ICES.	Sensitive biomarker, widely conserved. Inexpensive	May be repaired rapidly, not chemical specific.	Microscopy or by alkaline unwinding.
Histopathological changes (Hinton, 1989)	Physiological Organic and inorganic compounds, radiation.	In use in both academic and regulatory sectors.	Suitable for monitoring change in a wide variety of organisms.	Observations can be subjective. Other factors confound causal links being established.	Histological staining. Microscopy examination
Gonadal change e.g. imposex (Axiak <i>et al.</i> , 2003)	Morphometric TBT. Other endocrine disrupting chemicals.	In routine use in academic and regulatory sectors.	Specific for TBT, successfully used for monitoring.	May reflect previous exposure as is irreversible	Histological examination.
Scope for growth (Widdows and Johnson, 1988)	Whole organism energetics Many xenobiotic compounds.	In use by academic and regulatory sectors. Recommended for use by ICES.	Gives a holistic view of the functioning of an organism.	Response can be affected by other factors.	Many different methods required.
Vitellogenin protein induction (Riffeser and Hock, 2002)	Molecular Natural estrogens and their mimics.	In use by both academic and regulatory sectors. Recommended for use by ICES.	Monitors the effects of mixtures on the endocrine system of organisms.	Can be induced by wide variety of factors. Response not found in all species.	Spectrophotometric measure.
Presence of fluorescent bile metabolites	Biochemical PAHs	In use by both academic and regulatory sectors. Recommended for use by ICES.	Specific for PAHs.	Destructive.	Fluorimetric assay.
Lysosomal membrane stability (Wedderburn <i>et al.</i> , 2000)	Cellular	In use by both academic and regulatory sectors. Recommended for use by ICES.	Provides indication of general stress on organisms, linked to higher order effects.	Non specific and dependent on visual observation.	Light microscopy.
Changes in the immune system (Parry and Pipe, 2004)	Cellular	In use by academic and regulatory sectors. Promising ICES biomarker	Simple and inexpensive.	No data for linking effects at immune level to higher order effects.	Various methods available.
Transcriptomics	Molecular	Not in use at present. Microarray assessment in mussels is currently being tested.	Early detection of changes at the gene level.	Use is current limited. Bioinformaticians required. High cost.	Molecular biological determination.
Changes in protein expression. (Shepard and Bradley, 2000)	Biochemical	Not in routine use	Early detection of changes at the protein level.	Extensive optimization required. High cost.	Molecular biological determination.
Chemically activated luciferase gene expression (CALUX) and H4IIE bioassay	Molecular Organic lipophilic compounds.	H4IIE used in regulation CALUX in use in the academic sector. Promising ICES biomarker	Standard assays to determine presence of certain chemicals.	Only provides information on dioxin like chemicals.	Molecular biological determination.

Table 1. Selected Biomarkers, showing type, advantages, limitations, status and method. Example references given in parentheses. (After English Nature, 2004)

However, the use of biomarkers are limited by the following factors:

- Non specificity of response to chemical type, or class
- Induction of biomarker response by abiotic factors
- Some biological responses may be transient or only be part of a larger process
- They can be destructive in nature
- Lack of long term datasets to compare results against

The integration of biomarkers into the current regulatory framework would improve the ability of environmental monitoring to more accurately determine the consequences of pollutant presence. The biomarker concept has been presented, in this way, in by many authors (Handy, *et al.*, 2003; Wells *et al.*, 2001; Walker *et al.*, 2000; Livingstone *et al.*, 2000; Wu *et al.* 1999; Depledge *et al.*, 1993, 1994; Moore, 1985, 1990; Livingstone, 1993; McCarthy and Shugart, 1990) and has been investigated for more than 30 years. But biomarkers are still seldom used as ecotoxicological tools in marine management (Handy *et al.*, 2003). However, given the great wealth of data which exists there is the potential to apply this knowledge in a 'weight of evidence' approach, where multiple lines of evidence support decision making, which would integrate the biomarker approach into the environmental regulatory framework (Handy *et al.*, 2003).

Furthermore, it is likely that the future of world ocean management will require more, and increasingly subtle methods of monitoring, particularly as currently developing economies move into a more industrial phase and emergent technologies introduce new substances into the environment. Thus, the need for biological monitoring tools is unlikely to diminish.

1.3 Biomarkers Used in This Study

Cellular Pollutant Response – Lysosomal Biomarkers

Many studies have examined perturbation at the sub-cellular level, such as changes in membrane structure and cellular architecture, as a measure of pollutant effect. Pathological reactions to contaminants have been described at all levels of biological organisation ranging from the molecular and cellular level to the physiology of the whole animal (Moore, 1988; Lowe *et al.*, 1992, 1995; Widdows and Johnson, 1988). Initial studies, although examining sub-lethal responses, tended to be concerned with the responses of marine organisms exposed to relatively high (and presumably largely unrealistic) concentrations of commonly occurring environmental pollutants (Anderson, 1977; Johnson, 1977; Widdows *et al.*, 1982).

However, marine organisms have been shown to be sensitive to a wide range of environmentally realistic concentrations. Low and environmentally realistic concentrations of petroleum hydrocarbons have a significant adverse effect on the physiological and cellular condition of marine mussels *Mytilus edulis* (Widdows *et al.*, 1982). Simple relationships do not, however, exist between chemical measurements of contaminants and biological response. Anderson (1977) concluded that there was little agreement between sub-lethal responses of marine organisms and the level of hydrocarbon contamination in the tissues. Widdows *et al.* (1982) confirmed the lack of a simple correlation between individual physiological response and tissue hydrocarbon concentration. However, Lowe (1988) suggested that a lack of strict agreement between biological evidence of pollutant impact and the anticipated impact based on tissue chemistry may be due to the more subtle effects of complex pollutant cocktails and the toxic thresholds of individual pollutants.

The identification and quantification of cellular and sub-cellular responses to pollutant exposure has been shown to provide an early warning of pathological change in plants and animals (Bannasch *et al.*, 1989; Köhler, 1989; Köhler *et al.*, 1992; Moore *et al.*, 1994). Many different endpoints have been used in *in vitro* studies; however, the determination of the integrity of the plasma and internal membranes is widely viewed as an important indicator of cell viability (Lowe and Pipe, 1994). At the cellular level the lysosome has been identified as the critical target for the toxic effects of many contaminants (Moore, 1990).

Lysosomes are prominent amongst the many targeted organs and organelles chosen for biomarker study due to their unique ability to accumulate a diverse range of toxic metals and organic chemicals, including heterocyclic compounds and polychlorinated biphenyls (PCBs) (Lowe and Pipe, 1994; Lowe *et al.*, 1992; Moore, 1992; Rashid, 1991; Halliwell, 1997;). The sub-cellular lysosomal system in invertebrate and vertebrate cells has been confirmed as a target for the toxic action of xenobiotics and metals in a number of different studies (Moore and Clarke 1982; Moore *et al.*, 1985; Pipe and Moore, 1986; Nott and Moore, 1987; Axiak *et al.*, 1988; Moore, 1988; Cajaraville *et al.*, 1989; Hinton, 1989; Kohler, 1991; Viarengo *et al.*, 1992; Lowe and Pipe, 1994; Krishnakumar *et al.*, 1994; Moore, 1996). A number of laboratory studies have shown that lysosomal function and cell membrane stability are affected by exposure to organic (Moore *et al.*, 1985; Nott and Moore, 1987; Moore, 1992; Lowe and Pipe, 1994; Camus *et al.*, 2002) and inorganic substances (Viarengo *et al.*, 1987; Regoli, 1992; Shepard and Bailey, 2000). Exposure to a variety of contaminant effluents such as sewage sludge, pulp mill waste, oil spillage's and mixed wastes from industry have all been found to increase the fragility of fish and molluscan lysosomes (Moore, and Clarke, 1982; Moore, 1985, 1988, 1990;

Pipe and Moore, 1986; Nott and Moore, 1987; Köhler *et al.*, 1992; Lowe *et al.*; 1992; 1995a, 1995b; Krishnakumar *et al.*, 1994; Moore *et al.*, 1994a; Moore *et al.*, 1996; Petrov *et al.*, 2001).

In phagocytic blood cells, lysosomes have a role as a component of the immune response. They can perform a controlled release of the acid hydrolyses they contain to break down, for example, unwanted cellular components and foreign material that has entered the animal (Bayne and Moore, 1997). Lysosomes are also associated with cellular detoxication as a result of the lysosomal sequestration of metals (Nott and Nicolaidou, 1990).

Lysosomal perturbation is a significant cellular event as it is associated with pathological degeneration (Moore, 1988) and increases in lysosomal membrane permeability can lead to enhanced protein catabolism, cellular atrophy, and other pathological changes (Lowe *et al.*, 1981; Moore, 1985, 1988, 1990, 2002; Moore and Viarengo, 1987). Any damage to the lysosomal membrane could result in the leakage of the lytic enzymes into the cytosolic environment, the result of which may be severe cell damage or death (Deckers *et al.*, 1980). Changes at the sub-cellular level may spread rapidly into a complex network of associated secondary and higher order disturbances which become progressively more difficult for the cell to reverse or modify (Moore *et al.*, 1987). The destabilisation of lysosomes in the digestive cells of oil exposed mussels has been shown to be an important functional response that reflects general cellular disruption leading to enhanced autophagy (Widdows *et al.*, 1982; Moore and Clarke 1982). Enhanced autophagy will clearly have serious consequences for the maintenance of normal tissue function, causing digestive cell atrophy (Moore *et al.*, 1987; Moore, 1988). Damage to the lysosomal compartment of a cell will also affect the efficiency of intracellular digestion,

protein turnover, and other cellular systems. Impairment of the lysosomal system could, for example, cause the retardation in the rate, or even the failure, of endocytosis to such an extent that whole organism effects are observed.

Alterations in scope for growth have been shown to be functionally linked with impairment of the lysosomal system (Widdows *et al.*, 1982; Moore *et al.*, 1987; Allen and Moore, In Press). Bayne *et al* (1979) indicated a significant correlation between the labilisation period for β -N-acetyl hexosaminidase in mussel digestive gland cells and negative values for scope for growth.

The destabilisation of lysosomes in the digestive cells of oil exposed mussels is an important patho-physiological response that reflects general cellular disruption leading to enhanced autophagy (Widdows *et al.*, 1982). Lowe *et al.* (1995a) showed a correlation between lysosomal membrane fragility and total body burden of a range of organic and inorganic contaminants, while Moore *et al* (1987) demonstrated a correlation between digestive cell atrophy and lysosomal disturbances. These findings argue strongly for a mechanistic link from the lysosomal events through to the whole animal. Scope for growth is linearly correlated with the lysosomal stability of the digestive gland epithelial cells, indicating that lysosomal stability is also a good indicator of the overall health of the organism (Allen and Moore, 2004). Lysosomal membrane fragility is also associated with reproductive impairment. Release of lysosomal enzymes in eggs and embryos may also lead to chromosomal damage resulting in death or developmental abnormalities and a reduced capability for survival (Moore, 1985).

Molluscan digestive cells have been shown to demonstrate lysosomal destabilisation, after contaminant exposure, in a number of different studies

(Pipe and Moore, 1986; Nott and Moore, 1987; Axiak *et al.*, 1988; Cajaraville *et al.*, 1989; Winston *et al.*, 1991; Viarengo *et al.*, 1992; Lowe *et al.*, 1995a).

Moore *et al.* (1984) showed a reduction in lysosomal hydrolase latency in mussel digestive cells after exposures to copper and phenanthrene, while Grundy *et al.* (1996) showed an inhibition of phagocytosis and an increase in haemocytic lysosomal membrane fragility, in mussel haemocytes after poly aromatic hydrocarbon (PAH) exposure. Domouhtsidou *et al.* (2004) examined lysosomal response, in conjunction with metallothionein content in *M. galloprovincialis* exposed to a pollution gradient of metal contamination. Lysosomal destabilisation was observed in digestive gland cells, in correlation with metallothionein values (Domouhtsidou *et al.* 2004).

Grundy *et al.* (1996) showed a differential response in relation to the PAH the membrane was affected with and suggested that this could reflect the different physical and chemical properties of the PAHs under test. Increasing molecular weight increases aromaticity and hydrophobicity with a accompanying decrease in solubility (Grundy *et al.*, 1996). An alternative suggestion could be, however, that the structural characteristics of the PAHs influence how they behave in the mesocosm set up and therefore are more, or less, available as a result of any changes there, before they enter the organism. Grundy *et al.* (1996) examined the effects of PAHs on lysosomal membranes and phagocytic ability in *Mytilus* blood cells. Cells were exposed *in vitro* to 8.3, 16.6, 25 or 50 mg/l of anthracene, fluoranthene or phenanthrene, while whole animals were exposed to 250µg/l per day of single, 500 µg/l of double and 750 µg/l triple combinations of these PAHs (Grundy *et al.*, 1996). Total cell viability was measured using a 0.2% nigrosine solution which showed that overall cell viability was unaffected (Grundy *et al.*, 1996). However, the *in vitro* exposures of mussel blood cells

showed a reduction in lysosomal integrity following a 24 hr experiment with additive effects being observed when PAHs were used in conjunction with each other (Grundy *et al.*, 1996). Following a 14 day exposure to PAHs administered in mixture or alone the reverse was observed, with PAHs administered in isolation having more effect than those administered in mixture (Grundy *et al.*, 1996). Phagocytic activity was significantly reduced in the cells exposed *in vivo* to the highest levels of PAHs (Grundy *et al.*, 1996). Phagocytic activity was also most reduced in animals exposed to single administrations of PAH, mixture effects were less significant (Grundy *et al.*, 1996).

Burlando *et al.* (2002) demonstrated that exposure of molluscan lysosomes to 17 β -estradiol caused a destabilization of lysosomal membranes in association with an increase in Ca²⁺. Burlando *et al.* (2002) suggested that modification of cellular Ca²⁺ homeostasis could be responsible for lysosomal modification. Metals and oxidants have also been shown to affect Ca²⁺ homeostasis (Moore and Viarengo, 1987; Moore, 1985). Marchi *et al.* (2004) demonstrated significant lysosomal destabilization in *M. galloprovincialis* after *in vivo* exposure of haemolymph to mercury (Hg²⁺) and copper (Cu²⁺) at 50 μ m. Brown *et al.* (2003) reported cellular affects, after 68.1 μ g l⁻¹ copper exposure, in *M. edulis*.

PAHs, or their metabolites, may alter lysosomal membrane fluidity that will contribute to immune dysfunction (Grundy *et al.*, 1996). Shepard and Bailey (2000) also showed a reduction in lysosomal stability in mussel haemolymph in response to increasing levels of copper, in association with changing protein expression signatures, while Da Ros *et al.* (2002) showed changes in lysosomal integrity related to particular coastal environments. Furthermore, Ringwood *et al.* (1998) demonstrated a reduction in lysosomal integrity in haemolymph

lysosomes in oysters exposed to contaminated reference sites; and Nasci *et al* (1999) showed a reduction in lysosomal retention time after mussel exposure to contaminated sites in the Venice Lagoon. The lysosomes of molluscan haemocytes have been shown to be affected by contaminants (Lowe *et al*, 1995b, Lowe and Pipe, 1994, Moore *et al*, 1996).

Lowe *et al.* (1995) demonstrated no significant difference in the lysosomal response between molluscan digestive cells and blood cells following whole animal exposure to the hydrocarbon fluoranthene.

Several studies have utilised the lysosomal biomarker in bivalve molluscs to reflect the effects of chronic contaminant exposure (Moore *et al.*, 1982; Moore, 1988; Bayne *et al.*, 1988; Regoli 1992; Krishnakumar *et al.*, 1994; Lowe *et al.*, 1995; Gowland *et al.*, 2002; Da Ros *et al.*, 2002; Harding *et al.*, 2003; Castrol *et al.*, 2004).

Lysosomal integrity has been utilised in a number of field studies as a biomarker for the consequences of environmental contamination (Moore, 1986; Regoli 1992; Krishnakumar *et al.*, 1994; Lowe *et al.*, 1992, 1995a; Kohler *et al*, 1992; Moore *et al.*, 1996; Petrov *et al.*, 2001; Gowland *et al.*, 2002; Da Ros *et al.*, 2002; Harding *et al.*, 2003; Moleda *et al.*, 2005). Hwang *et al.* (2004) observed a recovery of lysosomal response in *C. virginica* after 28 day removal from a metal contaminated gradient.

The neutral red technique measures the retention of neutral red, a weak base dye, within the lysosomal compartment. Neutral red (NR) is a concentration dependent cytotoxic compound which has been shown to cause changes to the fine structure of rat and mouse lysosomes as well as inducing enlargement and vacuolation (McDonald and Koenig, 1965; Ohkuma and Poole, 1981).The dye

is added to isolated live blood cells and permeates into the lysosome in its unprotonated form where it becomes trapped by protonation (DeDuve *et al.*, 1974) inducing perturbations such as swelling and enhanced autophagy (Robbins *et al.*, 1964). Sub-cellular retention of the neutral red dye is measured at timed intervals using light microscopy. NR presents an additional stress to cells, the retention time being an integration of membrane response induced by both the NR and any additional stressors (Lowe and Pipe, 1994). In effect, the retention of the NR probe represents a measure of the adaptive ability of the cell after previous exposure to a contaminant (Lowe and Pipe, 1994). Thus, cells from animals exposed to environmental contaminants will exhibit reduced retention times for the neutral red probe (Lowe *et al.*, 1995b; Wedderburn *et al.*, 1998; Cheung *et al.*, 1998). Conversely, animals in relatively clean sites will be able to maintain lysosomal integrity against the challenge of the neutral red for longer periods of time. Retention times, of molluscan haemolymph, tends to be in the range of 100-160 minutes (Fernley *et al.* 2000; Wedderburn *et al.* 2000; Lowe *et al.* 1995; Grundy *et al.* 1996; Lowe and Pipe, 1994). Maximum values of reported retention time are partly a function of experimental design, and when observations are ceased.

Lowe and Pipe (1994) examined contaminant induced effect in mussel, *Mytilus edulis*, digestive cells by retention of the neutral red probe. The results showed that probe retention time was significantly reduced, after exposure of the mussels to fluoranthene (a polycyclic aromatic hydrocarbon) with a final concentration of 100µg/l, over a 7 day period. The failure of the lysosomal membranes to maintain their integrity against the additional stressor of the NR probe was observed in conjunction with elevated levels of lipid and of the lysosomal marker enzyme *N*-acetyl-β-D-hexosaminidase (NAH). Increases in

lipid level have been shown to be linked to contaminant exposure (Lowe, 1988; Lowe and Clarke, 1989) as have increased levels of NAH (Moore, 1980). Nicholson (2003) demonstrated a significant reduction in lysosomal retention time in *P. viridis* haemocytes following a 48 hour exposure to 150µg l⁻¹.

The neutral red retention technique has been shown to be a rapid and sensitive test for determining the lysosomal stability of indigenous invertebrate populations (Lowe and Pipe, 1994; Lowe *et al.* 1995a, 1995b; Cheung *et al.*, 1998; Shepard and Bradley, 2000; Fernley, *et al.*, 2000; Gowland *et al.*, 2002; Da Ros *et al.*, 2002; Harding *et al.*, 2003; Castro *et al.*, In Press) and to be relatively robust against environmental factors, such as salinity and temperature (Ringwood *et al.*, 1998; Nicholson, 2001). Harding *et al.* (2004) evaluated the use of the NR test as a response indicator in cultivated mussels. The objectives were to study mussel stress response in relation to post harvest conditions of handling, processing and storage practices (Harding *et al.*, 2004). Harding *et al.* (2004) found that neutral red retention was reduced in mussels that underwent washing and declumping as compared to unprocessed mussels. The use of the neutral red probe, to demonstrate lysosomal integrity, has been used on aquatic species such as dab *Limanda limanda* (Lowe *et al.*, 1992) and mussels *Mytilus edulis* (Lowe and Pipe, 1995). This technique has been used in a number of different studies on the haemolymph of marine invertebrates such as the mollusc *m edulis* (Lowe *et al.*, 1992; Lowe and Pipe, 1994; Moore *et al.*, 1996) and the common shore crab, *Carcinus maenas* (Wedderburn *et al.*, 1997). Apart from the fact that blood cells tend to be rich in lysosomes, the assay has the added advantage that there is no requirement for the animal under test to be sacrificed. This has ethical implications but also allows repeated, and long term, sampling of haemocytes. The simplicity of this

technique represents a significant departure from the complexity of more commonly used approaches to elucidate the lysosomal response, such as the histological sectioning and staining of tissue.

The neutral red method has been shown to have similar sensitivity to methods identifying lysosomal perturbation through the use of fluorescent molecular probes (Grøsvik *et al.*, 2000). The neutral red test has been shown to be more sensitive than the *N*-acetyl- β -hexosaminidase latency test for lysosomal fragility by Da Ros *et al* (2002) in mussels exposed to contaminated sites in the Venice Lagoon.

In only a few examples has the lysosomal fragility of haemocytes been assessed, using the neutral red method, in conjunction with other methods. Petrovic *et al* (2001) examined lysosomal response in relation to metallothionein levels; and Shepard and Bradley (2000) compared lysosomal response to protein expression signatures. Camus *et al* (2002) examined lysosomal response in tandem with a measurement of total oxyradical scavenging ability. Meiller and Bradley (2002) demonstrated a reduction in lysosomal stability in oysters exposed to zinc and an associated increase in specific protein signatures and faecal ribbons, a measure of physiological stress.

Variability of results when using the neutral red test could be induced by operator subjectivity, no mention is made of coding of samples in the literature which would remove the potential for bias. Further, the concentration of neutral red which is utilised will directly affect the retention time exhibited by the lysosomes. For example, Grundy *et al.* (1996) and Hwang *et al.* (2002) uses a different neutral red working solution than Lowe and Pipe (1994), Lowe *et al.*

(1995) and Harding *et al.* (2004). This could have consequences for the overall comparison of results between the different authors and could also affect data variability. Retention times, in control and exposed animals, are also shown to vary between different authors. Dailianis *et al.* (2003) reported extremely low retention times when compared to other authors such as Fernley *et al.* (2000) and Lowe *et al.* (1995). This may be due to differing protocol in processing the mussels; Dailianis (2003) immediately conducted testing whereas Fernley *et al.* (2000) and Lowe *et al.* (1995) implemented a recovery period after collection. Lower retention times, after handling and collection, are reported by Harding *et al.* (2004).

There are differences in the literature in how retention times are recorded. For example, Burlando *et al.* (2002) and Marchi *et al.* (2004) use a digital measurement of the ratio between cell volume and lysosomal volume to determine lysosomal stability after neutral red exposure. In contrast Fernley *et al.* (2000), Wedderburn *et al.* (1998), Lowe *et al.* (1992), Wedderburn *et al.*, (2000), Grundy *et al.* (1996) and Lowe and Pipe (1994) use operator recording methods.

No large scale comparative studies have been conducted examining differences in lysosomal retention of the neutral red probe between different species. Bekri and Pelletier (2004) examined the lysosomal responses of the polar seastar (*Leptasterias polaris*) to TBT and reported lysosomal destabilisation. However, without adequate baseline data it is difficult to compare these results with similar studies in other species and thus obtain any information on sensitivity. Domouhtsidou *et al.* (2004) examined lysosomal response, utilising a histological method, to examine digestive gland lysosomal response after exposure to a pollution gradient.

Physiological Response – Histopathological Biomarkers

Tissue and organ function and structure is an integration of the many biochemical, cellular, and physiological processes occurring within it, including any pathological disturbances to these processes (Moore *et al.*, 1994). Ultrastructural analysis of cells and tissues provides essential information on the pathological changes occurring in a variety of organelles which can be related to both biochemical changes at the cellular level and to tissue pathology (Klaunig *et al.*, 1979; Kohler, 1989; Moore, 1991).

Pathological alteration of mussel tissue, as a result of pollutant exposure, has been reported in a number of studies (Lowe *et al.*, 1981; Nott and Moore, 1987; Moore *et al.*, 1987; Lowe, 1988). Granular haemocytes (granulocytes) are phagocytic in nature (Lowe and Moore, 1979b; Carballal *et al.*, 1997) and their increased presence in mussels may be indicative of enhanced scavenging of damaged tissue. Increased vacuolation of bivalve digestive gland has been previously demonstrated after pollutant exposure (Lowe, 1988; Moore, 1988; Sarasquete *et al.*, 1992; Kela and Bowen, 1995); and Gold-Bouchot *et al.* (1995) showed a decline in connective tissue in *Crassostrea virginica* after exposure to cadmium. Bivalve epithelial thinning after pollutant exposure in bivalve molluscs has been shown in a number of studies (Lowe *et al.*, 1981; Moore *et al.*, 1987). This finding suggests a possible reduction in the ability of these animals to digest food material adequately and may therefore have consequences for general health and performance.

Histological examination of mussel tissue for symptoms such as digestive tubule atrophy has been shown to be a useful indicator of pollutant effect (Lowe, *et al.*, 1981; Lowe and Pipe 1986; Lowe 1988; and Moore, 1988).

Changes in mussel reproductive and storage tissue have also been investigated showing disturbance in lipid levels and internal structure after pollutant exposure (Bayne *et al.*, 1978; Rasmussen, 1982; Lowe and Pipe 1986, Lowe, 1988). Histopathological examination of bivalve digestive gland has also shown it to be a sensitive target for the injurious action of many pollutants (Lowe *et al.*, 1981; Moore *et al.*, 1987; Lowe, 1988; Syasina *et al.*, 1997). Alterations in secondary lysosome structure have also been observed, within digestive cells, which may be representative of enhanced autophagy of cytoplasmic contents (Lowe *et al.*, 1981).

Lowe (1988) observed alterations in mussel cell architecture by lipid accumulation and the formation of pathological enlarged heterogenous secondary lysosomes, following pollution exposure. Lowe and Pipe (1986) have also shown that exposure of mussels to hydrocarbons has a deleterious effect on gamete production and caused a reduction in nutrient storage cells of the mantle and gonad.

Physiological Response – Cardiac Activity Monitoring

Physiological responses should provide both an integration of the biochemical and cytological effects and an indication of the likely consequences of environmental change in a population (Bayne *et al.*, 1979). Variability in heart rate provides a sensitive measure of physiological competence in marine organisms such as crabs and mussels (Aagaard *et al.*, 1991; Aagaard, 1996; Depledge and Lundebye, 1996; Bamber and Depledge, 1997; Pirro *et al.*, 2001). Typically, heart rate is elevated in response to physical stress and this capacity for a rapid increase in respiration rate is presumably critical to the survival of crabs evading predation or environmental change (Bamber and Depledge, 1997). A range of standardised physiological assessments could

have a role to play in providing a clearer picture of the impact of chemicals in the environment (Bamber and Depledge, 1997).

Crustacea play an important part in the marine and aquatic environment, not only as a food source but also in the productivity of fisheries (Smith and Ratcliffe, 1978). The shore crab, *Carcinus maenas* is a convenient animal for study as it is readily available, easy to maintain in a laboratory aquarium culture and contains a large volume of cell-rich haemolymph.

Several physiological responses of molluscs and crustaceans have already been tested under laboratory and field conditions for their ability to detect environmental stressors (Akberali and Trueman, 1985; Baldwin and Kramer, 1994; Aagaard; *et al*, 1996; Lundebye and Depledge, 1998). Grace and Gainey (1987) demonstrated that 0.17ppm copper caused a 50% reduction in heart rate in *M. edulis*. Elevated heart rate in mussels is associated with periods of activity as indicated by valve gape, high rates of respiration, and filtering/pumping activity, whereas periods of inactivity are associated with low heart rate (Coleman, 1974). Heart rate data can thus provide useful information on the physiological status of the mussel (Baldwin and Kramer, 1994). Cardiac activity in mussels has typically been recorded using impedance cardiography (Trueman, 1967), which in itself can give rise to some degree of physiological disturbance. More recently, however, technological developments have permitted simultaneous recordings of cardiac activity from several invertebrates, for indefinite periods, without imposing undue stress (Depledge and Andersen, 1990). Physiological measurement of this type has been largely restricted to the study of crustaceans such as the shore crab (Bamber and Depledge, 1997). The use of invertebrate heart rate response in conjunction

with additional biomarkers has been addressed by a small number of authors (Depledge and Lundebye, 1996; Astley *et al.*, 1999).

1.4 Test Species

The use of animals living within an environment and the examination of the biological responses they exhibit to determine environmental health status is a well established approach. Given the behaviour of organisms, the nature of the marine environment and the behaviour of pollutants, both in the environment and in organisms, some marine species and trophic levels are more susceptible than others to environmental change (Stark, 1998).

As a result of this, key species have emerged as being ideally suited as 'sentinel' organisms for the measurement of pollutant effects. Many marine species have been selected for examination including, mussels, polychaetes, crabs, shrimps, pelagic and demersal fish and birds. As a sessile filter feeder the mussel has to filter large quantities of water and is thereby exposed to soluble and particle associated toxic substances (Granby and Splid, 1995). Mussels also have the attribute of being relatively tolerant, but not insensitive to, a wide range of environmental conditions (Gosling, 1992).

Because of this bioaccumulation of contaminants, extensive studies have been conducted to monitor levels of contaminants in the tissue of mussels in estuarine and coastal environments (Goldberg, 1975; Goldberg *et al.*, 1978; Phillips, 1980; Widdows *et al.*, 1982; Widdows and Donkin, 1992; O' Conner, 1992; Livingstone *et al.*, 2000)). However, the importance of including biological measurements, in concert with chemical measurements, has been emphasised (Bayne *et al.*, 1988; Bayne, 1989; Gray, 1992; Krishnakumar *et al.*,

1994; Widdows *et al.*, 1995) as providing greater information for the purposes of environmental management. Methods to determine pollutant-induced stress can include the examination of tissue concentration of pollutants, the perturbation of biological processes, and organism behaviour (Forbes and Forbes, 1994). Extensive studies have been undertaken to examine and determine the impact of a wide range of xenobiotics on various aspects of fish and invertebrate biochemistry, physiology, and population structure (Bayne *et al.*, 1988; Förlin *et al.*; 1995). These studies have been performed throughout Europe and the world to resolve various objectives, such as toxicity determination or environmental impact assessment (Lawrence and Hemingway, 2003).

***Mytilus edulis* (Common blue mussel)**

Mytilus edulis is of the phylum Mollusca (which includes snails, slugs and clams) and the class Pelacypoda (which are bivalves, also including clams, cockles, oysters and scallops). In the UK *Mytilus* has a very widespread distribution. Globally a number of different species are dominant in most intertidal and subtidal regions. *Mytilus edulis* is commonly found in dense populations on the rocky shores of open coasts, attached to surfaces by fibrous byssus threads, as well as dock pilings or off-shore structures. *Mytilus edulis* is not only a dominant member of coastal ecosystems but also has a commercial value, being farmed extensively. Commonly, specimens can vary in length from 2 – 10 cm. *Mytilus edulis* has a roughly triangular shell, usually blue or purple, which protects the soft internal tissues (Tyler-Walters, 2002).

Mytilus edulis has been widely studied, due to its extensive distribution, intertidal habit (which aids collection), ecological abundance and widespread

use as a biological indicator of environmental condition. Furthermore, it is relatively easy to maintain in the laboratory. For further general information on its biology, ecology and physiology the reader can refer to Bayne (1976) and Gosling (1992).

The major limiting factor in the distribution of *Mytilus*, other than the presence of sea water, is temperature (Stubbings, 1954). *Mytilus* is tolerant of temperatures in the range of -10°C (Williams, 1970) and +29 °C (Almada-Villela *et al.*, 1982). *Mytilus edulis* is tolerant of a wide range of salinities, with growth still possible in brackish conditions (Kautsky, 1982). Furthermore, *Mytilus edulis* is tolerant of a wide variety of environmental variables, including salinity but also oxygen tension as well as temperature and desiccation (Seed and Sucanek, 1992).

Mytilus edulis is a broadcast spawner, with peaks of spawning in spring and opportunistic spawnings when circumstances allow. Fecundity varies according to food supply (Thompson, 1979). Larval growth to metamorphosis, in 10 °C water can take between 2-4 weeks (Bayne *et al.*, 1975). Once settled, after the free swimming larval stage, larvae are known as spat. *Mytilus edulis* are relatively long lived and specimens have been reported as reaching 18-24 years (Thiesen, 1968). *Mytilus edulis* is capable of some small scale position readjustment although, for all intents and purposes, is sedentary. *Mytilus edulis* is a filter feeder capable of removing particles down to the 2-3µm range with an efficiency in excess of 80% (Mohlenberg and Riisgård, 1977). Mussels can pump approximately 4 litres of water an hour (Fisheries and Oceans, Canada 2003). Primary food sources are organic and bacterial material, adhered to particles or resuspended material. However, there are at least three species of

Mytilus and variability in response between these may confuse monitoring programmes.

The use of *Mytilus edulis* in biological monitoring programmes, and in the study here can be summarized as:

- Widespread and commonplace (global distribution)
- Easily collected in large numbers
- Sedentary (after a free swimming larval stage)
- Filter feeders, thus passing large quantities of water over surfaces
- Accumulate pollutants continuously
- Ecologically important member of the coastal zone
- Clearly identifiable stress responses
- Well characterized biology

***Carcinus maenas* (Common shore crab)**

Carcinus maenas is of the phylum *Crustacea* (which also includes barnacles, shrimps and other crabs) and of the class *Eumalacostraca* (which includes crabs, shrimps and lobsters). It is extremely common, and found on all shores of Britain and Ireland. *Carcinus maenas* is primarily a shore and shallow water species. It tolerates a wide range of salinities and is especially abundant in estuaries and salt marshes (Neal and Pizzolla, 2005). The common shore crab has a shell up to 8cm across with the first two legs possessing a well developed set of pincers (Neal and Pizzolla, 2005).

Carcinus females lay eggs which are attached to abdominal appendages, after hatching they become *zoea* larvae which moult to become *megalopa* larvae

(Little, 1996). These larvae sink to the bottom and metamorphose into miniature versions of the adult, moulting can occur many times and shore crabs can become as large as 86mm across the carapace (Little, 1996).

Carcinus predate on a variety of intertidal invertebrates but specializes in mussels (*Mytilus*) and dog whelks (*Nucella*). *Carcinus* moves freely around the shoreline, moving up and down with the rising and falling tide (Little, 1996). *Carcinus maenas* exhibits intrinsic internal rhythms responding to diurnal and tidal patterns (Little, 1996).

The rationale for the use of *Carcinus maenas* can be summarized as:

- Active predator of *Mytilus edulis*.
- Important member of the coastal zone
- Widely distributed
- Clearly identifiable responses to stress
- Well characterized biology
- Role in environmental monitoring needs to be further validated

1.5 Summary and prospectus

Biomarker studies have been implemented in a large number of different locations and organisms; and have utilised a battery of tests to identify pollutant exposure and effect. Biomarkers have been used at different levels of biological organisation and have certainly proved capable of identifying symptoms related to pollutant effect. There is, however, still a lack of information regarding the longer-term consequences of the expression of biomarkers within an organism and the effect this may have on an ecosystem. Distress signals from different levels of organisation within an animal should be capable of providing early warning prognostic biomarkers of reduced

performance, impending pathology and damage to health (Depledge *et al.*, 1993; McCarthy and Shugart, 1990; Moore *et al.*, 2002). For example, scope for growth indicates a reduction in fitness and adverse effects on gametogenesis (Bayne *et al.*, 1978; Lowe *et al.*, 1982)

The consequences of biomarker activity for biological fitness has not yet been fully established and a greater understanding of the relationship between 'early warning' biomarkers and more serious consequences at the population and community level is required (Goksøyr *et al.*, 1996; Parrett, 1998; Kenny *et al.*, 2002).

A large number of studies have only examined one biomarker in isolation and generally only in one species, rather than considering a multiple biomarker approach in a range of different species. Future efforts must focus on a more integrated approach to the validation of biomarkers that are, ultimately, prognostic for population and community endpoints (Depledge *et al.*, 1993; Moore *et al.*, 1994). By examining a number of biomarkers together it can become possible to identify associated responses and a greater understanding of pollutant effect. For example, the research to date on the lysosomal response in mussel digestive cells has shown it to be related linearly to scope for growth, a good indicator for general animal health (Allen and Moore, 2004). By integrating such robust biomarker data with higher level measures of ecosystem change it will become possible to use biomarkers as direct measures for evaluating ecosystem health (Allen and Moore, 2004). Such an approach requires a better understanding of particular biomarkers, as they relate to health status, in order to improve their interpretative value in monitoring (Moore, and Simpson, 1992; Moore *et al.*, 1994); but with the caveat

that biomarkers must be rapid and robust as a tool for environmental assessment (Depledge *et al.*, 1993; Moore and Simpson, 1992; Moore *et al.*, 1994). Finally, there is a continuing need within the global community for more guidance for the deployment of ecotoxicological approaches (Wells *et al.*, 2001).

1.6 Aims and Objectives

One of the crucial elements that techniques used in environmental monitoring studies require is the ability to be routinely applied in the field and to provide relevant data that relates to ecologically significant processes. Much of the past biomarker work has been restricted to the laboratory or has relied on the application of only one, or a few techniques, which do not provide a linkage to higher level effects. The determination of causal relationship is, of course, crucial to the understanding of pollutant effect; but it does not provide all the necessary data for making informed decisions regarding bio-monitoring activities and the interpretation of data.

The main objective of this work is to examine the biomarker approach, for the purposes of biomonitoring, and to explore its use as an *in situ* tool for establishing environmental quality. By examining the results of multiple biomarker tests used in the field, and laboratory, the efficacy of the chosen biomarkers can be assessed. An emphasis has been placed on attempting to link techniques between levels of biological organisation and, thus, provide data that can then be used to predict the ecological significance of pollutant exposure and effect. The following aims will be specifically addressed:

- Further validate the use of the Neutral Red Test in *Mytilus edulis*
- Examine the use of the Neutral Red Test in *Carcinus maenas*

- Utilise the Neutral Red Test in conjunction with other biomarkers
- Explore any linkages between lysosomal perturbation and higher order effects
- Validate the use of the Neutral Red Test for environmental monitoring

By examining techniques in this context and by using them on established, as well as previously unexamined, test organisms in a wide range of field and laboratory scenarios, valuable information will be gained on the application of the biomarker approach for real world bio-monitoring.

2.0 METHODS AND MATERIALS.

2.1 Neutral Red Test

The ability of cells to take up and retain the cationic vital dye, neutral red, has been used in several studies as a measure of cytotoxicity (Finter, 1969; Babich and Borenfreund, 1987, 1988; Dierickx and Van De Vijver, 1991). More recent work by Lowe *et al.* (1992) demonstrated the use of the neutral red assay as an indicator of contaminant exposure in fish. Further work developed the technique for use on invertebrate cells (Lowe and Pipe, 1994; Lowe *et al.*, 1995, 1995a, 1995b).

2.1.1 Neutral Red Test – *Mytilus edulis*

The procedure used here is that developed by Lowe and Pipe (1994). To maintain live molluscan haemocytes it was necessary to prepare a physiological saline (20 mM HEPES, 436 mM NaCl, 53 mM MgSO₄, 10 mM KCL, 10 mM CaCL₂) (Lowe, *et al* 1995). This was adjusted to a pH 7.3 with 1 M NaOH. The neutral red stock was prepared by dissolving 20mg of neutral red in 1ml of DMSO, 10µl of the stock solution was then made up to 5ml with the physiological saline for the working concentration. The mussel shell was prised partially open and held in position while 0.5 ml of haemolymph was withdrawn from the anterior adductor muscle into a 2.5 ml syringe, fitted with a 25 gauge needle, containing 0.5 ml of a mussel physiological saline.

In order to reduce shearing forces the needle was first removed before the contents of the syringe were ejected into a 2 ml siliconised Eppendorf.

The neutral red stock solution was made by dissolving 20 mg of dye (C. I. 50040, Sigma) in 1 ml of DMSO (dimethyl sulphoxide). The working solution

was prepared by diluting 10 μl of the stock solution with 5 ml of the mussel physiological saline. A 50 μl aliquot of the cell suspension was dispensed onto a 76 x 26 mm microscope slide and placed into a light proof humidity chamber for 15 minutes to allow the cells to attach. Excess solution was then tipped off, 40 μl of the neutral red working solution added, then a 18 x 18 mm coverslip placed on the slide.

After a further 15 minutes incubation in the humidity chamber the preparations were removed and inspected under a microscope. The preparations were examined again 15 minutes later and systematically, thereafter, at 30 minute intervals to determine at what point in time there was evidence of dye loss from the lysosomes to the cytosol. The test for each slide was terminated when dye loss was evident in 50 % (numerically assessed within each field of view) of the granular haemocytes and the time was recorded when this occurred. The mean retention time was then calculated for each sample set.

2.1.2 Neutral Red Test - Crabs

The technique described here is adapted from Lowe, *et al* (1995a) from the technique developed on mussels. . Haemolymph (100 μL) was extracted from the arthrodal membrane using a Drummond pipette and mixed with 100 μL of anticoagulant (0.45 M NaCL, 0.1 M glucose, 30 mM trisodium citrate, 23 mM citric acid, 10 mM EDTA, pH 4.6; Smith and Ratcliffe, 1978). 40 μL of this mixture was then placed on a slide. Physiological saline (0.5 M NaCL, 11 mM KCL, 12 mM $\text{Ca}_2 \cdot 6\text{H}_2\text{O}$, 26 mM $\text{MgCL}_2 \cdot 6\text{H}_2\text{O}$, 45 mM Tris, 0.1 M HCL (38ml l^{-1}), pH 7.4; Smith and Ratcliffe, 1978) was added and cells incubated for 15 minutes at 10⁰C. Excess was removed and 40 μL of the dye working solution was added. Cells were scored according to Lowe, *et al* (1992).

2.1.3 Neutral Red Test – Freshwater Mussel

The technique described here is an adaptation of that previously described by Lowe, *et al* (1995). The physiological saline (NaCl 0.936 g, KCL 0.019 g, CaCL₂ 1.0 g, Mg Cl₂ 0.07 g, per litre of distilled water) used to maintain the haemolymph was formulated for freshwater mollusca. The test for each sample was terminated when dye loss was evident in over 50% of the granular haemocytes. The slides were examined blind, the animals being coded to prevent the operator from having prior knowledge of the animals exposure. The bivalves were briefly removed from their tanks for bleeding, before being replaced. Due to the size of the shellfish and the comparative ease in locating the adductor muscle successful bleeding can take place in a time period of ten seconds.

2.2 Cardiac Activity Monitoring

Heart rates were recorded using the CAPMON system developed by Depledge and Anderson (1990). Non-invasive, light weight infra red emitter/detectors were used to transduce heart beat by attachment to the shell directly above the pericardial cavity. During the heart's cycle of action, the amount of infra-red light reflected back to the detector allows the organism's heart rate to be monitored. The detectors are interfaced with a personal computer. This recording system permitted 16 animals to be monitored simultaneously. Infra-red transducers were glued (Loctite 314) directly to the shell, on the mid-dorsal line just behind the posterior termination of the hinge. The infra-red transducers monitored the heart's cycle of action and heart rate was determined every minute and stored on disk for later analysis. The emitter/detectors are housed

on a collar on the animal which was stuck down with adhesive and then carefully removed later, without causing shell damage.

2.3 Metallothionein Analysis

Quantification of metallothionein (MT) was achieved using the indirect method described by Pederson and Lundebye (1996) and Viarengo *et al.* (1997).. Frozen whole tissue was placed in a mortar, filled with liquid nitrogen and ground to fine consistency. Approximately 1 g of sample was transferred to a 10ml beaker, cold distilled water containing 1mM DTT (dithiothreitol) and 0.1mM PMSF (phenylmethylsulfonylfluoride) was added. The suspension was sonicated and centrifuged for 70 min at 55,000 rpm at 4°C.

The supernatant was prepared for a direct method for MT quantification (using spectrophotometry) as described by Dieter *et al.*, (1987) and Viarengo *et al.*, (1997). A two step ethanol/ chloroform precipitation of the supernatant (with centrifugation) was performed and the resulting pellet was resuspended in 5mM Tris-HCl, 1mM EDTA buffer, pH 7, followed by reaction with DTNB (5,5-dithiobis-2-nitrobenzoic acid) as described by Viarengo *et al.* (1995). Sample absorbency was read at 412nm in a UNICAM UV/VIS spectrophotometer, and the MT concentration was quantified using glutathione (GSH) as a reference standard (Viarengo *et al.*, 1995). Statgraphics 5.0 was used to test differences between sites using one-way ANOVA and multiple range tests (LSD). Bartlett's test was applied to check the homogeneity of the variance.

2.4 Measurement of Osmoregulatory Ability

Measurement of osmoregulatory ability was performed as described in Bamber and Depledge (1997). However, after haemolymph samples were taken crabs were transferred to tanks containing distilled water. Samples were taken from

each crab three hours later and frozen in liquid nitrogen. Osmolality was measured by osmometer (Wescor 5500) requiring 10 μ l of sample per evaluation.

Site differences were examined by Kruskal-Wallis 1-way analysis of variance and multiple range tests on means (Fishers LSD procedure).

2.5 Rapid Source Inventory Technique

National reports were prepared by the littoral states involved in the Black Sea Working Party for Routine Pollution Monitoring. The studies were carried out in accordance with the World Health Organisation's (WHO) Guideline "Assessment of Sources of Air, Water, and Land Pollution/A guide to Rapid Source Inventory Techniques and their Use in Formulating Environmental Control Strategies. Part One: Rapid Inventory Techniques in Environmental Pollution, 1993". The scope of the study was limited to the point sources of wastewater discharges including domestic as well as industrial discharges. The inputs through rivers were also included. Solid wastes directly or indirectly discharged or dumped into the Black Sea were also surveyed and included in this study. Data was collected and compiled by Sarikaya *et al.* (1997) through extensive surveying of the littoral states through the use of detailed questionnaires for the assessment of domestic and industrial sources.

2.6 Tissue Analysis

Pesticides and PCBs were analysed by gas chromatography on a MEGA2 8560 with a FISIONS capillary column and ECD. The calibration for this was conducted using pesticides and PCB standards SE-52 MESL IAEA. The analysis was performed in accordance with "Measurements of organochlorine and petroleum hydrocarbons in environmental samples" IAEA-MEL/MESL

(Villeneuve, 1995) by participating laboratories in the UNESCO/IOC funded Black Sea Mussel Watch.

2.7 Histopathology

Each mussel was uniquely identified, shell length measured, dissected and tissue preserved in 4°C Baker's formol calcium fixative. Mussels were dissected to provide a mid-cross section through the digestive gland, gill, gonad and kidney. The foot and associated musculature was removed prior to fixation. After 24 h fixation, tissues were dehydrated through an alcohol series before embedding in paraffin wax. Wax sections (7µm thick) were cut and stained in Papanicolaou. Relative pathologies were determined using a semi-quantitative method where specific abnormalities (gonad maturity, presence of adipogranular cells, oocyte atresia, granulocytoma, granulocyte number; digestive tubule vacuolation, luminal integrity, duct epithelium, phasic activity, and the presence of brown cells) were scored on a scale of 1 – 5, lower numbers indicating the increased presence or relative severity of the particular pathology.

Slides were generally coded, prior to observation, to remove any opportunity for operator bias.

2.8 Animal Husbandry

Mussels were collected at low tide from Whitsand Bay in Cornwall, South West England, a relatively clean reference site in a size range of 4.0 - 5.0 cm. Obviously damaged mussels were discarded and scissors were used to cut the mussel free from the rock, to avoid damaging the mussels' internal tissue. Mussels were allowed to equilibrate in the laboratory for 24 hours + before experiments began. Mussels were held in UV-irradiated recirculating seawater

at a constant temperature of 12°C. Mussels were placed in 20 L aerated static tanks and fed a diet of algae. Mussels were fed on alternate days on a diet of algae. Water changes were conducted on a 24 hour cycle after any bleeding for neutral red testing. Mussels that were observed to be spawning, identified by the release of gametes, were discarded.

Intermoult male crabs were collected from the Yealm estuary using baited drop nets. Crabs were held in the laboratory for two days, prior to use, in recirculating filtered seawater. Crabs were fed and exposed to a 12 hour light/dark cycle. Crabs were fed on alternate days and care taken to avoid overfeeding and fighting between crabs. Crabs were placed in 20 l closed aerated tanks and fed strips of mackerel. Animals were bled before feeding occurred. Water changes were conducted on a 24 hour cycle after bleeding for neutral red testing.

3.0 DOSE RESPONSE AND METHOD SENSITIVITY

3.1 ABSTRACT

To gain an understanding of the relative sensitivity of the different biomarker techniques being used in this study a range of laboratory studies was undertaken to examine biological response and obtain dose response information. This will be useful to aid in the interpretation and validation of future field-derived data. *Mytilus edulis*, the blue mussel, and *Carcinus maenas*, the shore crab, were collected from clean reference sites and exposed in a range of laboratory based experiments to a variety of common environmental contaminants. Statistically robust data were obtained ($p < 0.05$, $n \geq 10$) showing dose related differences. The neutral red technique proved capable of demonstrating biological response. A sperm viability assay and the use of image analysis for quantifying lysosomal dysfunction proved difficult to apply for technical reasons.

3.2 Lysosomal response of *Mytilus edulis*, after pollutant exposure, using the neutral red technique

3.2.1 Introduction/Aims

The marine mussel is commonly used as a sentinel organism in laboratory and field tests. The neutral red test has been previously identified as a measure of lysosomal dysfunction. To further examine and validate the value of this test as a measure of pollutant induced damage, mussels were exposed to common environmental pollutants at a range of concentrations. By being able to identify toxicological response, and determine differential response to different doses it will be possible to better interpret environmental data. Further, given that the neutral red test can identify morphological change within the lysosomal system of the cell, it may be possible to observe responses that are linked to specific pollutants or doses.

3.2.3 Method

The neutral red test was conducted as described in section 2.1.1.

Mussels were exposed to copper sulphate (CuSO_4) (Sigma Chemicals), benzo [a] pyrene (BaP) (Sigma Chemicals), and a known disrupter of lysosomal function, chlorpromazine. (Sigma Chemicals) Exposure periods were conducted for 2 and 6 day periods.

2 Day Exposure	6 Day Exposure
D	Day 0: Dose
Day 1	Day 1
Day 2 Bleed	Day 2: Bleed
	Day 3
	Day 4: Dose/ Change
	Day 5
	Day 6: Bleed

Table 2. Table showing dosing and water change regime for each exposure regime.

The 2 and 6 day exposure experiments were conducted on different dates. Exposure tanks were prepared in triplicate, with carrier vehicle controls also being utilised. An unexposed control was utilised. Given the time dependent nature of the neutral red test it was impractical to analyse all the mussels immediately. Three assays were conducted, one immediately after the other, each using a sub-set of mussels from each tank.

Mussels were not fed during the exposure period and water changes and dosing were conducted every two days. Mussels were held in 20 L of seawater with 12 mussels to a tank. Replicate exposures and controls were utilised. In each case 3 doses were investigated 1 mg l^{-1} , 0.5 mg l^{-1} and 0.1 mg l^{-1} . Stock Cu and chlorpromazine were dissolved, at the appropriate concentration, in to a 500ml aliquot of seawater from the tank before addition. DMSO was used as a solvent for BaP. Tanks were aerated throughout the exposure period.

After haemolymph extraction slide preparations were prepared independently of the operator and coded in such a way as to prevent prior knowledge of the exposure regime. Given the semi-quantitative nature of this test, this is vital to remove operator bias that could confound experiment design and subsequent data analysis. The neutral red test was conducted as described in Section 2.1.1.

3.2.4 Results – 2 Day Exposure

BaP Exposure – 2 Day Exposure

The 1 mg l⁻¹ exposure group showed a significantly reduced retention time for the neutral red probe compared to the other exposure groups and the controls ($p < 0.05$, $n=17$; fig. 1). There was no significant difference between the controls. All the exposure groups showed significantly reduced retention times to the neutral red probe compared to the controls.

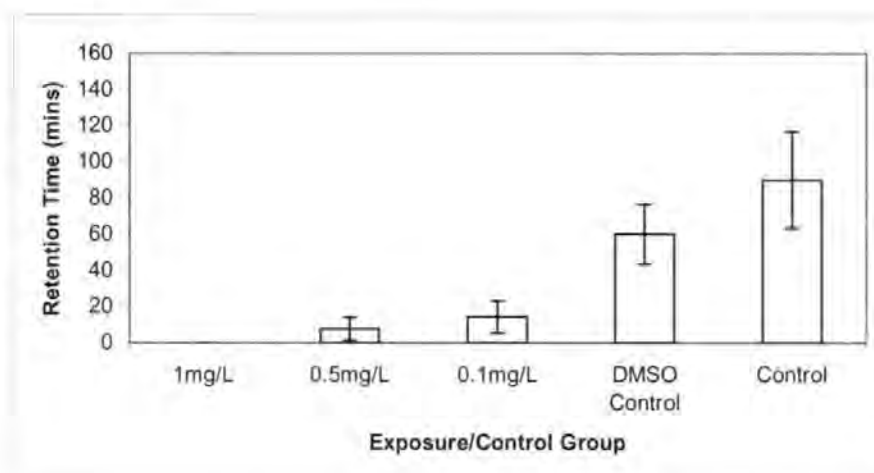


Figure 1. Neutral red retention times for mussels exposed to BaP for a two-day period. Bars represent the 95% CI, significant differences exist between the exposure groups and the control.

Copper Exposure – 2 Day Exposure

There were no significant differences in lysosomal retention of the neutral red probe between any of the exposure groups ($p > 0.05$, $n = 17$; fig 2). The exposure groups all showed reduced lysosomal retention compared to the controls ($p < 0.05$, $n=17$).

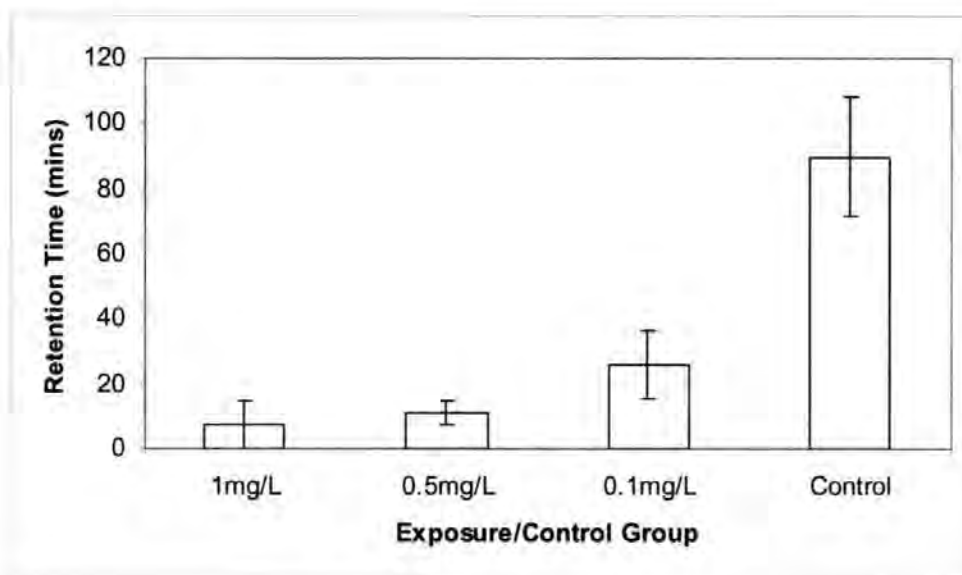


Figure 2. Neutral red retention times for mussels exposed to CuSO_4 for a two-day period. Bars represent the 95% CI, significant differences exist between the exposure groups and the control.

Chlorpromazine – 2 Day Exposure

The neutral red retention was significantly reduced in the 1 mg l^{-1} exposure group compared to the other exposures ($p < 0.05$, $n=17$; fig 3). The exposure groups all exhibited significantly reduced retention times compared to the control ($p < 0.05$, $n=17$). There was no significant difference between the 0.5 mg l^{-1} and 0.1 mg l^{-1} exposure groups.

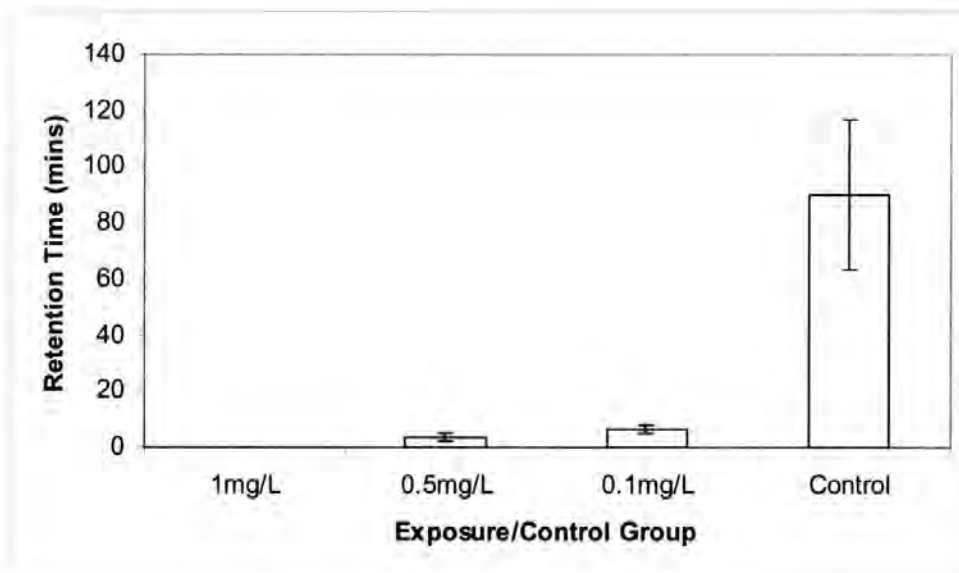


Figure 3. Neutral red retention times for mussels exposed to chlorpromazine for a two-day period. Bars represent the 95% CI, significant differences exist between the exposure groups and the control.

3.2.5 Results - 6 Day Exposure

BaP Exposure – 6 Day Exposure

Significant differences were shown, on the day 2 sampling, between the different doses, and between the doses and the controls. The mussels exposed to the 1 mg l⁻¹ dose had a significantly reduced retention time compared to the mussels exposed to the 0.5 and 0.1 mg l⁻¹ doses ($p < 0.05$, $n=18$; fig. 4). There was no significant difference between the 0.5 and 0.1 mg l⁻¹ doses ($p > 0.05$, $n=18$). The exposure groups were all significantly reduced compared to the control groups ($p < 0.05$, $n=18$). There was no significant difference between the carrier control and the seawater control ($p < 0.05$, $n=18$). The retention time of the mussels exposed to 0.1 mg l⁻¹ was shown to increase at day 6 compared to day 2 ($p < 0.05$, $n=18$).

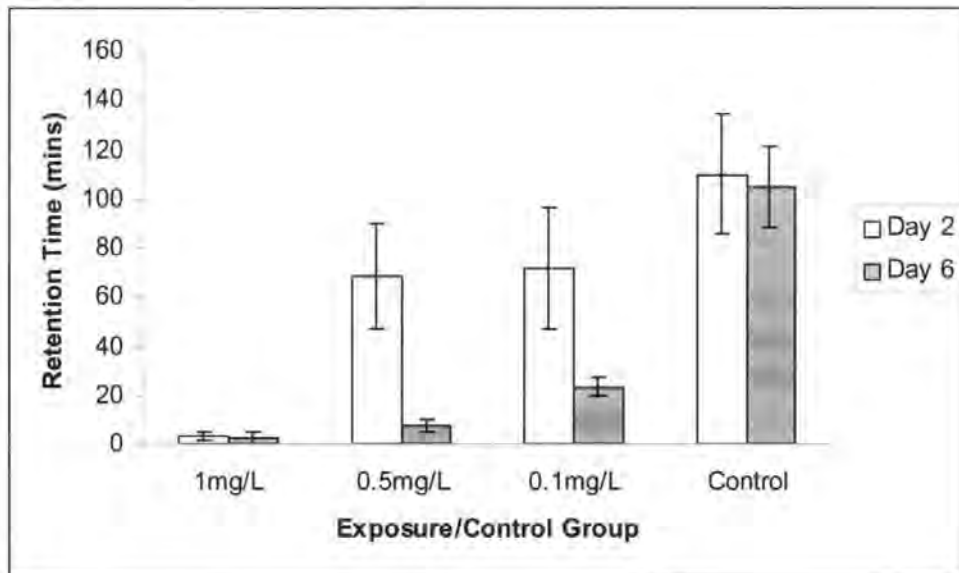


Figure 4. Graph showing neutral red retention in mussel haemocytes at days 2 and 6 during a 6 day exposure to BaP. Significant differences exist between groups, the 95% CI is represented by the error bars.

Copper Exposure – 6 Day Exposure

The mussels exposed to $1 \text{ mg l}^{-1} \text{ CuSO}_4$ were shown to have significantly reduced retention times for the neutral red dye compared to the other doses and the control ($p < 0.05$, $n=18$; fig 5). At day 2 there was no significant difference between the 0.5 mg l^{-1} and the 0.1 mg l^{-1} exposure groups and the control ($p > 0.05$, $n=18$). At day 6 there were significant differences between all the exposure groups and the controls ($p < 0.05$, $n=18$). The retention time of mussel haemocytic lysosomes exposed to 0.1 mg l^{-1} was significantly greater at the 6 day period compared to the other exposure groups ($p < 0.05$, $n=18$). There was a significant reduction in lysosomal retention time at day 6, compared to day 2 ($p < 0.05$, $n=18$).

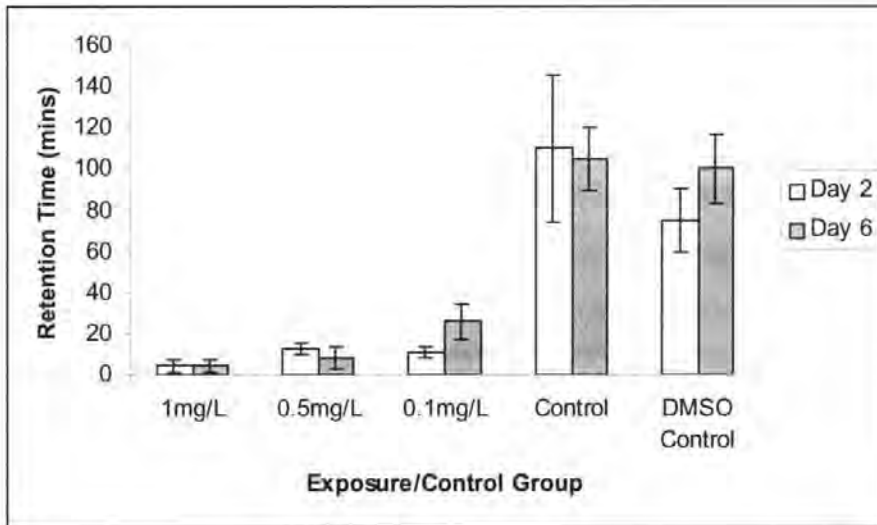


Figure 5. Graph showing neutral red retention in mussel haemocytes at days 2 and 6 during a 6 day exposure to CuSO_4 . Significant difference exist between groups, the 95% CI is represented by the error bars.

Chlorpromazine Exposure – 6 Day Exposure

Neutral red retention was shown to be significantly difference between the exposure groups ($p < 0.05$, $n=18$; fig 6). Mussels exposed to the 1 mg l^{-1} concentration were shown to have significantly reduced retention times compared to the 0.5 and 0.1 mg l^{-1} exposure groups. There was a significant reduction in retention time at day 6 compared to day 2 ($p < 0.05$, $n=18$).

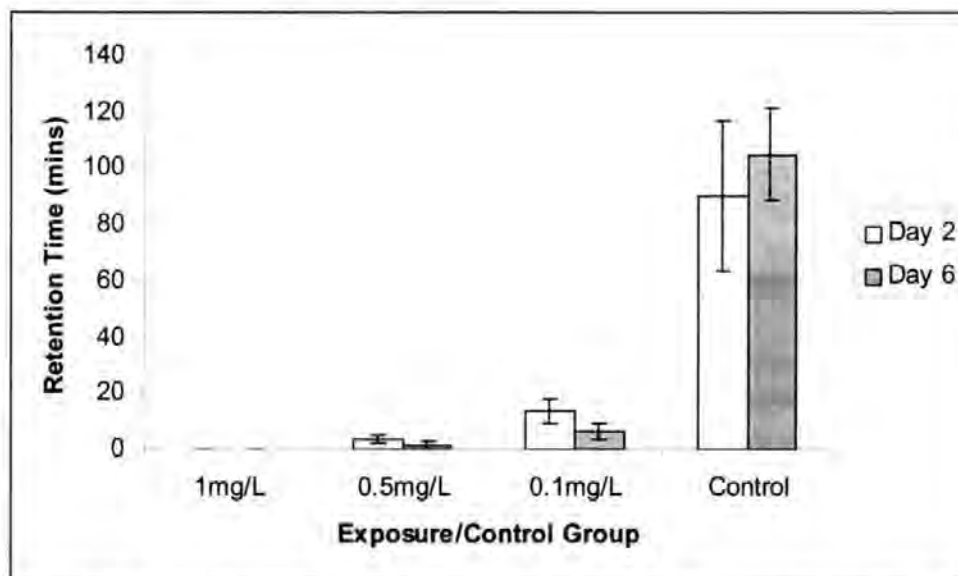


Figure 6. Graph showing neutral red retention in mussel haemocytes at days 2 and 6 during a 6 day exposure to chlorpromazine. Significant differences exist between groups, the 95% CI is represented by the error bars.

3.3 Dose response of *Carcinus maenas*, after exposure to common environmental pollutants using the neutral red test for lysosomal dysfunction.

3.3.1 Introduction/Aims

The lysosomal component of molluscan haemolymph has been demonstrated to have value in the interpretation of the consequences of environmental pollution but there has been little extension of this technique to other organisms. If lysosomal response can be demonstrated in *C. maenas* this presents a further opportunity to elucidate environmental responses in a potential sentinel animal. Further, given the greater range of physiological and behavioural responses which *C. maenas* exhibits it could be possible to further develop mechanistic linkages of pollutant effect by using this test in conjunction with other biomarkers. This work was designed to identify the presence and location of lysosomes within crab blood cells, develop a protocol for haemocyte removal and then examine lysosomal response to pollutant dose.

3.3.2 Localisation of lysosomes in crab blood cells (haemocytes)

3.3.3 Method

C. maenas haemolymph was extracted using a Drummond pipette from the arthrocoxal membrane between the leg and shell. The membrane was pricked with a needle and the pipette then placed over the wound. Haemolymph was withdrawn steadily, the aim being to prevent any air bubbles in the pipette.

In the absence of any form of anticoagulant or physiological solution immediate clotting was noticed and cell preparations could not be adequately prepared. The anticoagulant and physiological saline described in section 2.1.2 were utilised to improve the capture and recovery of *C. maenas* haemolymph.

A 50:50 ratio of the haemolymph to the crab anticoagulant was attempted, within the Drummond pipette. After removal haemolymph was placed into an Eppendorf tube. Physiological saline was then added at a 50:50 ratio, and the Eppendorf tube held on ice to reduce any reduce cellular activity and prevent the risk of clotting. A pipette was used to extract 50 µl of haemolymph solution and placed on a microscope slide. Slides were treated with poly-L-lysine solution to improve cell adhesion.

The fluorescent probes BODIPY-FL-verapamil (BFLV) and acridine orange were used to verify the presence and location of lysosomes within the *C. maenas* haemolymph. 1 µl of BFLV was added to 500 µl of *C. maenas* physiological saline. The Bodipy fluorophore has spectral characteristics similar to those of fluorescein. After an exposure period of 15 minutes in a darkened humid incubation chamber at 10°C slides were examined using fluorescence microscopy. Acridine orange was prepared in *C. maenas* physiological saline

(1 μ l/ 500ml) and slides treated in the same manner. Slides were examined at 15 minute intervals.

3.3.4 Results

Molecular Probe/Concentration	Time: 15 mins	Time: 60 mins
BFLV 1ul/500ml	Granular bodies visible	Granular bodies visible
Neutral Red	Granular bodies visible	Granular bodies visible/cytosolic staining
Acridine Orange 1ul/500ml	Granular bodies visible	Granular bodies visible

Table 3. Table showing the staining patterns of three molecular probes, BODIPY-FL verapamil, neutral red, and acridine orange, after a 15 and 60 minute incubation.

Granular spherical bodies were clearly visible in both treatments. Quenching of the fluorescence response was observed after approximately 200 minutes; and different cell types could be observed on the basis of presence and absence of granular bodies. BFLV was observed to give a clear response with little background fluorescent. Acridine orange treated slides had a greater level of background fluorescence.

Granulocytic cells were observed containing numerous (>100) stained organelles. A smaller population of cells were observed commonly containing a smaller number of stained organelles (<20) and being of smaller size. Neutral red was observed to locate in the same organelles as BFLV and there was little interference with the fluorescent signal. Neutral red was observed to leak into the cellular cytosol after approximately 60-90 minutes. The presence of neutral red and BFLV within the same organelles was verified by the exclusion of fluorescent light to the slide followed by examination with light microscopy.

3.4 Crab haemocyte lysosomal response after pollutant exposure

3.4.1 Introduction/Aims

If the lysosomal response of *C. maenas* haemolymph is to have a use as a biomarker of pollutant induced effect it is necessary to determine the sensitivity of the cellular response to pollutant exposure. The aim of this work was to expose *C. maenas* haemolymph to a known cellular toxicant to determine if there is any measurable lysosomal response.

3.4.2 Method

Crabs were obtained and maintained as described in section 2.8.

Blood was extracted according to the protocol given in Section 2.1.2. The physiological saline and anticoagulant was prepared according to the method given above in section 2.1.2. Three crabs were bled and haemolymph aliquoted to enable incubation at each of the concentrations under test.

Haemolymph solution was added to 1 ml Eppendorf tubes containing 500 μl of physiological saline containing 10 mg L^{-1} , 1 mg l^{-1} , and 0.5 mg L^{-1} concentration of chlorpromazine. The haemolymph was incubated at 10°C for 15 minutes. 25 μl of haemolymph was removed and placed onto a poly-L-lysine treated microscope slide. 25 μl of neutral red solution was then added. Slides were allowed to incubate for a further 15 minutes in a darkened incubation chamber at 10°C. Slides were then examined immediately and then at 15 minute intervals thereafter. Untreated controls were utilised and slides coded to remove any opportunity for operator bias.

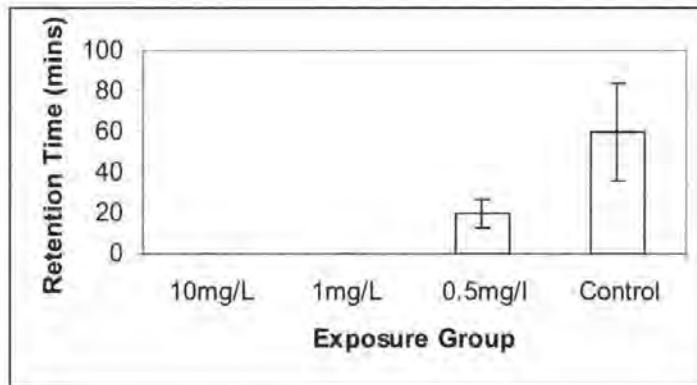


Fig 7. Mean data of lysosomal retention time for the neutral red probe in *C. maenas* haemolymph following 15 minute exposure to chlorpromazine at 10, 1, and 0.5 mg l⁻¹ concentration. Cells were completely disrupted in the 10 and 1 mg l⁻¹ exposure groups. There was a significant difference between the 0.5 mg l⁻¹ exposure group and the control ($p < 0.05$, $n=15$). Bars represent the 95% CI.

3.4.3 Results

Using a basic pollutant exposure it proved possible to quantify crab haemocytic lysosomal response using the neutral red probe. The amphiphilic cation chlorpromazine induced significant differences in lysosomal retention time for the neutral red probe ($p < 0.05$, $n=15$; fig. 7).

3.5 *C. maenas* and *M. edulis* lysosomal response after pollutant exposure

3.5.1 Introduction/Aims

Carcinus maenas has not been previously used in conjunction with lysosomal integrity tests. By examining the responses of crab haemolymph after exposure to environmental contaminants it will be possible to determine the possible use of *C. maenas* haemolymph lysosomes as tool for environmental testing, in a similar way to that already suggested for *M. edulis*.

The aim of this work was to determine the applicability of the neutral red test on crab haemolymph and examine any differences in sensitivity between crab and mussel lysosomal response.

3.5.2 Method

Mussels and crabs were obtained and maintained as described in section 2.8.

Neutral red tests were conducted as described in 2.1.2 and 2.1.3.

Exposures were undertaken using Cu, BaP, and chlorpromazine. Crab haemolymph was exposed to 10 mg l⁻¹, 1 mg l⁻¹, and 0.5 mg l⁻¹ for 30 minute incubation periods before beginning the neutral red test (as described earlier). Cu SO₄ and chlorpromazine stock were prepared in the appropriate physiological saline before use. Benzo-[a]-pyrene (BaP) stock was prepared in ethanol. Serial dilution was utilised to obtain the appropriate concentration for the working solution. Haemolymph was extracted according to the protocols described in Section 2. Immediately after haemolymph removal, exposures were started in 1 ml Eppendorfs, held on ice, in darkened conditions. Haemolymph was collected from 5 crabs and then each sample of blood exposed used in the 10, 5, and 1 mg l⁻¹ exposure groups. Exposures were conducted for 30 minutes.

3.5.3 Results

All chemicals under test, at the highest dose, caused an immediate breakdown of the lysosomal membrane. The endpoint of the neutral red test, i.e. complete cytosolic staining, was observed immediately at 10mg l⁻¹ in all cases (figs. 8 and 9). There was no significant difference between 5 and 1 mg l⁻¹ with any of the chemicals. There was a highly significant difference between the exposure groups and the control groups. There were also significant differences between the *C.maenas* and *M. edulis* control groups (fig. 8).

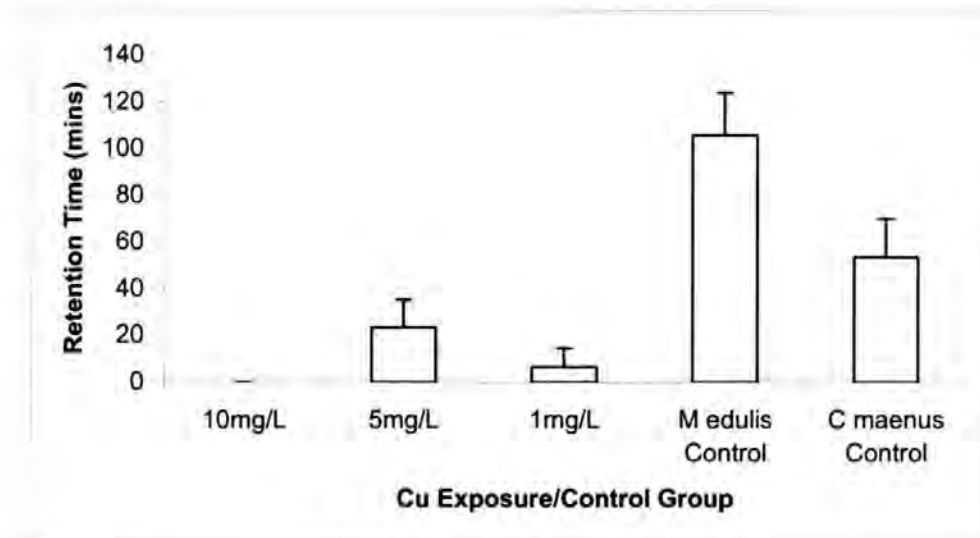


Figure 8. Graph showing dose response of *C. maenas* haemolymph after exposure to 10, 5, and 1mg l⁻¹ Cu. A control of unexposed *C. maenas* cells was also examined. *M. edulis* haemolymph cells were also examined for comparative purposes. There is no significant difference between doses ($p < 0.05$, $n = 5$ per exposure group) 5 and 1. No intact lysosomes were observed in the highest dose. There is a significant difference between exposure groups and the control groups ($p < 0.05$, $n = 5$ per exposure group). There is a significant difference between *M. edulis* and *C. maenas* control groups ($p < 0.05$, $n = 5$ per exposure group). Bars represent 95% CI.

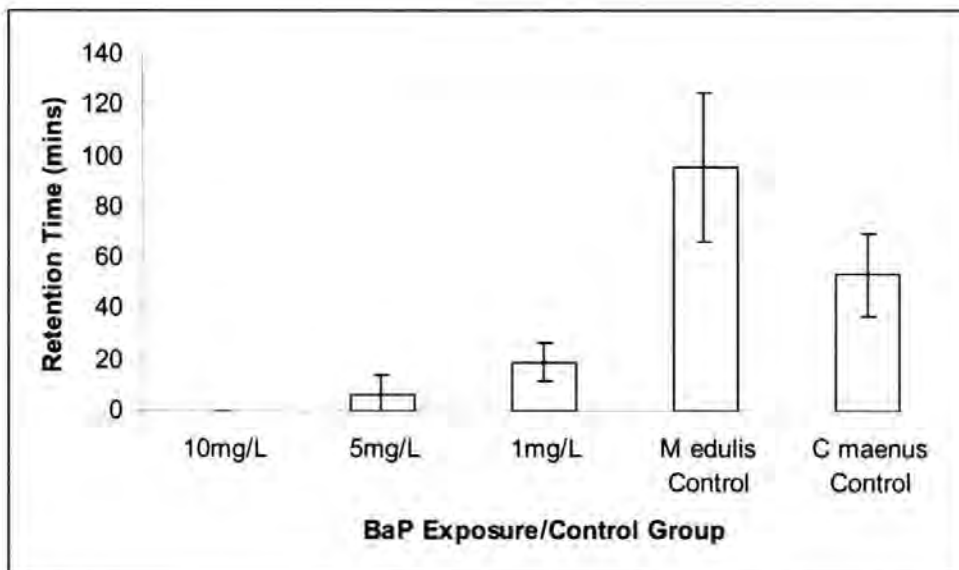


Figure 9. Graph showing dose response of *C. maenas* haemolymph to 10, 5, and 1mg l⁻¹ BaP. A control of unexposed *C. maenas* cells was also examined. *M. edulis* haemolymph cells were also examined for comparative purposes. There is no significant difference between doses ($p < 0.05$, $n = 5$ per exposure group) 5 and 1. No intact lysosomes were observed in the highest dose. There is a significant difference between exposure groups and the control groups ($p < 0.05$, $n = 5$ per exposure group). Bars represent 95% CI.

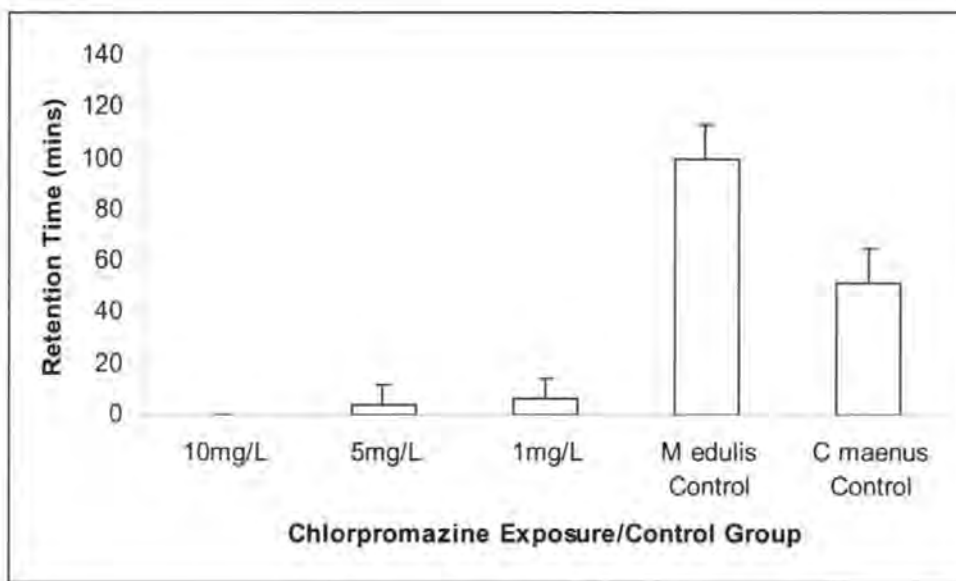


Figure 10. Graph showing dose response of *C. maenas* haemolymph to 10,5, and 1mg l⁻¹ chlorpromazine. A control of unexposed *C. maenas* cells was also examined. *M. edulis* haemolymph cells were also examined for comparative purposes. There is no significant difference between doses ($p < 0.05$, $n = 5$ per exposure group) 5 and 1. No intact lysosomes were observed in the highest dose. There is a significant difference between exposure groups and the control groups ($p < 0.05$, $n = 5$ per exposure group). There is a significant difference between *M. edulis* and *C. maenas* control groups ($p < 0.05$, $n = 5$ per exposure group). Bars represent 95% CI.

3.6 Comparison of Mussel and Crab lysosomal response after a 12 day exposure to copper.

3.6.1 Introduction/Aims

Although the lysosomal response of mussels has been documented there is a lack of evidence examining longer-term response in *Carcinus maenas* and *M. edulis*. By conducting a comparison between lysosomal membrane responses in these two different marine species it will also be possible to gather data on baseline toxicity data on lysosomal response to a common marine pollutant and a comparison between the lysosomal response in two commonly utilised marine species

3.6.2 Method

Mytilus edulis

Mussels, *Mytilus edulis*, were collected from Whitsand Bay. Mussels in the size range of 3.5 – 5.0 cm were chosen. See section 2.8 for husbandry details.

Carcinus maenas

Crabs, intermoult *Carcinus maenas*, were collected from the Yealm Estuary using baited drop nets. Only crabs with a carapace size of 50-60 mm were utilised. See section 2.8 for husbandry details.

Day	Crab Regime	Mussel Regime
0	Collection of crabs from the Yealm Estuary	Collection of Mussels from Whitsand Bay
1	Exposure	Exposure
2	Bleed/Feed	Bleed/Feed
3	Water Change/Redose	Water Change/Redose
4	Bleed/Feed	Bleed/Feed
5	Water Change/Redose	Water Change/Redose
6	Bleed Feed	Bleed Feed
7	Water Change/Redose	Water Change/Redose
8	Bleed/Feed	Bleed/Feed
9	Water Change/Redose	Water Change/Redose
10	Bleed/Feed	Bleed/Feed
11	Water Change/Redose	Water Change/Redose
12	Bleed	Bleed

Table 4. Showing Crab/Mussel Exposure and Bleeding Regime

Exposure/Bleeding Regime

Bleeding, of both organisms, was conducted as described in Section 2. All blood samples were stored on ice until bleeding was complete. The neutral red test was conducted as described in Section 2.1.1 and 2.1.2.

3.6.3 Results

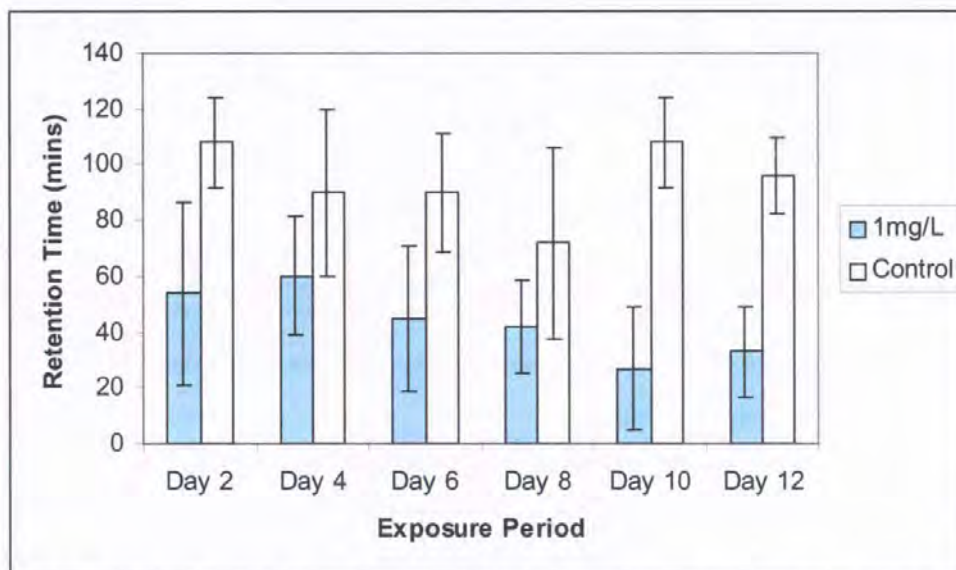


Figure 11. Graph showing *M. edulis* haemocytic lysosomal response to 1mg l^{-1} Cu during a 12 day exposure. Significantly reduced lysosomal retention time was observed in exposure groups at days 2, 10 and 12 ($p < 0.05$, $n = 20$). Bars represent 95% CI.

There was a significant reduction in lysosomal retention integrity in the 1 mg l^{-1} copper exposed mussels at days 2, 10, and 12 compared to the control groups.

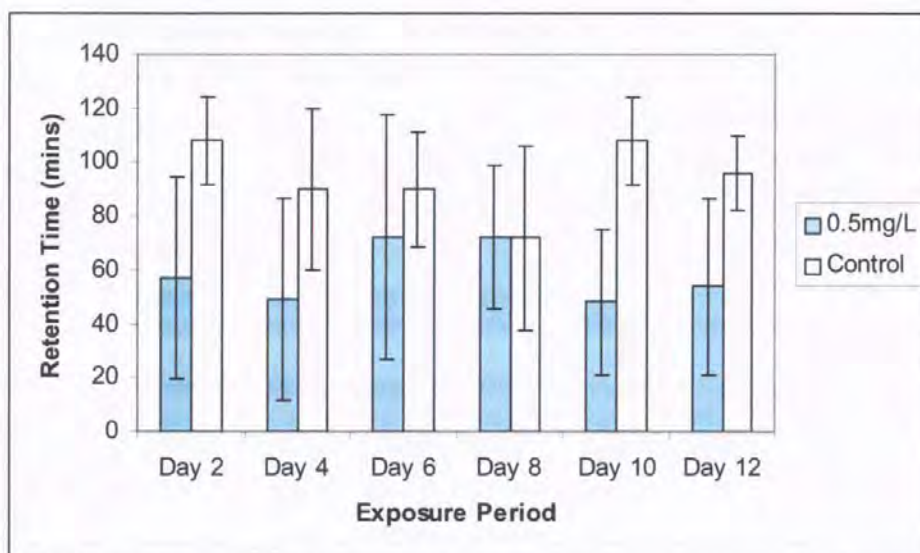


Figure 12. Graph showing *M. edulis* haemocytic lysosomal response to 0.5 mg/L Cu, during a 12 day exposure. Significantly reduced retention times were observed between control and exposure groups at day 10 ($p < 0.05$, $n = 10$). Bars represent 95% CI.

There was a significant reduction in lysosomal integrity at day 10 in the mussels exposed to 0.5 mg l^{-1} Cu. There was a significant reduction in lysosomal retention of the neutral red dye on days 4, 6, and 12 in those mussels exposed to 1 mg l^{-1} BaP. There was a significant reduction in lysosomal retention on days 4, 6, 8, 10 and 12 in those mussels exposed to 0.5 mg/l^{-1} BaP. There was a significant reduction in lysosomal integrity on day 4 in those mussels exposed to 0.1 mg l^{-1} BaP. There were no significant differences between the solvent control and controls.

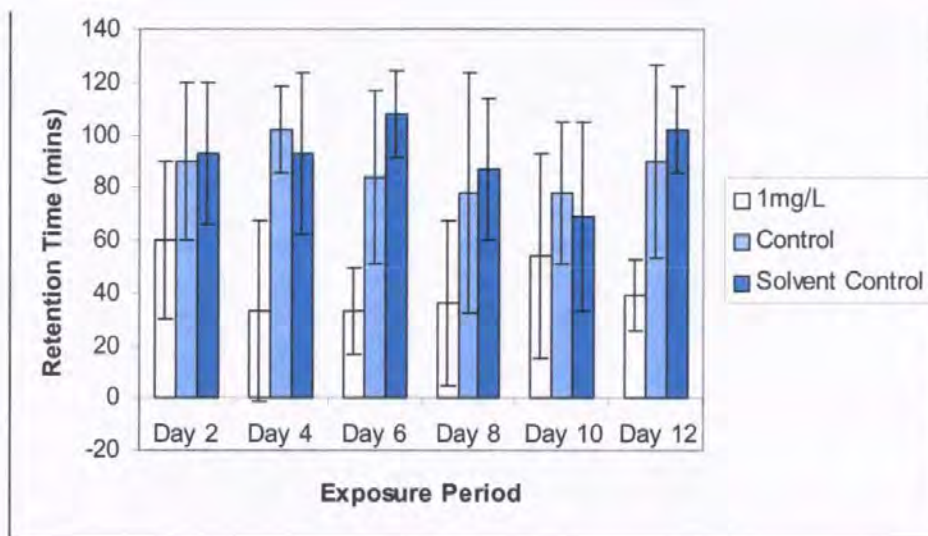


Figure 13. Graph showing *M. edulis* haemocytic lysosomal response to 1mg/L BaP, during a 12 day exposure. Significant reduction in lysosomal retention time was observed at days 4 (control vs exposure group, $p < 0.05$, $n = 20$), 6 (control/solvent control vs exposure group, $p < 0.05$, $n = 20$), and 12 (control/solvent control vs exposure group, $p < 0.05$, $n = 20$). Bars represent 95% CI.

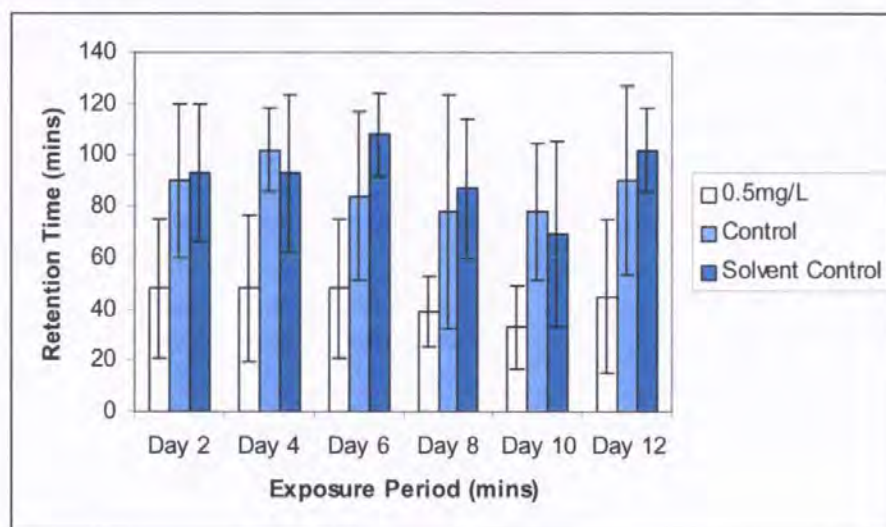


Figure 14. Graph showing *M. edulis* haemocytic lysosomal response to 0.5mg/L BaP, during a 12 day exposure. Significant reduction in lysosomal retention time was observed on days 6 (solvent control vs exposure group, $p < 0.05$, $n = 20$), 8 (solvent control vs exposure group, $p < 0.05$, $n = 20$), 10 (control vs exposure group, $p < 0.05$, $n = 20$) and 12 (solvent control vs exposure group, $p < 0.05$, $n = 20$). Bars represent 95% CI.

The copper exposed crabs showed no significant reduction in lysosomal integrity at the 0.1 mg l⁻¹ and 0.5 mg l⁻¹ doses. There was a significant reduction in lysosomal integrity in the crab haemocytic lysosomal response on day 12 compared to the control in the 1 mg. l⁻¹ exposure group ($p < 0.05$, $n = 10$)

C. maenas exposed to BaP showed significant reductions in lysosomal retention of the neutral red dye on days 6, 8, and 12 at the 1 mg/l⁻¹ exposure (p

< 0.05, n=10). Exposure to 0.5mg/l^{-1} caused a significant reduction in lysosomal integrity on day 12 ($p < 0.05$, n=10). There was no significant reduction in lysosomal integrity in the 0.1mg/l^{-1} exposures ($p > 0.05$, n=10).

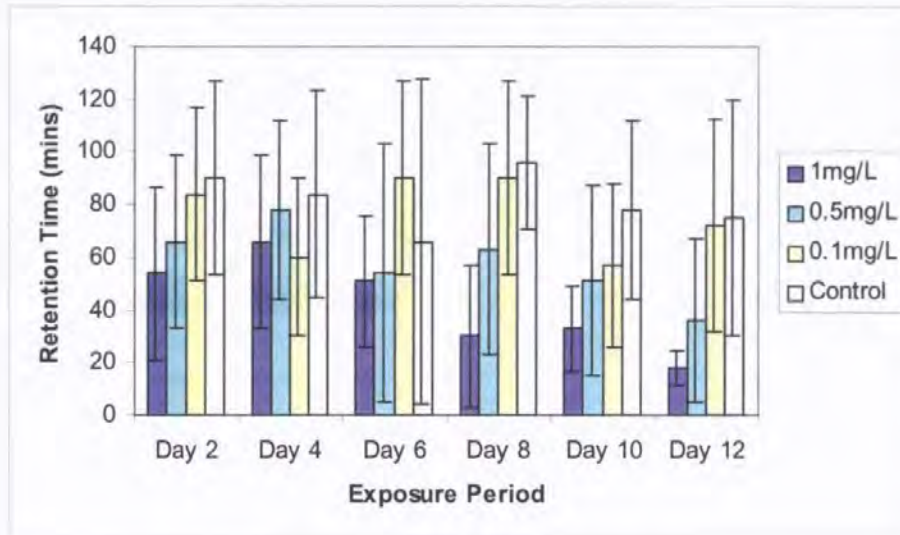


Figure 15. Graph showing *C. maenas* response to Cu exposure following 12 day whole animal exposure. There are no significant differences between control and exposure groups ($p > 0.05$, n=10) with the exception of day 12 which shows a significantly reduced lysosomal retention in the 1mg l^{-1} exposure group. Bars represent 95% CI.

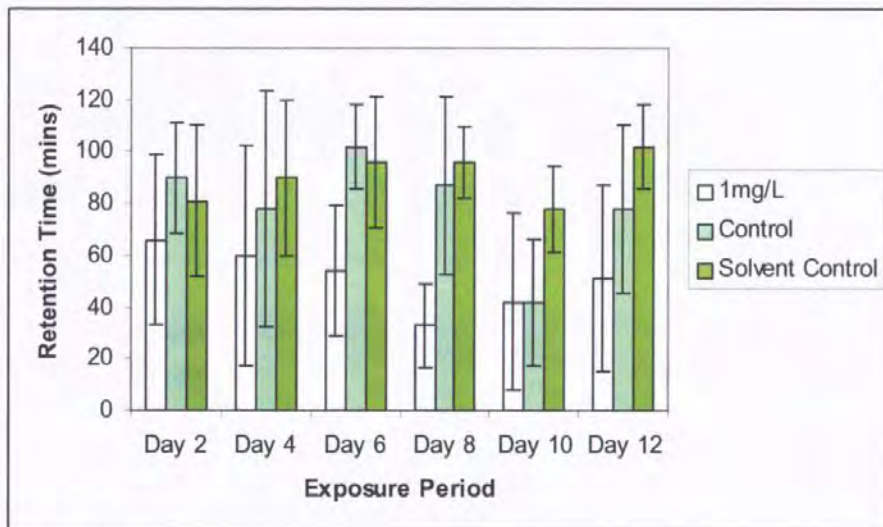


Figure 16. Graph showing *C. maenas* haemocytic lysosomal response to 1mg/L BaP, during a 12 day exposure. Significant differences exist between the solvent control and the exposure group were observed on day 8 ($p < 0.05$, $n = 10$).

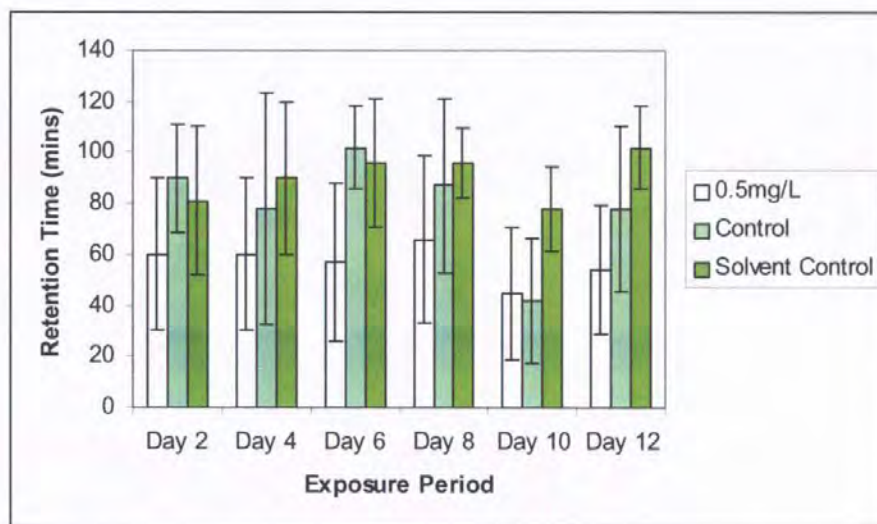


Figure 17. Graph showing *C. maenas* haemocytic lysosomal response to 0.5 mg/L BaP, during a 12 day exposure. Significant differences were observed between the control and exposure groups on day 12 ($p < 0.05$, $n = 10$).

3.7 Discussion

This study has attempted to examine and quantify mussel and crab lysosomal response using dye based retention assays. The verification of lysosomal number and location in crab haemocytes was accomplished with a fluorescent dye. There are several advantages to using fluorescent dyes in *in vitro* toxicology; they are safer alternatives to radioactive tracers and also allow analysis using fluorescent microscopy (Essig-Marcello and Buskirk, 1990). The application of molecular probes to identify cellular processes and to aid in the understanding of pollutant effect on a mechanistic basis is a useful means understanding the basis of pollutant exposure (Bach, 1989). The development of probes, such as Bodipy – FL verapamil, has enabled the identification of specific cellular configurations to take place which, with developments in image analysis and laserscan confocal microscopy, forms a powerful new technology for the investigation of pathological problems (Moore, 1992). Previous studies have confirmed that Bodipy-verapamil, a fluorescent derivative of verapamil, is a useful agent for the imaging of lysosomes in drug- sensitive cells (Lelong *et al.*, 1991).

In mussel haemolymph Moore *et al* (1996) demonstrated the accumulation of Bodipy-FL-verapamil only in lysosomes. Lelong *et al* (1991) found no other sites of accumulation within cloned drug sensitive human carcinoma cells and there was no evidence of any other accumulation in this study. Lelong *et al* (1991) observed that Bodipy verapamil is a substrate for P glyco-protein in multi-drug resistant (MDR) cells and that this derivative is preferentially concentrated in lysosomes of drug-sensitive cells only. It is therefore possible that crab haemolymph cells also express the MDR phenotype. This could be tested by experimental challenging the crab haemolymph to cytotoxic substances in the presence and absence of verapamil, an inhibitor of multi-drug

transport activity (McFadzen *et al.*, 2000). Observed crab blood cells (haemocytes) were noted to be intensely granulated and amoeboid in nature, as reported by previous workers (Williams and Lutz, 1975; Johnston *et al.*, 1973; Smith and Ratcliffe, 1978). Cells could be distinguished on the basis on the presence or absence of lysosomes, as reported by Smith and Ratcliffe (1978). Such divisions are probably arbitrary since intermediate forms between the two cells types have been observed, suggesting a maturation series or a dependence on the physiological state of the animal (Williams and Lutz, 1975; Smith and Ratcliffe, 1978). High numbers of lysosomal rich cells were observed which supports the theory that they have a role in the clearance and degradation of foreign matter from the haemolymph (Smith and Ratcliffe, 1978). The identification of lysosomal rich cells in crab haemolymph is encouraging for their continued use for the determination of sub-cellular pollutant effect. However, the difficulties in maintaining the live haemocytes for the periods of time sometimes necessary to quantify the pollutant response may pose a confounding factor to their eventual use.

The neutral red test distinguished between different doses of contaminants in both mussel and crab haemocytic lysosomes. Copper and Benzo-a-pyrene were chosen as they are common environmental pollutants. Metals and hydrocarbons have been shown to inhibit the sub-cellular activity of invertebrates (Moore *et al.*, 1984; Grundy *et al.*, 1996) and cause alterations in lysosomal function (Lowe *et al.*, 1981; Lowe and Pipe, 1994; Shepard and Bailey, 2000). The primary aim of this work was to test the application of the neutral red test in a novel species, the shore crab (*Carcinus maenas*) and to further verify the application of the neutral red test in the mussel (*Mytilus edulis*). The exposure concentrations may therefore be not directly related to

common environmental conditions but they enable a clear identification of the lysosomal response to contaminant induced stress. There was no examination of tissue residue for the contaminants under test but it is assumed that uptake was related to the conditions present in the water as both metals and PAHs are known to accumulate in mussel tissue (Moore *et al.*, 1984; Nott and Moore, 1987; Widdows and Donkin, 1992).

Mussels were shown to have reduced lysosomal stability after contaminant exposure, after both haemolymph and whole animal exposure. Shepard and Bradley (2000) showed reduced lysosomal stability in mussels after 24 hr exposure to copper sulfate, with an associated rise in specific protein expression. Harrison and Berger (1982) demonstrated that copper exposure caused adverse changes in the lysosomal latency of *Mytilus edulis* digestive cells. Copper has been shown to destabilise oyster digestive gland lysosomal membranes (Ringwood *et al.*, 1998). Reduced lysosomal stability has also been shown in mussels and oysters after zinc exposure (Lowe and Moore, 1979; Meiller and Bradley, 2002).

The lack of a significant lysosomal response to copper in the whole animal crab, other than to the highest dose exposures, demonstrates a different lysosomal sensitivity compared to mussels, which demonstrated a clear reduction in lysosomal fragility after copper exposure at significantly lower doses. Pedersen and Lundebye (1996) demonstrated a low sensitivity in crabs to environmentally high concentrations of copper using stress proteins and metallothionein as a marker of effect and suggested that crabs are able to pass copper through the cytosolic compartment for storage in granules. However, Lundebye and Depledge (1998) demonstrated irregularities in cardiac activity

and mortality in crabs exposed to comparable doses of copper for a four week period.

However, the exposure of crab haemolymph to copper caused significant perturbation of the lysosomal membrane. This may suggest that the effect of copper in blood is a direct effect on membrane function whereas whole animal metabolism can effect a detoxication that reduces the toxic consequences of exposure. Tissue residue would enable an examination of any such mechanisms in the crab and enable an identification of the fate of the copper. Pedersen and Lundebye (1996) showed an increase in copper concentrations in crab midgut gland after exposure to a copper contaminated site. Copper can effect metal requiring enzymes and proteins, and can cause the generation of hydroxyl radicals leading to oxidative damage (Moore, 1994b Ringwood *et al.*, 1998;).

Chlorpromazine is a known disruptor of lysosomal membrane by inducing lysosomal enlargement and autophagy and has been reported to cause lysosomal dysfunction in mussels (Moore *et al.*, 1996). Chlorpromazine was shown to cause a similar effect on crab haemocytic lysosomes and it is suggested that the generalised lysosomal response to stress is being conserved between the two organisms under test.

4.0 MEASUREMENT OF HAEMOCYTIC AND HISTOLOGICAL RESPONSES IN MUSSELS

4.1 ABSTRACT

The field application of sub-cellular and tissue level biomarkers was examined by conducting a 20-day transplantation experiment utilising the neutral red lysosomal integrity test on the blue mussel *Mytilus edulis*. Mussels were collected from a relatively uncontaminated site, the mouth of the Exe Estuary. Mussels were then relocated in specially prepared baskets to the Sutton harbour site. Mussels were also set up in static tanks at the University of Plymouth, in constant conditions and a 12 hour light/dark cycle. Laboratory mussels, were fed a diet of cultured algae. Positive controls were also prepared in the laboratory in static aerated tanks.

Lysosomal neutral red retention was assessed in both groups after 10 and 20 days. A sub sample of haemolymph was taken and used for micronucleus analysis. After haemolymph extraction mussels were sacrificed and processed for histopathological analysis. After 20 days the mussels remaining at the Sutton Harbour site were returned to the laboratory to examine any recovery. Neutral red data from the control groups in the recirculating system showed no significant difference between control replicates. Positive controls showed a significant difference to controls. There was no significant difference between neutral red control mussels at the beginning and end of the experiment. There was no significant difference between positive control mussels at the beginning and end of the experiment. There was a significantly reduced retention time in the mussels transplanted to Sutton Harbour compared to the controls ($p < 0.05$, $n=20$). In the digestive gland tissue there were significant alterations in phasic

activity, epithelial thickness of the tubules, and gonadal atresia in the mussels transplanted to Sutton Harbour, compared to the controls ($p < 0.05$, $n = 20$). The micronucleus assay showed no significant difference between the mussels transplanted to Sutton Harbour and the controls ($p > 0.05$, $n = 20$). There was, however, a trend in an increased number of abnormalities of the nucleus of mussels taken from the Sutton Harbour mussels, but there were too many artifacts present to allow robust measurements to be made. The recovery of the mussels was also examined, using the neutral red retention assay to measure lysosomal fragility. These results show a potential linkage between cellular and pathological response and demonstrate the value of a multi-biomarker response to identify regions of possibly poor environmental quality.

4.2 Method

Mussels, in the size range 3.5-5.0cm, were collected from the Exmouth mussel beds during low tide. Damaged or deformed mussels were discarded and care taken not to tear the byssel threads during collection. Mussels were held in the laboratory in static aerate tanks for 24 hours before exposures began, moribund mussels (as identified by excessive shell gape) were removed. Epibionts were removed by scraping. Mussels were maintained as described in section 2.8.

Laboratory Controls

Mussels were held in static aerated tanks, utilising UV filtered seawater, and subjected to a 12 hour light dark/cycle. Mussels were fed on alternate days on algal culture and water was changed on non-feeding days. Three tanks were utilised with 15 mussels in each: mussels were taken from each tank for sacrifice.

Laboratory Positive Controls

Mussels were held as described above. Mussels were exposed to 0.1 mg l⁻¹ of BaP, delivered using DMSO as a solvent. Redosing was conducted after each water change; and three additional tanks were utilised as a DMSO control.

Field Transplanted Mussels

Mussels were relocated to a permanently submerged pontoon strut in specially constructed plastic net bags. Salinity measurements of the site showed a range of 29-35ppt. Three bags were utilised, each containing 15 mussels, to facilitate collection. On day 20 the remaining mussels were removed from Sutton Harbour, taken from the bag, and placed in laboratory conditions as described above, for a further 10 days.

Immediately after collection from the Exmouth reference site mussels, were bled and sacrificed. Tissue and haemolymph was preserved (as described in Section 2) for analysis. The neutral red test was conducted immediately. Haemolymph and tissue collection was then conducted according to the regime given below.

The neutral red retention test was conducted as described in Section 2.0. Pathology scores were interpreted also as described in Section 2.0. In both cases, slides were coded to prevent operator bias.

Day	Activity
0	Collection from Exmouth Haemolymph collection/sacrifice
10	Haemolymph collection/sacrifice n=10 (lab, field, and +ve control)
20	Haemolymph collection/sacrifice Return of transplanted mussels to laboratory n=10 (lab, field, and +ve control)
30	Haemolymph collection/sacrifice n=10 (lab, and +ve control)

Table 5. Table showing experimental schedule

4.3 Results

Neutral Red Retention

Neutral red retention, as a measure of lysosomal fragility, showed significant differences in lysosomal retention time between mussels maintained in the laboratory and those relocated to the Sutton Harbour site, after 10 and 20 days ($p < 0.05$, $n=10$; fig. 18). The positive controls, maintained in the laboratory, also showed a significant reduction in the lysosomal retention time of the neutral red probe compared to the controls ($p < 0.05$, $n=10$; fig. 18). Although there was a trend of reduced retention time in the positive controls compared to the relocated mussels there was no significant difference ($p > 0.05$, $n=10$; fig.

18). There was no significant change in the retention values of the mussels relocated back to the laboratory, and allowed to recover for a ten day period, following the 20 day field exposure. There were no significant differences in the retention time of the neutral red probe in the lysosomes of those mussels maintained in the laboratory. There was, however, an apparent trend of declining retention times.

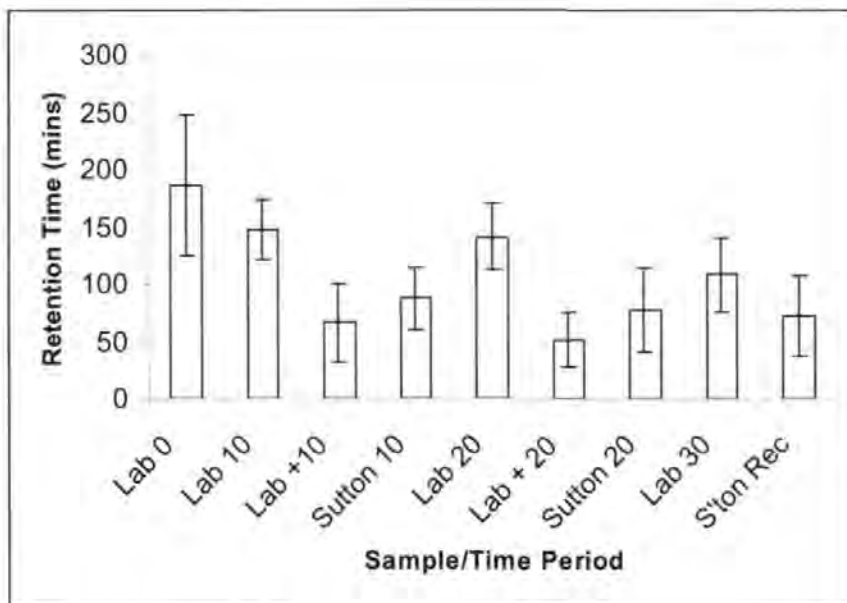


Figure 18. Neutral red response in mussels held in the laboratory (Lab 0, 10, 20, and 30), positive controls held in the laboratory (Lab +10 and Lab +20), mussels relocated to Sutton Harbour (Sutton 10 and Sutton 20), and mussels returned from Sutton Harbour to the laboratory (S'ton Rec (Sutton Recovery)). Significant differences exist between Sutton 10 and Lab 10 ($p < 0.05$, $n = 10$). Significant differences exist between Lab+10 (positive control) and Lab 10 ($p < 0.05$, $n = 10$). Significant differences exist between Sutton 20 and Lab 20 ($p < 0.05$, $n = 10$). Significant differences also exist between Lab+20 and Lab 20 ($p < 0.05$, $n = 10$). There is no significant difference between Sutton Recovery and Lab 30 ($p > 0.05$, $n = 10$).

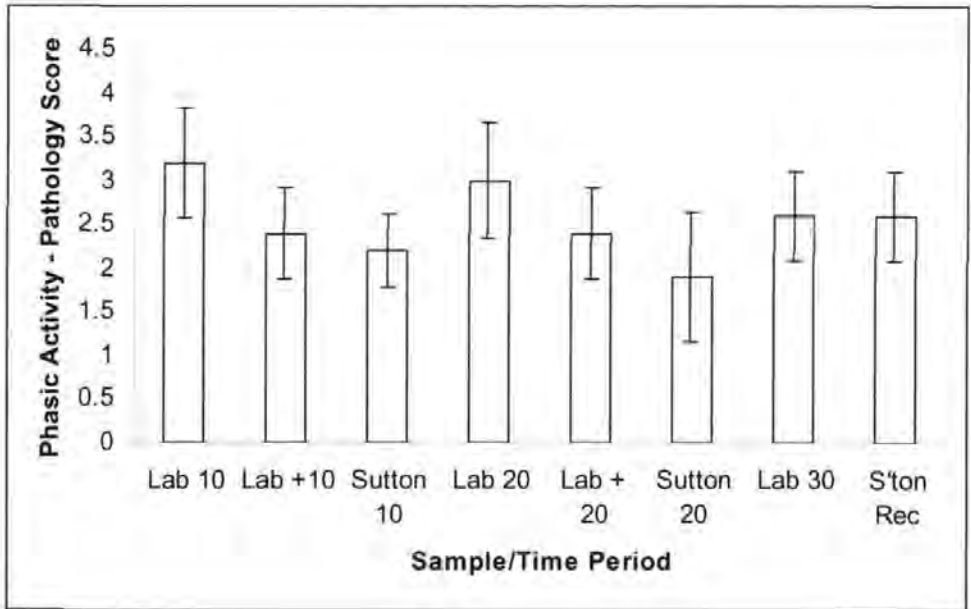


Figure 19. Graph showing phasic activity in mussels held in the laboratory (Lab 0, 10, 20, and 30), positive controls held in the laboratory (Lab +10 and Lab +20), mussels relocated to Sutton Harbour (Sutton 10 and Sutton 20), and mussels returned from Sutton Harbour to the laboratory (S'ton Rec (Sutton Recovery)). Significant differences exist between Sutton 10 and Lab 10 ($p < 0.05$, $n=10$).

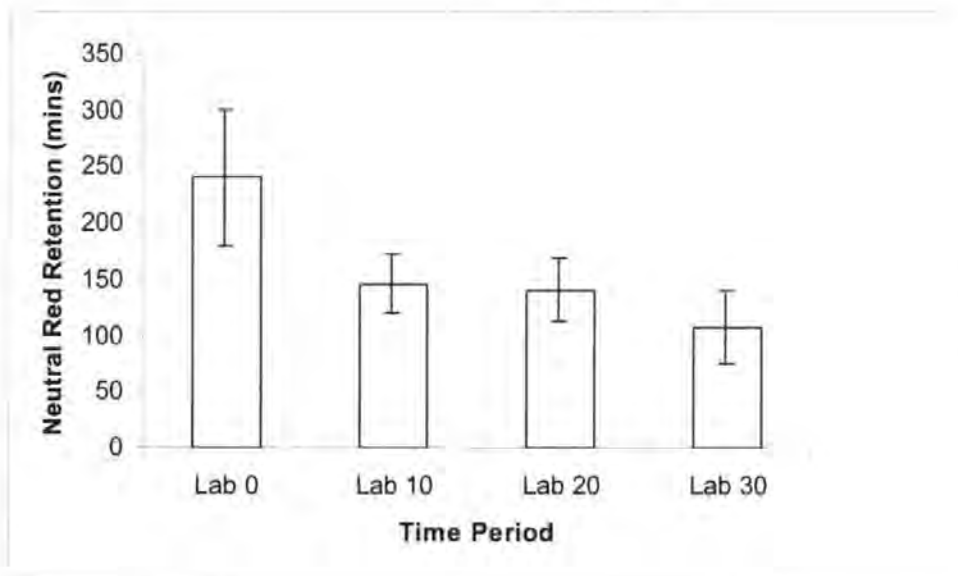


Figure 20. Neutral red retention in mussels, *Mytilus edulis*, maintained in static aerated tanks for a 30 day period. Mussels were exposed to a 12 hour light/dark cycle. Water change and feeding (mussels were given an algal broth) were conducted on alternate days. Lab 0 is significantly different to Lab 10, 20, and 30 ($p < 0.05$, $n=10$).

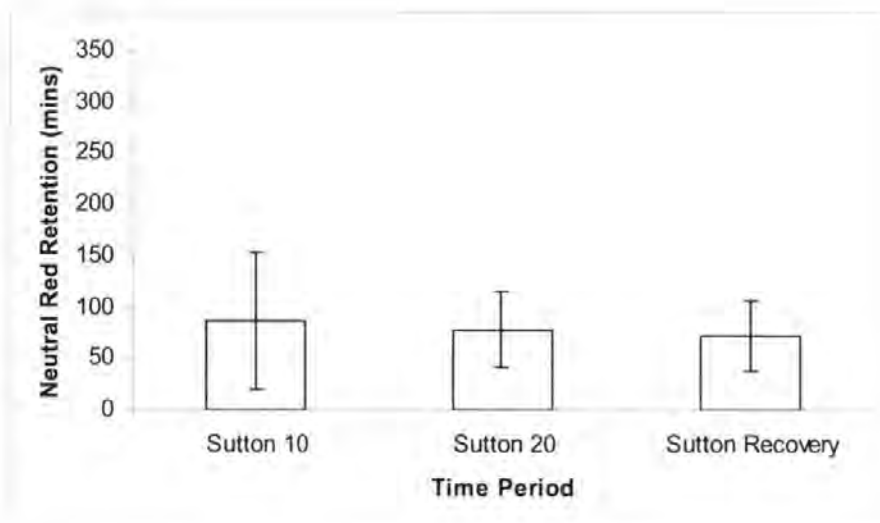


Figure 21. Neutral red retention in mussels, *Mytilus edulis*, after 10 and 20 day exposure to a pollutant impacted field site, Sutton Harbour. Data is also shown for the neutral red retention of those mussels returned to the laboratory for a 10 day period, after 20 days of field site exposure. No significant differences exist ($p > 0.05$, $n=10$).

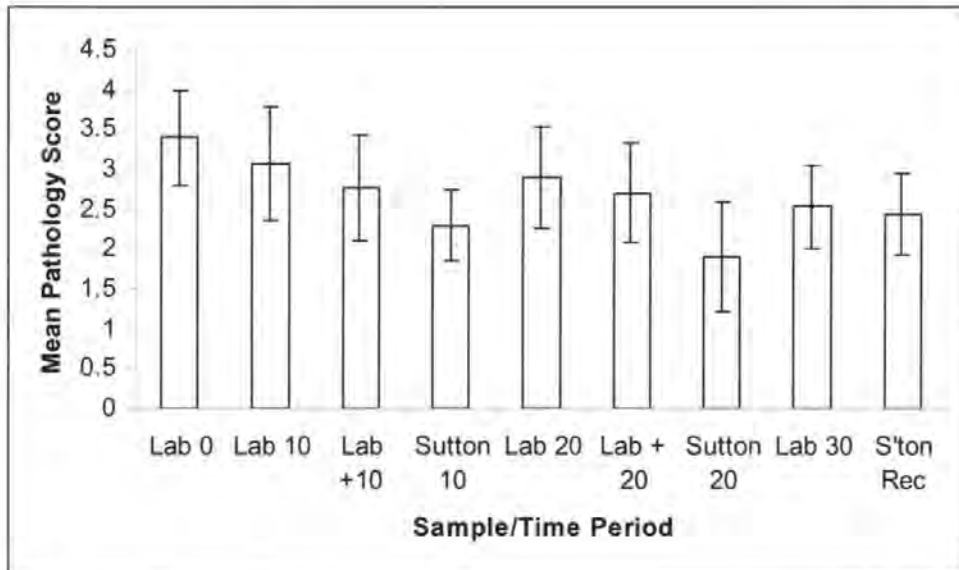


Figure 22. Mean values of all the pathological endpoints scored (Gonad maturity, digestive tubule phasic activity and tubule epithelial thinning) in mussels, *Mytilus edulis*, held in the laboratory (Lab 0, 10, 20, and 30), positive controls held in the laboratory (Lab +10 and Lab +20), relocated to Sutton Harbour (Sutton 10 and Sutton 20), and returned from Sutton Harbour to the laboratory (S'ton Rec (Sutton Recovery)). There is a significant difference between Sutton Harbour 10 and Lab 0 ($p < 0.05$, $n = 10$).

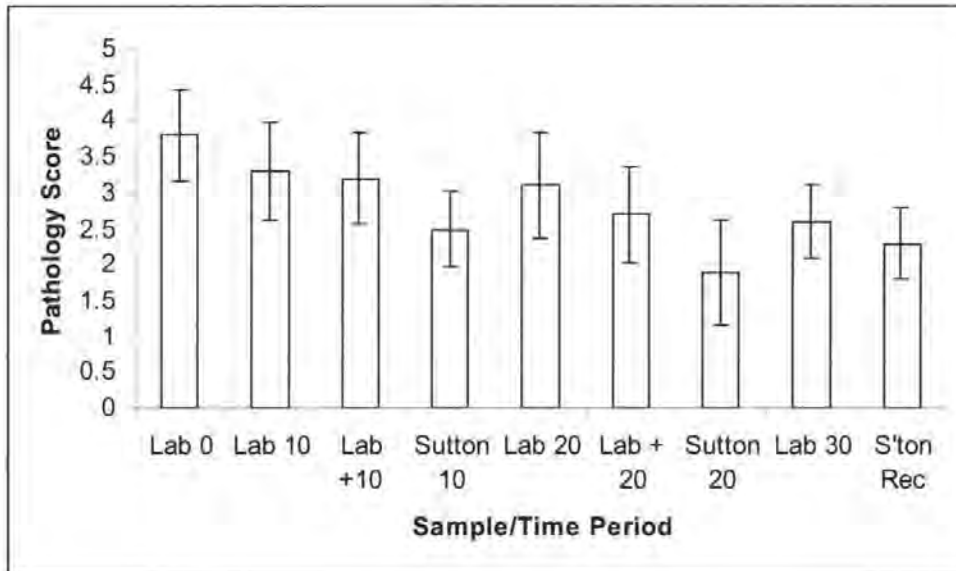


Fig 23. Severity of epithelial thinning in mussels, *Mytilus edulis*, held in the laboratory (Lab 0, 10, 20, and 30), positive controls held in the laboratory (Lab +10 and Lab +20), relocated to Sutton Harbour (Sutton 10 and Sutton 20), and returned from Sutton Harbour to the laboratory (S'ton Rec (Sutton Recovery)). There is a significant difference between Sutton Harbour 10 and Lab 0 ($p < 0.05$, $n = 10$).

Pathology

Pathological endpoints, the incidence of gonad abnormalities, phasic activity and epithelial thinning of the digestive tubules were examined individually and in combination. The combination of all the pathology scores did not show any significant differences, though there was a clear trend of increased pathological abnormalities in mussels relocated to Sutton Harbour. There was a significant reduction in tubule phasic activity in mussels relocated to Sutton Harbour after a 10 day period, compared to laboratory maintained mussels ($p < 0.05$, $n = 10$). There was also a significant increase in digestive tubule epithelial thinning in the same mussels ($p < 0.05$, $n = 10$). There were no significant alterations in the gonad of the mussels ($p > 0.05$, $n = 10$).

Micronucleus

The micronucleus test data was not considered robust enough for inclusion. High levels of artifacts were detected which led to erroneous results being

recorded. The presence of the artifacts was considered to be a by-product of the staining protocol.

4.4 Discussion

These results show differences in biomarker response between those mussels maintained in the laboratory and those exposed to the Sutton Harbour environment, a polluted marina with a poor flushing rate. The incidence of greater lysosomal membrane fragility, as indicated by the lower retention times, in the haemocytes of mussels relocated to Sutton Harbour is indicative of pollutant induced stress. Lysosomal response to pollutant stress has been demonstrated using the neutral red test in a number of field studies (Lowe, *et al.*, 1995a; Cheung *et al.*, 1998; Nasci *et al.*, 1998; Ringwood *et al.*, 1998). However only a few studies have examined the neutral red test in association with other biomarkers of pollutant effect. Da Ros *et al* (2002) examined the neutral red test for lysosomal stability in conjunction with the lysosomal latency test and found that the neutral red test was more sensitive for identifying pollutant-induced stress. Wedderburn and Hornsby (2002) used the neutral red test in conjunction with a histological examination of mussel tissue for pathological alteration and reproductive status, demonstrating fluctuations in lysosomal response associated with reproductive condition. Shepard and Bailey (2000) examined lysosomal response, using the neutral red test, in conjunction with measurements of protein expression.

The pathological responses of the mussels showed an overall reduction in tissue integrity after relocation to the contaminated field site from measured values taken immediately after collection. Da Ros *et al* (2000) showed a reduction in digestive gland epithelial thickness in mussels translocated from a

clean reference site to an area affected by industrial pollution. Lowe *et al* (1981) showed structural changes in mussel epithelial cells and lysosomal disruption after exposure to North Sea crude oil. Mussels accumulate chemical contaminants, particularly in the digestive gland (Krishnakumar *et al.*, 1994) and this makes them a target for pollutant effect.

Mussels maintained in the laboratory environment showed a gradual decrease in lysosomal integrity, and an increase in the incidence of tissue showing pathological alteration. Lysosomal responses in the digestive cells of mussels are not contaminant specific and can be induced by changes in temperature, salinity, and food availability (Moore *et al.*, 1987). It is possible that laboratory conditions will exert a gradual stress on captive mussels that can be expressed in lysosomal fragility, for example. The positive controls showed an immediate reduction in lysosomal integrity. However, there was no evidence of significant pathological change in the tissue in BaP exposed mussels.

Return to the laboratory environment did not cause any immediate or significant recovery. There was, however, no indication of worsening condition and a possible trend within the neutral red data for an improvement in lysosomal integrity. The application of control organisms in the laboratory and the use of positive controls enabled a greater interpretation of experimental results to be achieved. The linkage between the impairment of lysosomal membrane integrity and dysfunction at the physiological level, as demonstrated by changes in phasic activity and epithelial thickness suggests a mechanistic link between perturbing events at the sub-cellular level and higher order pathology. The use of positive controls in the laboratory enabled the testing of the chosen methods to identify pollutant based responses. As only one dose of Benzo-[a]-

Pyrene was used it is impossible to use this data to rank the field data, although this could be a consideration for future studies.

This work demonstrated the generalised response exhibited by the biomarkers under test as no differences in the type of response were noted. Further, the apparent worsening condition of the mussels maintained in the laboratory, as well as the expression of injury in the field mussels was similar to that of the positive controls. In all cases, the pathological response was exhibited in a similar way, although field mussels did show increased levels of granulocytes and parasitic incidence in their tissue.

The difficulties in obtaining meaningful micronucleus (MN) data were caused by the high incidence of artifacts after the staining process. This led to a greater than expected number of MN being counted which, after consultation with published literature regarding the 'normal' incidence of nuclear abnormalities, led to the rejection of these data as being insufficiently robust for inclusion. There was an apparent trend of increased abnormalities in the field exposed mussels but this cannot be supported by statistical analysis and must, therefore, be treated only as an anecdotal observation.

This work demonstrates the value of linking lysosomal stress response with high order effects as this gives greater robustness to the overall data set for identifying the status of the environment under study. Further, it provides further evidence that the lysosomal response, although generalised, is indicative of whole animal stress and could therefore be prognostic for reduced overall performance.

5.0 SIMULTANEOUS NON-DESTRUCTIVE MEASUREMENT OF HEART RATE ACTIVITY AND LYSOSOMAL INTEGRITY IN *ANODONTA CYGNEA*, AFTER EXPOSURE TO MALATHION AND COPPER.

5.1 ABSTRACT

The use of the freshwater bivalve mussel *Anodonta cygnea* as a sentinel organism for pollutant monitoring was investigated. Animals were exposed to either copper, a neurotoxic trace metal, or malathion, an insecticide. Biomarkers of lysosomal integrity and cardiac activity were measured in a non-destructive manner. Lysosomal integrity was assessed using the retention time of the supravital dye neutral red within the haemocytic lysosomes. Heart rate activity was measured using the CAPMON system which emits infra-red light onto the heart, the reflected light is measured and the heart rate activity can be recorded. Lysosomal integrity was significantly compromised after exposure to malathion ($P < 0.05$, $n=9$), copper exposure caused a similar trend ($P < 0.05$, $n=9$). The addition of copper caused a significant decrease in the mean and maximum heart rate ($P < 0.05$, $n=8$), as did the addition of malathion ($P < 0.05$, $n=8$). This demonstrates the potential use of these biomarkers in assessing contaminant induced stress in freshwater systems, using *Anodonta cygnea* as a sentinel organism.

5.2 Introduction

Much work has been accomplished on the biological monitoring of pollutants in the marine environment; but relatively little attention has been given to the study of freshwater systems, into which many contaminants first enter. The aim

of this work was to investigate the potential use of the freshwater swan mussel *Anodonta cygnea* as a sentinel organism for the bio-monitoring of freshwater systems.

Selected biomarker responses of *Anodonta cygnea* were examined after short term *in vivo* exposure to two potential pollutants, the metal copper and the insecticide malathion. Previous work using freshwater mussels has examined the acute toxicity of trace metals and pesticides (Keller and Ruessler, 1997; Moulton *et al.*, 1996 ; McKinney *et al.*, 1996) or else, utilised destructive methods to elucidate sub-lethal pathological responses (Tallendini *et al.*, 1986; Hemelraad *et al.*, 1990). The investigation of the effects of these contaminants has not been fully examined in the context of non-destructive sub-lethal biomarker responses in *Anodonta cygnea*.

Fresh water mussels can reach sizes of over 200 mm in length and more than 10 years of age. The swan mussel is the largest member of the molluscan family Unionidae. It is found in the muddy bottoms of slow moving rivers or canals, lakes and ponds. Keller and Ruessler (1997) reported *Anodonta cygnea* are among the most imperiled fauna in the United States as a result of the possible effects of pesticides and other contaminants.

The environmental impact of neurotoxic pesticides has been of great concern for many years because of their potency and inevitable release into the environment, especially when used in agriculture (Nimo and McEwan, 1990). Malathion is a broad-spectrum non-systemic organophosphate insecticide and is used extensively in the agricultural and forestry industries. Malathion acts as an irreversible acetylcholinesterase inhibitor which causes lethal accumulation of acetylcholine at nerve endings. The use of pesticides in agricultural activities can cause the acute accumulation of contaminants in local water courses which

could result in the exposure of non-target organisms (Keller and Ruessler, 1997).

Effluents, which include trace metals, such as copper, can enter the aquatic environment and cause serious metabolic, physiological and structural impairments in living organisms (Rajalekshmi and Mohandas, 1992). Copper is a common contaminant in marine and freshwater environments, as a result of its extensive use in anti-fouling agents (Clark, 1986). Copper can effect metal requiring enzymes and proteins, and can cause the generation of hydroxyl radicals leading to oxidative damage (Ringwood *et al.*, 1998; Moore, 1994b).

The application of sub-lethal biomarkers in freshwater systems, for the monitoring of such contaminants is limited. Physiological biomarkers, such as the measurement of heart rate, can allow the integration of the effects of toxicity on different target tissues into the context of whole animal response and are particularly useful since the elicited responses can be related to Darwinian fitness parameters (Depledge and Lundebye, 1996).

Biomarkers of cellular perturbation involving structure and function can be considered as 'early warning' markers of continuing and potentially increasing pathological damage. There is considerable evidence for damage to lysosomal membrane stability by environmental xenobiotics in aquatic organisms (Moore, 1990), yet only a small number of studies have investigated the nature of the lysosomal response in freshwater organisms (Svendsen and Weeks, 1994).

Several physiological responses of molluscs and crustaceans have been tested under laboratory and field conditions for their ability to detect environmental stressors (Akberali and Trueman, 1985; Baldwin and Kramer, 1994; Aagaard; *et al*, 1996; Lundebye and Depledge, 1998; Astley *et al.*, 1999). Heart rate data have been shown to be useful in that they can provide an indication of the well

being of a mollusc. Technological developments have permitted the cardiac activity of several organisms to be monitored in a simultaneous manner over long periods of time, without imposing undue stress (Depledge and Andersen, 1990) on the organism under test.

The examination of specific biomarker responses in selected "sentinel" species, such as *Anodonta cygnea*, allows comparisons to be made between various field sites, with a range of relative environmental quality. By examining several biomarker responses at different levels of biological organisation within an organism mechanistic linkages between different pathological responses can begin to be established.

5.3 Materials and methods

Experimental Animals

Anodonta cygnea were collected in hand trawl nets from the sediment of the Exeter ship canal on the south coast of Devon, UK. Only samples with undamaged shells and which were greater than 80 mm in length were retained for experimental work. The mussels were transferred to constant laboratory conditions (12:12 light dark regime, water temperature 15°C, dechlorinated tap water) within 4 hours of collection.

Before use the mussels were allowed to acclimate to laboratory conditions for 48 hours. 24 animals had collars adhered to their shells for the heart rate monitoring, a further 27 animals were coded for the lysosomal assay. Both groups were maintained in the same tanks. The mussels were held in 20 l glass tanks filled with aerated dechlorinated tap water (16°C).

Copper and Malathion Exposure

Copper stock solution ($10 \text{ mg}\cdot\text{ml}^{-1}$) was made up by dissolving copper sulphate (Sigma) in 1 l of water. Cu SO_4 (0.5 mg l^{-1} , $500 \mu\text{g l}^{-1}$) was added to the holding tank by carefully pipetting from the stock solution. Pure malathion has a solubility limit of 145mg/L in distilled water and is very slow to dissolve unless a carrier is used. A stock solution (400 mg l^{-1}) of malathion (98.5 % m/v) was made up by diluting malathion (20 μl) in ethanol (20 ml) and water (30 ml). Malathion (0.1 mg l^{-1} , $100 \mu\text{g l}^{-1}$) was also pipetted into the appropriate tanks. The tanks were then securely covered and aerated.

Statistics

Analysis of variance was conducted for all the data sets using a 95% confidence interval. All calculations were performed on the Statgraphics package.

5.4 Results

Lysosomal Activity

Granulocytic cells were observed with lysosomes retaining the neutral red dye. Retention times were observed which were similar to those recorded in other bivalve molluscs in studies reported elsewhere. Retention times were recorded 72 hours before exposure, 24 hours after exposure, and then 72 hours after recovery in clean water.

There was no significant ($P = 0.5325$, $n = 25$) differences between the control animals over the 120 hour period during which the experiment was conducted (see figure 24). Values were in the range of 15 to 105 minutes, the average retention time being 75 minutes. There were no significant differences between any of the pre-exposure values for the different groups.

Heart Rate Activity

Anodonta cygnea heart rates recorded over a range of 7 days (Bloxham, unpublished) exhibited a range from 7 to 35 bpm, with a mean heart rate of 15 bpm (See figure 25). Marine molluscs exhibit a heart rate in the range of 20-28 bpm (Bayne, *et al.*, 1976). The larger size of *A. cygnea* compared to marine molluscs allowed efficient placement of the emitter/detector.

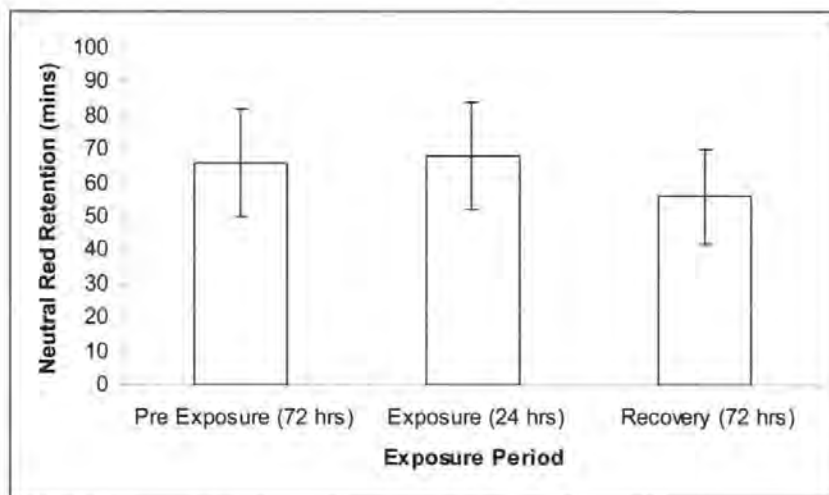


Figure 24. Mean lysosomal retention time of control (i.e. unexposed) *Anodonta Cygnea* haemolymph during the experimental period.

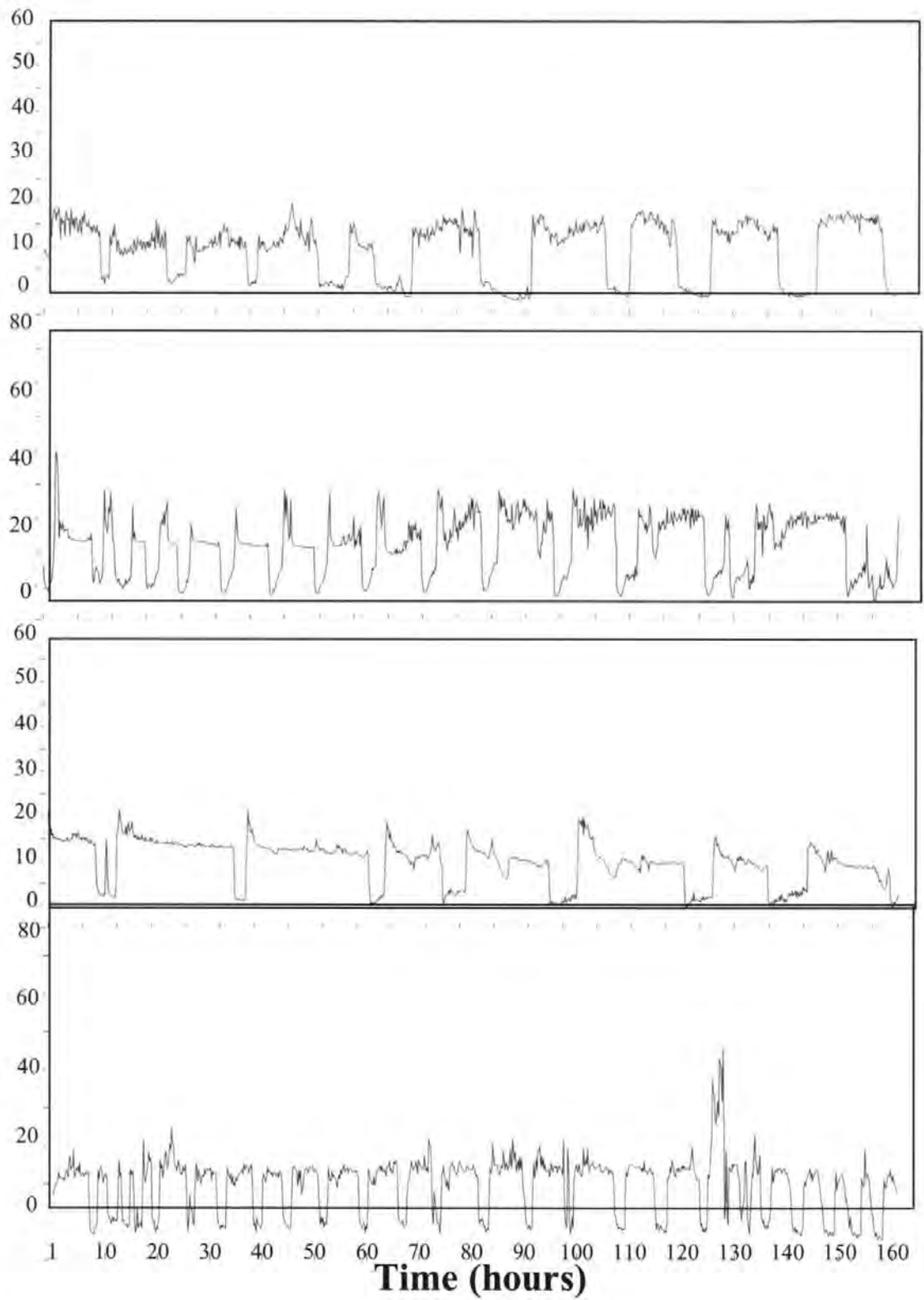


Figure 25. Heart rate recordings (beats per minute) of *Anodonta cygnea* (n=4) over a 7 day (160 h) period during conditions of high food concentrations (Bloxxham, unpublished).

Heart rate (bpm)

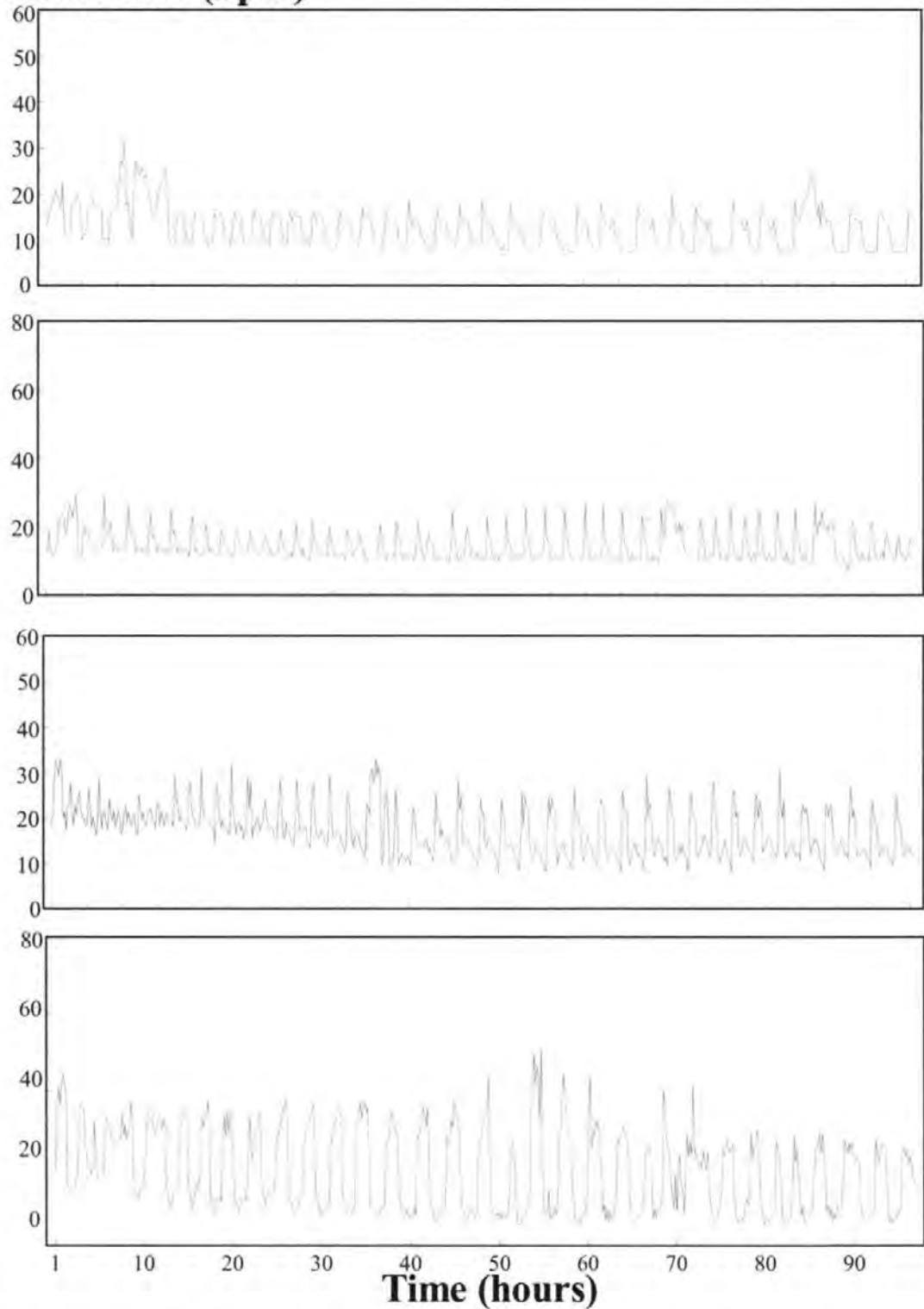


Figure 26. Heart rate recordings (beats per minute) of *Anodonta cygnea* (n=4) over a 4 day (98 h) period during conditions of no food (Bloxxham, unpublished).

Copper Exposure – Lysosomal Retention

There was a significant difference between the pre-exposure and the exposure lysosomal retention values ($P = 0.048$, $n = 27$; see figure 27). The mean retention for the pre-exposure group was 101 minutes. The mean retention time

for the copper exposure was 56 minutes. The mean retention time for the post exposure period was 61 minutes. There was no significant difference between the post exposure retention values and the exposure or pre-exposure values ($P > 0.05$, $n = 27$). Values were in the range of 0 to 135 minutes.

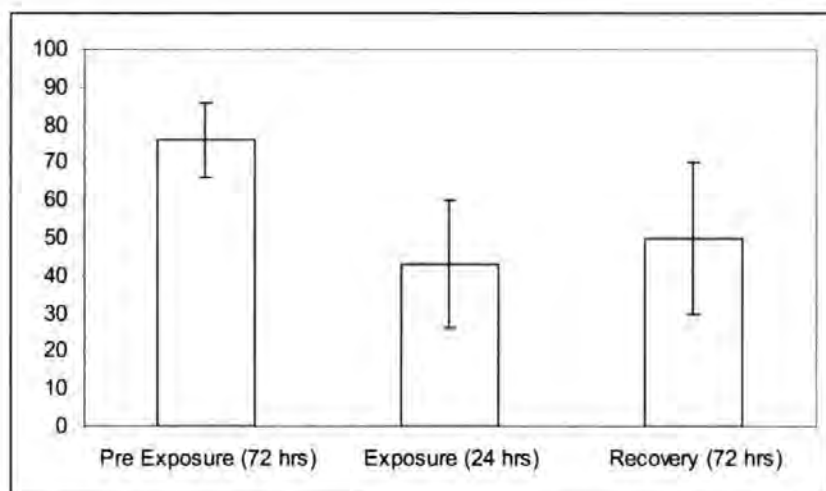


Figure 27. Mean lysosomal retention time of copper exposed (0.05 mg l^{-1}) *Anodonta Cygnea* haemolymph during the experimental period.

Malathion Exposure – Lysosomal Retention

There was a significant difference between the pre exposure and exposure values ($P = 0.04$, $n = 27$), there was also a significant difference between the post exposure and pre exposure values ($P = 0.01$, $n = 27$; see figure 28). There was no significant difference between the exposure and post exposure values ($P > 0.05$, $n = 27$). The mean retention time for the pre exposure group was 95 minutes. The mean retention time for the exposure group was 35 minutes, and the retention time for the post exposure group was 36 minutes. Values were in the range of 0 to 135 minutes.

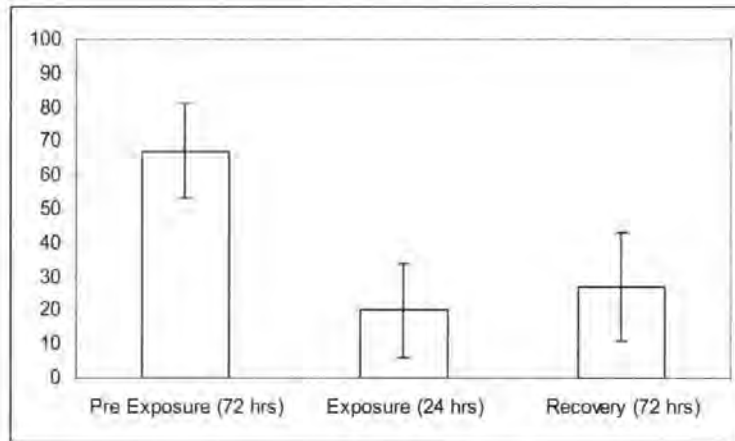


Figure 28. Mean lysosomal retention time of malathion exposed (0.05 mg l^{-1}) *Anodonta Cygnea* haemolymph during the experimental period.

Copper Exposure – Cardiac Activity

The mean heart rate ($n=8$) decreased from 7 bpm to 4 bpm during the 24 h exposure period (see figure 29). During the post exposure period the mean heart rate increased to 9 bpm.

The mean maximum heart rate decreased during the exposure period from 19 bpm to 14 bpm. The mean heart rate increased to 26 bpm during the 72 h post exposure period (see figure 30). This trend was also reflected in the standard deviation of the mean (2.9, 2.5 and 4.7 respectively). Analysis of variance showed that the heart rate during the period of exposure was significantly different than during pre- and post exposure ($P = 0.0027$). The maximum heart rate and the standard deviation of the mean heart rate were also significantly different during exposure to copper compared with 72 h pre and post exposure. ($P = >0.001$ and, $P=0.0015$ respectively; see figures 31 and 32).

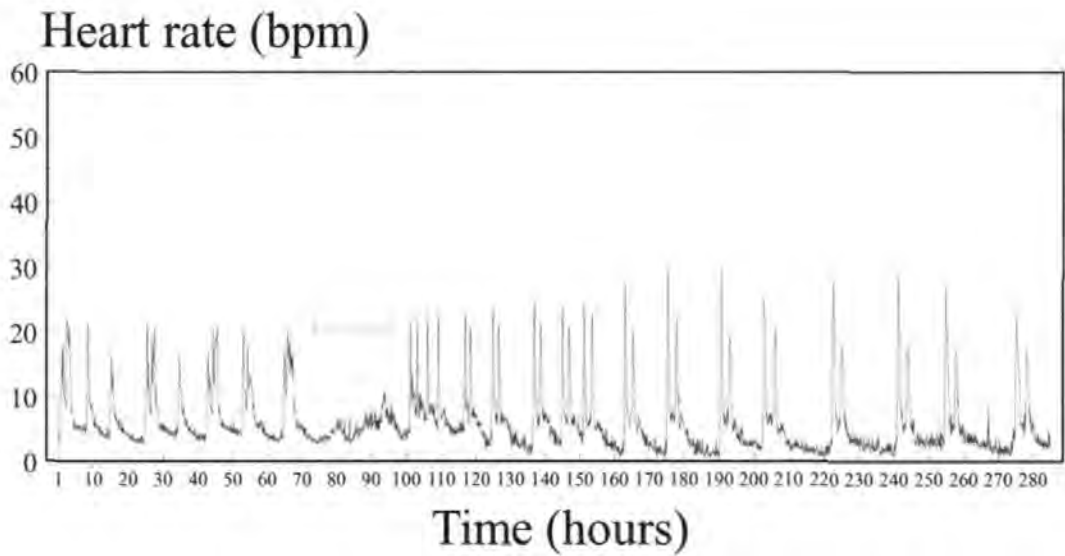


Figure 29. Twelve day (290 h) heart rate recording (beats per minute) of *Anodonta cygnea*. The arrow indicates the addition of copper (0.05 mg l^{-1}) for a 24 h exposure period (Bloxxham, unpublished).

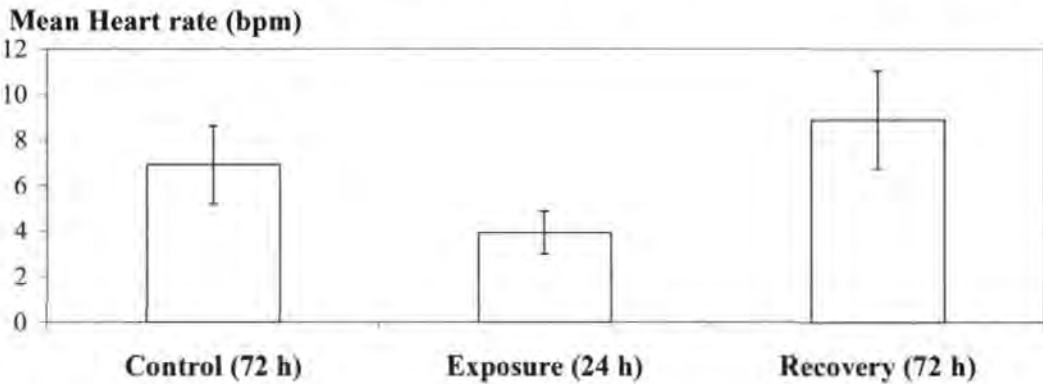


Figure 30. Mean heart rate of *Anodonta cygnea* before, during, and after exposure to copper (0.05 mg l^{-1}).

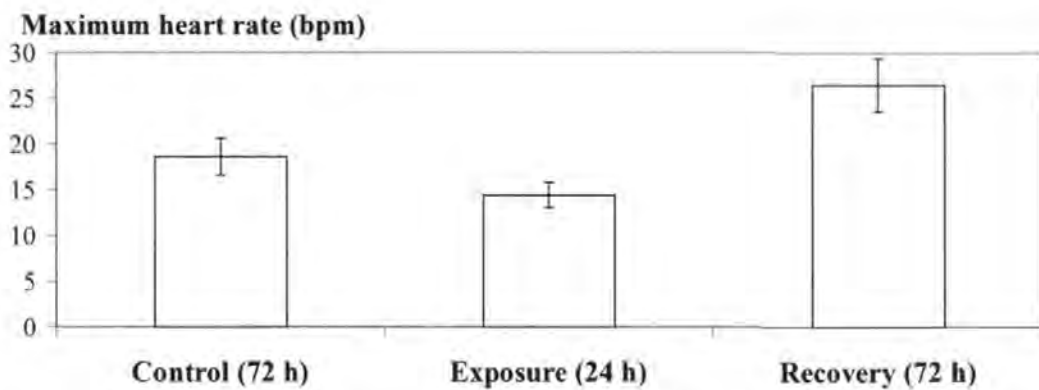


Figure 31. Maximum heart rate of *Anodonta cygnea* before, during, and after exposure to copper (0.05 mg l^{-1}). Note the increase in maximum heart rate in the recovery period, post exposure.

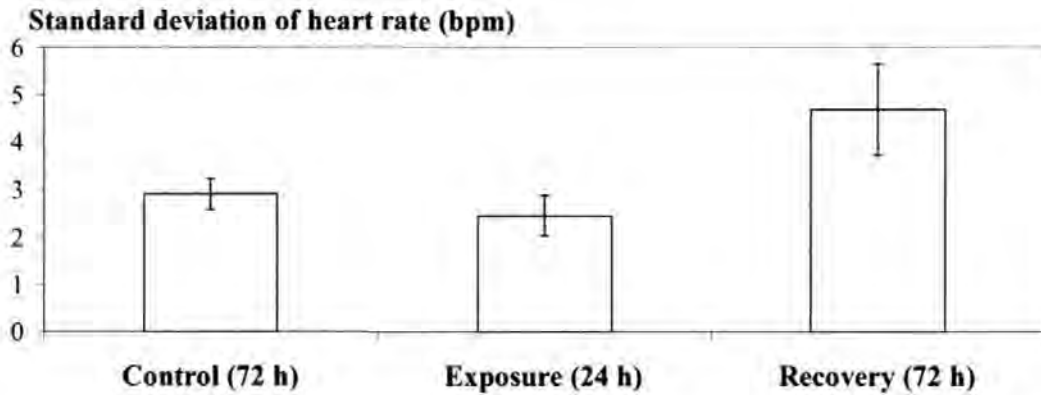


Figure 32. Standard deviation of the heart rate of *Anodonta cygnea* before, during, and after exposure to copper (0.05 mg l^{-1}). Note the increase in variability in the heart rate during the recovery period, post exposure.

Malathion Exposure – Cardiac Activity

Disruption of the basal cardiac rhythm occurred during the 24 h period of exposure to malathion ($100 \mu\text{g l}^{-1}$). Figures 33, 34 and 35 show the mean and maximum heart rate together with the standard deviation of the mean heart rate for the pre-exposure, exposure and post exposure periods. The mean heart rate ($n=8$) decreased from 4.6 bpm during the period prior to the addition of the contaminant to 2.5 bpm during the exposure period. During the post exposure period, the mean heart rate increased to 3.9 bpm. The same trend was apparent for the maximum heart rate (16.8, 11.6 and 22 bpm respectively) and for the standard deviation of the mean heart rate (3.5, 2.0 and 4.2 bpm respectively).

Analysis of variance showed that the mean heart rate and the standard deviation of the mean was significantly different prior to the addition of the contaminant compared to the 24 h exposure period ($P=0.0123$ and, $P=0.0046$ respectively). No statistical significant difference of the maximum heart rate was shown for the pre-exposure compared with the exposure period although there was a statistically significant difference between the 24 h exposure period and the 72 h post exposure period ($P=0.0287$).

Mean heart rate (bpm)

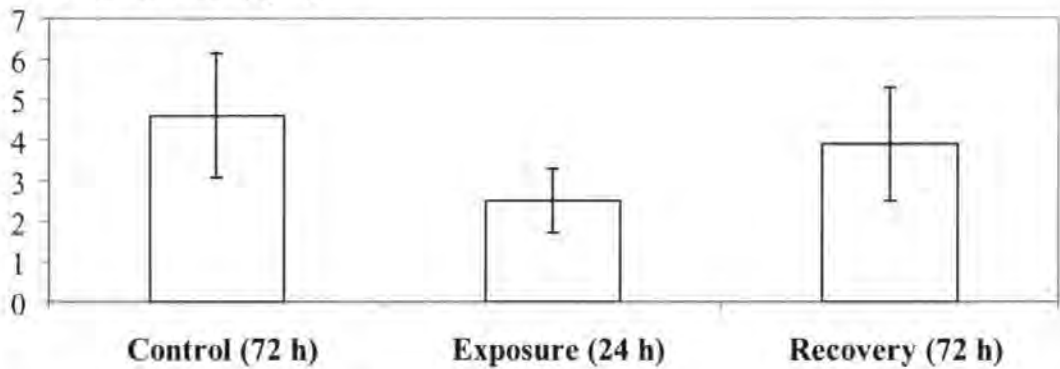


Figure 33. Mean heart rate of *Anodonta cygnea* before, during, and after exposure to malathion (0.05 mg l^{-1}). Note the increase in maximum heart rate in the recovery period, post exposure.

Maximum heart rate (bpm)

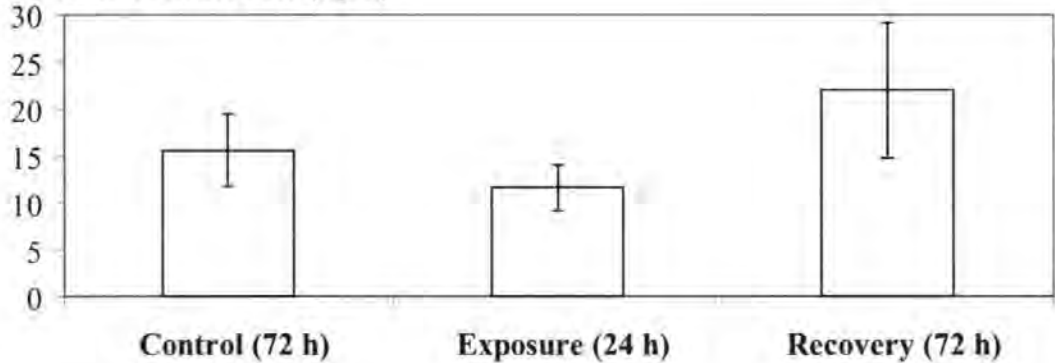


Figure 34. Maximum heart rate of *Anodonta cygnea* before, during, and after exposure to malathion (0.05 mg l^{-1}). Note the increase in maximum heart rate in the recovery period, post exposure.

Standard deviation of heart rate (bpm)

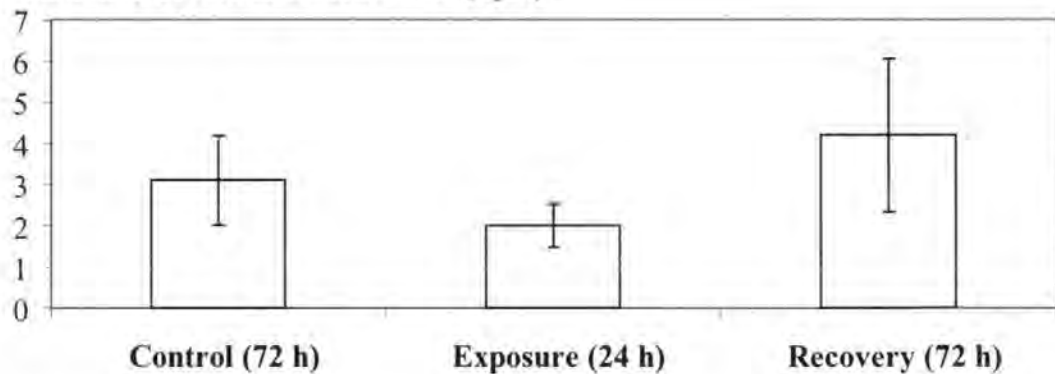


Figure 35. Standard deviation of the heart rate of *Anodonta cygnea* before, during, and after exposure to malathion (0.05 mg l^{-1}). Note the increase in variability in the heart rate during the recovery period, post exposure.

5.6 Discussion

The results show that the addition of copper and malathion coincides with a significant reduction in heart rate activity and lysosomal membrane integrity. There is an indication of a recovery in the heart rate after exposure for both the copper and malathion exposed animals. There is no significant recovery of the lysosomal integrity during the post-exposure period and a general trend of reduced lysosomal integrity.

Anodonta cygnea has been used in a number of studies to examine the toxicological effects of contaminants on freshwater bivalves (Salenki, 1976, 1977; Salenki and Varenka, 1976, 1978; Salenki and Balogh, 1989; Tallandini *et al.*, 1986; Hemelraad *et al.*, 1985; Hemelraad *et al.*, 1990) and their potential as sentinel organisms for biomonitoring has been suggested (Crane *et al.*, 1995; Shalanki, 1985). Other members of the Unionidae have also been examined in terms of pathological response to pollutants (Kulshrestha, *et al.*, 1970; Suzuki, 1986; Holwerda *et al.*, 1985; Hansten *et al.*, 1996). A number of studies have examined the effect of copper on *Anodonta cygnea* (Salenki and Balogh, 1989; Salenki and Varenka, 1976; Tallandini *et al.*, 1986). However, only Salenki and Varenka (1978) have examined the effects of pesticides on *Anodonta cygnea* and the effects of malathion have only been examined on other species of freshwater Unionidae (Keller and Ruessler, 1996).

Only a small number of studies have attempted to characterise the haemocytes of freshwater mollusca (Narain *et al.*, 1985; Eble and Sampson, 1994). This work has allowed the identification of granulocytic haemocytes in the blood of *Anodonta cygnea* by the application of both the neutral red probe and the examination of the blood cells using the molecular probe BODIPY-FI-verapamil.

This has confirmed the existence of lysosomes in the blood cells of the *A. cygnea* and a morphology of the haemocytes which is very similar to that found in marine species of mollusca.

The lysosomal response to contaminants has been examined extensively in fish (Lowe, *et al*, 1992), molluscs (Lowe *et al*, 1995) and to a lesser extent in crustaceans (Astley *et al.*, 1999). The findings shown here for the freshwater bivalve *Anodonta cygnea* show similar trends to those recorded in marine species. Reduced lysosomal integrity has been recorded, both in the laboratory and the field, as a response to contaminants in a variety of different invertebrate cell types. The lysosomal component of the Unionidae blood cells (haemocytes) has been shown to be responsive to a wide range of pollutants including organic xenobiotics (Suzuki, 1986; Holwerda *et al.*, 1985) and trace metals (Tallendini *et al.*, 1986; Hemelraad *et al.*, 1985, 1990). The lysosomal assay used here has been previously used on only one other freshwater species, the snail *Viviparus contectus*, by Svendsun and Weeks (1995). The lysosomal component of the haemolymph of *Anodonta cygnea* has not been examined in terms of pathological response to pollutants, nor in conjunction with physiological monitoring. Furthermore, the application of the neutral red assay to elucidate the lysosomal response represents a novel application of this technique, in terms of the chosen organism.

Little work has been carried out on the long term continuous recording of heart rate in bivalve molluscs. Generally heart rate monitoring has involved invasive impedance cardiography and recordings have been made for only a few hours at a time (Trueman, 1967; Coleman, 1974; Depledge, 1984), although longer-term recordings have been made. Long term heart rate monitoring has been

investigated to a greater extent in crustaceans such as the common shore crab, *Carcinus maenas*, and the fresh water crayfish *Astacus astacus*.

Copper has been shown to cause increases in crab heart rate. (Bamber and Depledge, 1997; Lundebye and Depledge, 1998) Davenport (1977) reported that marine mussels exposed to copper for 6 hour periods exhibited decreased heart rates. However, these always coincided with valve closure and it was concluded that bradycardia rather than the direct effect of copper was causing the alteration in cardiac rhythmicity (Davenport, 1977). Only Ramana-Rao *et al.* (1983) have examined the effects of contaminants on the heart rate of freshwater bivalves. Salenki and Varenka (1978) examined the rhythmicity of valve movement as a response to copper in *Anodonta cygnea*.

Valve movement has also been used to examine the effects of environmental factors on *Anodonta cygnea* (Salenki, 1977). The addition of sub-lethal doses of copper to *Anodonta cygnea* was observed to cause a reduction in the duration of active periods (Salenki and Varenka, 1976). Salenki and Balogh (1989) reported that $100 \mu\text{g l}^{-1}$ of copper caused a shortening of the active periods of *Anodonta cygnea*. These observations are in agreement to the results shown here, where heart rate activity was observed to fall after copper exposure.

A number of different insecticides were observed to cause a reduction in survival and pumping behaviour of *Anodonta cygnea* (Salenki and Varenka, 1978). Keller and Ruessler (1997) reported a 96-h LC50 value for three species of adult Unionid mussels greater than 350 mg l^{-1} for malathion. The 48-h LC50 for *Daphnia magna* is $1 \mu\text{g l}^{-1}$. Malathion was observed to cause an inhibition in the rate of heart beat in the freshwater mussel *Lamellidens marginalis*

(Ramana-Rao *et al.*, 1983). Ciliary activity in *Lamellidens marginalis* is also inhibited by malathion (Kabeer-Ahamad *et al.*, 1979).

Donkin *et al.* (1997) showed a marked reduction in acetylcholinesterase activity in the gill of the marine bivalve *Mytilus edulis* after a three day exposure to 0.1mg l⁻¹ dichlorvos, a compound very similar to malathion in terms of solubility and log octanol/water coefficient (log P). At this concentration dichlorvos was also shown to cause a reduction in feeding rate in *Mytilus edulis* (Donkin *et al.*, 1997). After exposure to carbaryl, another acetylcholinesterase inhibitor, Donkin *et al.* (1997) showed that reduction in feeding rate was possibly a result of the narcotic effect of this pesticide, with only a small enhancement of toxicity as a result of its neurotoxic properties.

Only a small number of studies have examined the effect of pesticides on the lysosomal system of freshwater organisms (De and Sur, 1990; Baby-Shaikila *et al.*, 1993; Oulmi *et al.*, 1995). De and Sur (1988) found that chronic exposure of aldrin to the freshwater crab *Paretelphusa spinigera* caused changes in the internal composition of midgut gland lysosomes. Oulmi *et al.* (1995) and Baby-Shaikila *et al.* (1993) both reported lysosomal disruption in freshwater fish after pesticide exposure. Prevot and Gobillard (1985) reported that malathion caused the formation of lysosomes and lipidic vesicles after exposure to the dinoflagellate *Prorocentrum micans*. Banerjee *et al.* (1978) reported the degeneration of tissues, and changes in protein levels, in fish from malathion contaminated environments.

A number of studies have examined the effect of copper on the lysosomal component of marine organisms (Harrison and Berger, 1982; Viarengo *et al.*, 1984, 1985; Moore *et al.*, 1984; Ringwood *et al.*, 1998; Shepard and Bradley, 2000). A smaller number of studies have investigated the lysosomal response

to copper in freshwater organisms (Svendsen and Weeks, 1995; Suresh *et al.*, 1993); and Tallendini *et al.*, (1986) reported the accumulation of copper in the lysosomal component of *Anodonta cygnea*. A reduction in membrane stability, a symptom of lysosomal pathology, was observed here following copper exposure and this trend has been reported for marine bivalves (Etxeberria *et al.*, 1994; Ward, 1990). The lysosomal compartment of marine bivalves has been shown to be involved with the detoxication of copper by the autophagic sequestration of metallothionein (Viarengo *et al.*, 1985). Metallothionein has also been shown to occur in the tissues of *Anodonta grandis grandis* after cadmium exposure (Malley *et al.*, 1993). Thomson *et al.* (1985) showed the selective accumulation of copper in the lysosomes of blood haemocytes in the oyster *Crassostrea gigas*. It is suggested that copper is being selectively taken up via the lysosomal autophagic pathway, as a result of Cu-detoxication, in *Anodonta cygnea* and that this is contributing to the destabilisation of the lysosomal membranes. Copper toxicity is caused by reactive oxygen species (ROS) that probably attack the lysosomal membrane (Moore, 1994b). Harrison and Berger (1982) demonstrated that copper exposure caused adverse changes in the lysosomal latency of *Mytilus edulis* digestive cells.

No previous studies have attempted to use heart rate monitoring and lysosomal integrity in tandem nor in a non-invasive mode. The results indicate a pathological response as a result of the sub-lethal exposure to copper and malathion. The use of a physiological and a sub-cellular biomarker suggests that alteration at the sub-cellular level is associated with changes in physiological activity. If, through damage to the lysosomal component, fusion and enzymatic processes are impaired then nutritional functions and normal levels of autophagy may be perturbed (Moore, 1994). The significance of this

lysosomal disruption, and whether it is causing physiological change or occurring simultaneously with it cannot be demonstrated from these data. However, there is considerable evidence that lysosomal dysfunction and damage is a good predictor for higher level pathologies (Moore, 1990, 2002) and these data support this. During the post exposure period there was a significant return to pre-exposure cardiac activity but no significant recovery of lysosomal membrane retention for the neutral red probe. This could represent a difference in sensitivity between the two techniques and would suggest that, although associated, there is no direct linkage. However, the lysosomal failure to recover may have implications for the general health of the organism as lysosomal function has been shown to be directly involved with the cellular immune process in marine mussels (Grundy, 1996)

The future use of *Anodonta cygnea* as a freshwater analogue of *Mytilus edulis* in biological monitoring programmes is proposed. Shalanki (1985) suggested the use of *Anodonta cygnea* as an indicator of trace metal pollution by the examination of metal accumulation in its tissues. However, this is providing information only on tissue residue and not on biological response to pollutant exposure. This work has shown that it will be possible to use *Anodonta cygnea* as a sentinel organism for the non-destructive, long-term, monitoring of freshwater regimes. The results obtained here are also encouraging in that they demonstrate the conservation of certain biological responses between different species of mollusc and the positive implication that this has for environmental monitoring programmes generally.

6.0 BIOMARKERS OF BIOCHEMICAL AND CELLULAR STRESS IN SHORE CRABS (*CARCINUS MAENAS*): AN *IN SITU* FIELD STUDY.

6.1 ABSTRACT

Biomarkers are potentially useful diagnostic tools for identifying exposure to physical, chemical, and environmental stresses. Although various biomarker techniques have been developed in the laboratory their application in the field has been limited. In the present study a suite of techniques; measurements of metallothionein induction, lysosomal integrity, and osmoregulatory ability, have been used in an attempt to provide general assessment of environmental quality. An emphasis was placed on *in situ* application. Five estuarine sites were studied, two clean sites, two relatively contaminated urban sites, and one intermediate. Shore crabs, *Carcinus maenas*, were collected and haemolymph samples taken for the lysosomal and osmoregulatory assays. A further set of animals were sacrificed and tissue preserved for the metallothionein analysis. Lysosomal integrity was significantly different in crabs from clean sites and the urban sites ($n=40$, $p<0.05$). The potential value of the biomarker approach is that it could provide data for preliminary environmental impact assessment and is easily applicable in the field.

6.2 Introduction

Biomarkers of pollution exposure and effect have been suggested as a means of establishing the status of marine ecosystems. However, finite resources and possible immensity of the problem force a rationalisation of the spending powers of monitoring bodies. Consequently, there is a need for sensitive yet

cost effective biomarkers that might be used in ecological risk assessment (Depledge, 1994).

The objective of this study was to rapidly discern the “biological health” of a number of different field locations, in a manner which could aid the initial stages of a risk assessment. Relative environmental quality at these sites was to be determined by the comparison of the biomarker responses. An emphasis was placed on evaluating the *in situ* application of the biomarker techniques and, provide useful biological information.

Three techniques were chosen for study, a measurement of haemolymph lysosomal stability, an assay for metallothionein concentration, and an assessment of osmoregulatory ability.

The common shore crab, *Carcinus maenas*, was utilised in this study as it is widely distributed throughout most estuaries. This study provided the opportunity to further investigate its role as a sentinel organism. Lysosomes are highly conserved multifunctional cellular organelles containing a variety of hydrolases. Metallothionein (MT) has had limited use as a biomarker in crustaceans (Pedersen *et al*, 1996). Metallothioneins are low weight proteins capable of binding metals in the cells of a wide range of organisms. Field studies have shown that MT concentrations may be a useful indicator of an organism's exposure to metals (Hogstrand and Haux, 1990). The lysosomal system has been shown to be involved in the metabolism of many metals, either through sequestration and binding within the lysosomes, or as a target of their toxicity (Lowe and Moore, 1979; Viarengo *et al*; 1987).

Measurement of crustacean haemolymph osmolality has previously been investigated as an indicator of exposure to chemical stress (Bamber and Depledge, 1997). Crabs hyper-regulate their haemolymph in response to falling external salinity. Using a challenge protocol, (i.e. exposing crabs to low salinity) deleterious effects on physiology associated with osmoregulation can be identified by measurement of haemolymph osmolality. The gross effect of water quality on the process of osmoregulation in crabs can thus be established.

6.3 Materials and Methods

Five sites around the south west of the United Kingdom were chosen for study. The Plym Estuary and Pomphlett Creek receive diffuse and point sources from a number of industrial and urban sources. The Restronguet site is rich in metals from past and present mining activity. The Erme and the Avon are impacted by a smaller number of domestic sources and some boating activity.

Sixteen intermoult male crabs of carapace width 50 - 60 mm were obtained from baited drop nets. The neutral red and osmoregulation challenge tests were conducted *in situ* on eight crabs. Eight further crabs were sacrificed, dissected, and the midgut gland snap frozen for metallothionein analysis. A van was suitably equipped so that techniques could be carried out *in situ* and tissue safely stored.

Methods for neutral red, metallothionein and osmoregulatory ability were conducted as described in section 2.0.

6.3 Results

Neutral red retention values were in the range of 0 to 100 minutes. These are typical values for crabs haemocytes as obtained in laboratory studies (Wedderburn, unpublished). There was a significant difference between the

lower mean retention time of Pomphlett Creek (41 minutes, $n=16$, $P < 0.05$) and the higher values of the Erme (81 minutes) and Avon (77 minutes) estuaries. There was also a significant difference ($P < 0.05$) between the Plym (56 minutes) and the Erme (81 minutes) estuary (Fig. 36).

There were no statistically significant differences in metallothionein concentration. Metallothionein values (Cheung, unpublished) for the Pomphlett site were the highest (301 $\mu\text{g/g}$ wet weight tissue), and the Erme estuary had the lowest (228 $\mu\text{g/g}$ wet weight tissue). The Avon and Restronguet sites had values of 230 and 238 $\mu\text{g/g}$ wet weight tissue, respectively.

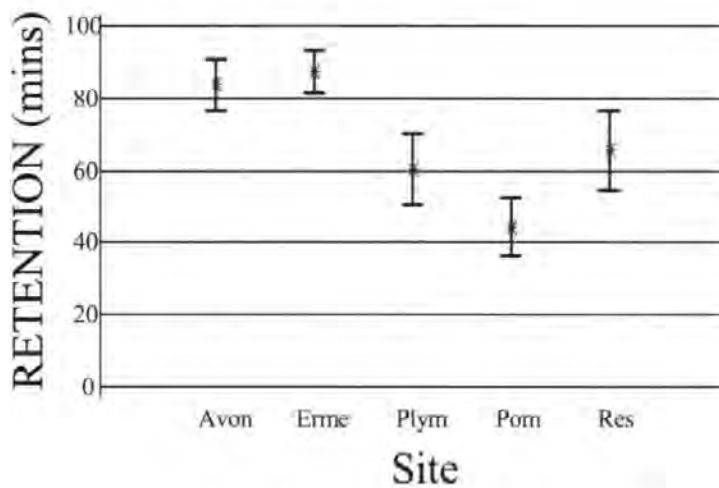


Figure 36. *Carcinus maenas*. Box and whisker plot showing lysosomal neutral red retention, in minutes, by sampling sites. Significant differences exist between the Pomphlett creek ('Pom') and the Avon and Erme sites, and the Plym and Erme sites ($P < 0.05$, 95% confidence interval, Kruskal-Wallis). Res= (Restronguet Creek).

Significant differences in osmolality values ($P < 0.01$) exist between the Avon and Erme pre-stress values and the Plym, Pomphlett, and Restronguet pre-stress values. There was no significant difference between any of the osmolality end points ($P > 0.05$) (Figs. 37 and 38).

The location and number of lysosomes in the circulating haemocytes of crabs was previously determined using the fluorescent probe BODIPY-FL-verapamil (Molecular Probes, Oregon; Wedderburn, unpublished).

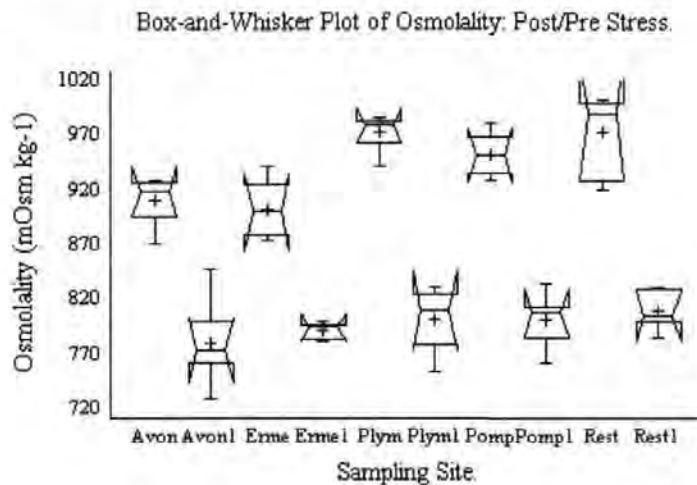


Figure 37. *Carcinus maenas*. Osmolality values in haemolymph, pre/post-exposure of three hours to distilled water. "1" ending indicates values obtained after three hour challenge to distilled water. A significant difference exists between the Avon and Erme values to other sites ($n=40$, $p<0.05$, 95% confidence interval, Kruskal-Wallis). Rest=Restronguet.

6.4 Discussion

The neutral red lysosomal integrity assay showed that animals from the Pomphlett and Plym sites had significantly reduced retention times for the neutral red probe in their haemocytic lysosomes. The Restronguet, Avon, and Erme exhibited increasing retention times and thus a decrease in lysosomal perturbation. Reduced retention times have been shown to be associated with inputs of urban runoff, waste water and leachate discharges (Lowe *et al.*, 1992,1995).

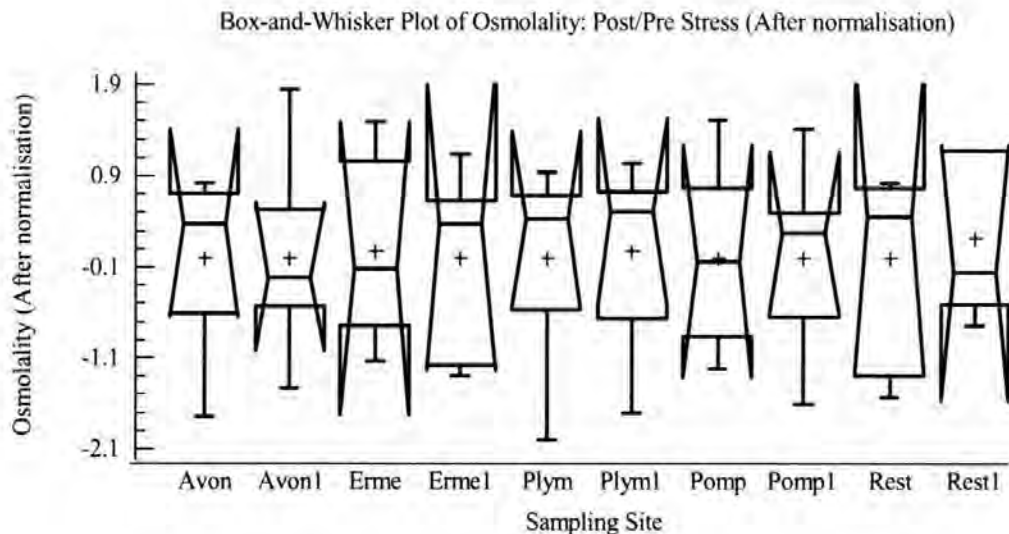


Figure 38. *Carcinus maenas*. Osmolality values in haemolymph, after data transformed by normalisation to remove difference in salinity starting values. No significant differences exist between the data ($n=40$, $p>0.05$, 95% confidence interval, Kruskal Wallis).

The MT data presented here are consistent with the findings of other authors, using the same sampling sites and technique. Pedersen *et al.* (1996) found no significant difference in the levels of MT in crab midgut between the Restronguet site, which is contaminated with metals, and the less contaminated Yealm, and Avon sites.

The osmolality values reported here are within the expected range for crabs (Siebers *et al.*, 1982). The osmolality data obtained from the five sites does not allow conclusions to be drawn concerning site related osmoregulatory ability of the crabs. The results are confounded by the significant difference in the osmolality value for the haemolymph, between sites, at the beginning of the experiment. This could be a result of hydrographic differences in salinity at the sampling sites.

The neutral red technique has been shown to perform well when used with haemolymph from *C. maenas*. This use of crabs is significant, given the important role that this organism has in many estuarine ecosystems. It is curious, however, that the heightened levels of metals at the Restronguet site

are not associated with a similar increase in the levels of midgut metallothionein or a reduction in lysosomal stability. The sensitivity of the technique used here, for detecting levels of metallothionein, probably needs further investigation to understand the principles underlying its use. Metallothionein concentration has been found to be highly variable among species and differs among tissues and as a function of age (Forbes and Forbes, 1994). Furthermore, Nott (1992) indicated that metals can be rendered biologically unavailable, and non-toxic, by the detoxication activities of marine mussels on an environmental scale. This may be a contributing factor in reducing metallothionein induction in the crabs.

The *in situ* use of osmoregulation ability needs to be more closely examined and may require further adaptations to fully demonstrate the potential which has been indicated in other studies (Bamber and Depledge, 1997).

Although the lysosomal data obtained here cannot be related to one particular contaminant or group of contaminants, it indicates those sites where there is a greater biological effect, as indicated by increased lysosomal perturbation. The trends shown in the lysosomal data presented here are reflected in other studies using the same technique. Reduced retention times, evidence of lysosomal membrane dysfunction, have been shown to be sufficiently sensitive to discern site-related differences in environmental quality (Lowe *et al.* 1995). Krishnakumar *et al.* (1994) reported a reduction in lysosomal stability in mussels collected from urban sites. This technique's sensitivity, using the haemolymph of *C. maenas*, needs to be further addressed, though the results found here are encouraging as an indicator of gross environmental quality. The lack of any significant lysosomal response from the animals collected from the

Restronquet site could possibly be due to an adaptation of these animals to cope with the historically high background levels of metals, or the lack of significant levels of biologically available pollutants.

In conclusion, by rapidly characterising a site using *in situ* methods, biological effects monitoring programs can be more focused and as such become more cost effective, whilst still providing useful environmental information.

7.0 Metallothionein Levels, Lysosome Integrity and Cardiac Activity in Blue Mussels (*Mytilus edulis*) deployed along the Mersey and Tees Estuaries (UK).

7.1 ABSTRACT

In this study attempts were made to measure metallothionein levels, lysosomal integrity and cardiac activity in *Mytilus edulis* deployed along the Mersey and Tees estuaries to determine whether these biomarkers used together might be utilised in the determination of general environmental quality. In conjunction with the biomarker studies a number of toxicity tests were conducted on the Tees and Mersey estuaries. These can be divided into those examining water column toxicity or sediment toxicity. Those examining water column toxicity were the ECLOX test (a rapid *in vitro* chemiluminescent screening procedure) and the Microtox test (measuring light production resulting from the metabolism of the marine organism *Vibrio fischeri*) (Environment Agency unpublished data). The MT analysis showed no clear trends nor was there any meaningful correlation between the sites. Lysosomal retention was shown to be severely depressed at several sites on the Tees estuary, although no significant differences were observed between sites on the Mersey. Significantly reduced heart rates were observed from mussels from the Mersey and Tees. Strong correlations were found for the neutral red results and the Microtox, *T. battagliai*, *C. Volutator* and oyster bioassays carried out on the Tees estuary. However, for the Mersey estuary, the comparison of the neutral red assay and the toxicity bioassays revealed only extremely weak correlations.

7.2 Introduction/Aims

This study aimed to measure metallothionein levels, lysosome integrity and cardiac activity in mussels deployed along the Mersey and Tees estuaries to determine whether these biomarkers used together might be utilised in the determination of general environmental quality. In conjunction with the biomarker studies a number of toxicity tests were conducted on the Tees and Mersey estuaries. These can be divided into those examining water column toxicity or sediment toxicity. Those examining water column toxicity were the ECLOX test (a rapid *in vitro* chemiluminescent screening procedure) and the Microtox test (measuring light production resulting from the metabolism of the marine organism *Vibrio fischeri*) (Environment Agency unpublished data). Studies were also made of oyster embryo larval development (conducted on the Pacific oyster *Crassostrea gigas*) and *Tisbe battagliai* reproduction (Environment Agency unpublished data). Tests conducted on the estuarine sediment were *Corophium volutator* growth and lethality and lug worm, *Arenicola marina*, cast formation and lethality (Environment Agency unpublished data).

The nature of this study allows several issues to be addressed. In the first instance the neutral red assay can be compared to the cardiac and metallothionein assays allowing some comparison of the ability of these techniques to relate to one another and discriminate between sites. The results of these putative biomarkers can also be compared with the more 'classical' toxicity techniques using model laboratory organism responses to sediment and water samples. The comparison of the chosen biomarkers with the lethality tests will allow important information to be gained regarding the sensitivity of the biomarker techniques. The neutral red test has not been previously compared with a study comprising such a range of toxicity tests or differing sites. Further, by comparing the biomarker techniques with the results of

biological abundance data, the effectiveness of these techniques will be ascertained. The result of such a comparison will be extremely useful as it will add to the debate on the ecological relevance of these techniques. The combination of all these approaches makes this a uniquely novel study.

7.2 Materials and Methods

7.2.1 Experimental animals

Metallothionein

64 mussels (*Mytilus edulis*) were deployed at eight separate sites along the Tees estuary (8 mussels per station; 4 opposite sites on either side of the estuary) between 30th Aug - 27th Sep 1996 (mussels from site 5 were not collected because of tidal conditions). Similarly, 64 mussels were deployed along the Mersey estuary between 25th Sep - 23rd Oct 1996 (mussels from site 8 were not collected because of tidal conditions). Following deployment, the mussels were dissected (on the same day as collection) and the whole tissues immediately frozen in liquid nitrogen. These samples were stored at -80°C until MT analysis.

Lysosomal integrity and Cardiac Activity

136 mussels (shell length 4.15-4.87 cm) were deployed along the Mersey estuary (same sites as MT) between 28th Aug - 25th Sep '96. Similarly, 136 mussels (shell length 4.11-5.19 cm) were deployed along the Tees estuary (same sites as MT) between 27th Sep - 25th Oct '96. 9 animals were used per station for the neutral red assay and 8 animals per station for heart-rate measurements. Following deployment, the mussels were transferred, to an aquarium (on the same day as collection under cool conditions) and maintained

in aerated seawater from their respective sites at 15°C in 2 L tanks (mussels from site 3 on the Tees were not collected because of tidal conditions). The animals were not fed during a one day holding period or during subsequent experiments.

7.2.2 Metallothionein analysis

Metallothionein analysis was conducted as described in section 2.3 by V.V. Cheung (Research assistant, University of Plymouth).

7.2.3 Neutral red assay

The neutral red test was conducted as described in section 2.0, with no modifications.

7.2.4 Cardiac activity

The mean resting heart rate of each individual was measured over 7 h using the technique described in section 2.2. Care was taken to ensure that the mussels were not disturbed during the recording periods. Bartlett's variance check indicated that mean heart rates of individuals were not normally distributed (because the heart stops beating periodically). Therefore, distribution-free non-parametric statistics were used (i.e. Kruskal-Wallis test).

7.3 Results

7.3.1 Metallothionein Concentrations

Mussels from sites 1, 4 and 7 on the Mersey had significantly elevated mean MT concentrations when compared with the other stations ($P < 0.05$; see figure 39). Mean MT concentrations ranged from 86 - 114 $\mu\text{g/g}^{-1}$ for these sites. The other sites had mean MT concentrations ranging from 60 - 68 $\mu\text{g/g}^{-1}$.

Figure 39. shows the mean levels of MT in mussels transplanted in the Tees. There were no significant differences in mean MT concentrations among the sites ($P > 0.05$), although all MT levels were all greater than $86.6 \mu\text{g/g}^{-1}$.

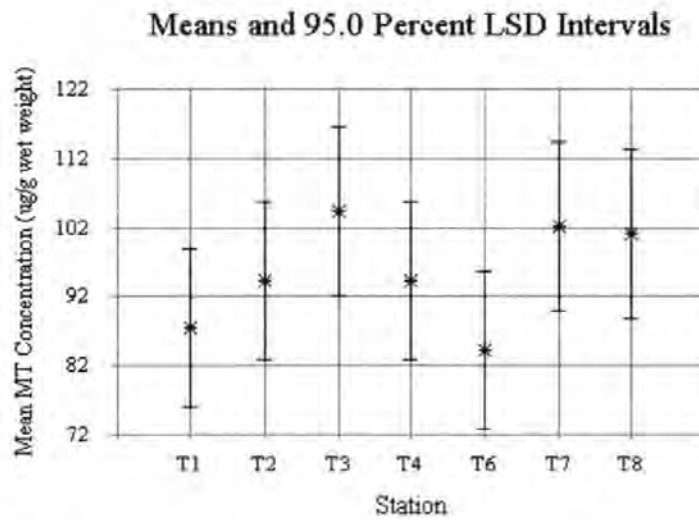


Figure 39. Mean levels of metallothionein in mussel tissue collected from animals deployed at sampling stations 1-8 on the Tees estuary. One way ANOVA shows that there is no significant difference between any of the sites ($p > 0.5$).

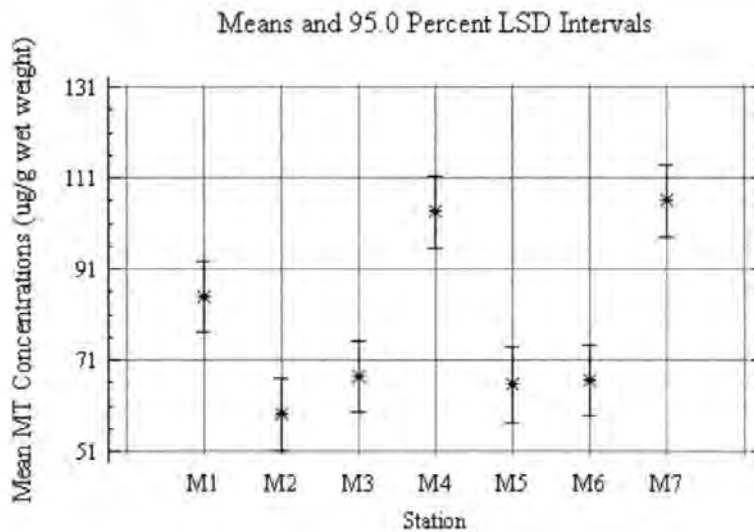


Figure 40. Mean levels of metallothionein in mussel tissue collected from animals deployed at sampling stations 1-7 on the Mersey estuary. One way ANOVA shows that there are significant differences between sites ($p < 0.5$).

7.3.2 Lysosome integrity

Overall, there were no significant differences among the mean retention times for any of the stations on the Mersey ($P > 0.05$; see fig. 41). However, multiple range tests indicated that mussels from site 5 had significantly higher mean retention times (59 minutes) than sites 6, 7 and 8 (ranging from 38-42 minutes; $P < 0.05$).

Sites 1, 2 and 8 on the Tees had significantly higher mean retention times compared with the remaining sites ($P < 0.05$; see fig 42). There were no significant differences between sites 4, 5, 6 and 7 ($P > 0.05$). Sites 5 and 6 had the lowest mean retention times (41 and 39 minutes, respectively).

Average retention times were lower in the Mersey mussels when compared with the Tees.

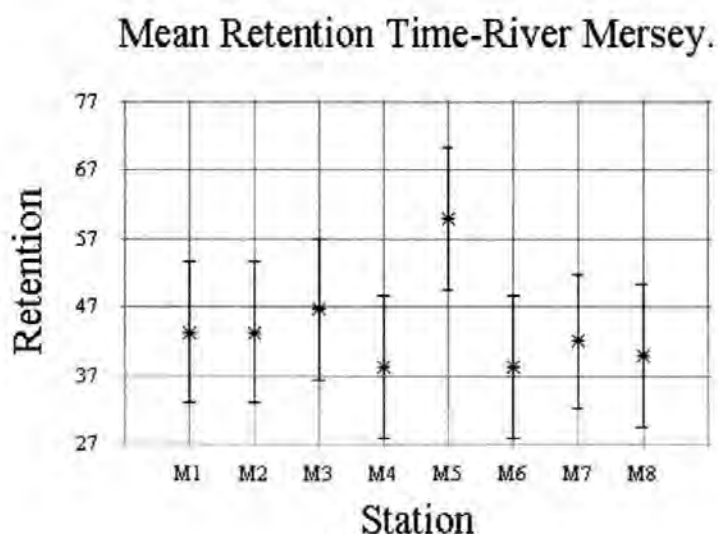


Figure 41. Mean retention time values and 95% confidence intervals for *M. edulis* relocated into the River Mersey. $n=9$ (per station), a one-way ANOVA shows no significant difference

between the means ($P>0.05$) and Kruskal Wallis analysis indicates no significant difference between the medians ($P>0.05$).

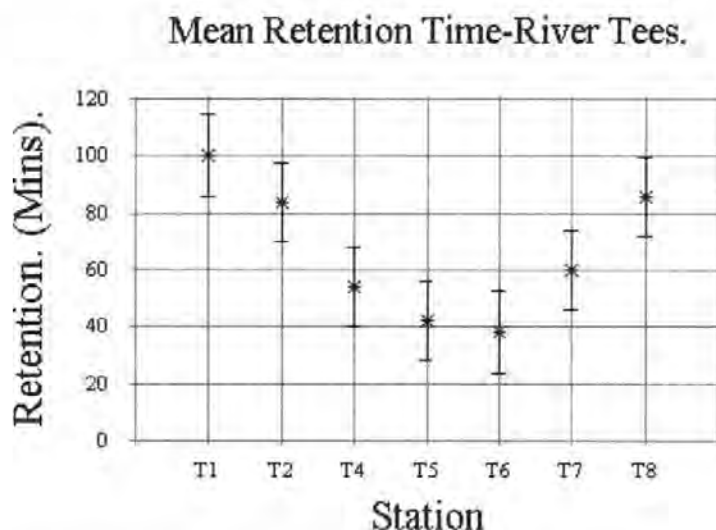


Figure 42. Mean retention time values and confidence intervals for *M. edulis* relocated to the River Tees. $n=9$ (per station), one-way ANOVA indicates a significant difference between the means ($P=0.0001$) and Kruskal Wallis indicates a difference between the medians ($p=0.0009$).

7.3.3 Cardiac Activity

Heart rates of mussels were found to be irregular over the first hour of cardiac recording, this was almost certainly due to handling stress caused by transducer attachment, and heart rates were observed to stabilize. Thus, the first hour of recordings were not included.

Statistical examination of the group of mussels from the Mersey indicate that median heart rates were significantly lower at site 8 ($P < 0.05$). Heart rates ranged from 2-6 beats per minute at this site. Median heart rates were not significantly different among the other sites ($P>0.05$). The majority of the mussels at these sites had median heart rates ranging between 20-28 beats per minute.

For the groups of mussels deployed in the Tees, there were significant differences in median heart rates among the different sites ($P<0.05$). Mussels from sites 1, 4 and 5 had significantly lower median heart rates than sites 2, 7 and 8 ($P < 0.05$; there was high inter-individual variability at site 6).

Nevertheless, with the exception of site 5, the majority of mussels had median heart rates ranging between 20-28 beats per minute. At site 5, 4 out of the 8 mussels had median heart rates lower than 15 beats per minute.

7.3.4 Toxicity Tests

At the estuarine sites the laboratory based water column assays were shown to discriminate between stations (Environment Agency unpublished data) The sediment based bioassays were also shown to discriminate between sites (Environment Agency, unpublished data). Of these, the *Corophium in situ* bioassay performed the best, in terms of discrimination and also correlation with the patterns seen in the other data. Generally, the OEL, ECLOX, Microtox, and the 10 day *C. volutator in situ* bioassays had very high rank correlations in their ability to identify possibly stressed sites (Environment Agency, unpublished data). Assays were performed by the Environment Agency using protocols described by Williams (1993) and Thai (1991).

7.3.5 Correlations

Strong correlations were shown to exist between the neutral red test results and a number of the bioassays conducted on the Tees estuary. These were with the comparison of the neutral red assay and, 28 day *C. volutator* ($r = 0.902$, see fig.43), Microtox ($r = 0.817$; see fig. 44), *T. battagliai* lethality ($r = 0.817$), and OEL ($r = 0.76$, see fig. 45). Weaker correlations were shown between the neutral red test and 10 day *C. volutator* lethality ($r = 0.617$), ECLOX ($r = 0.407$), *C. volutator* lethality ($r = 0.225$), and *C. volutator in situ* ($r = 0.032$). For the Mersey estuary the comparison of the neutral red assay and the toxicity bioassays revealed only extremely weak correlations ($r < .30$ in all cases).

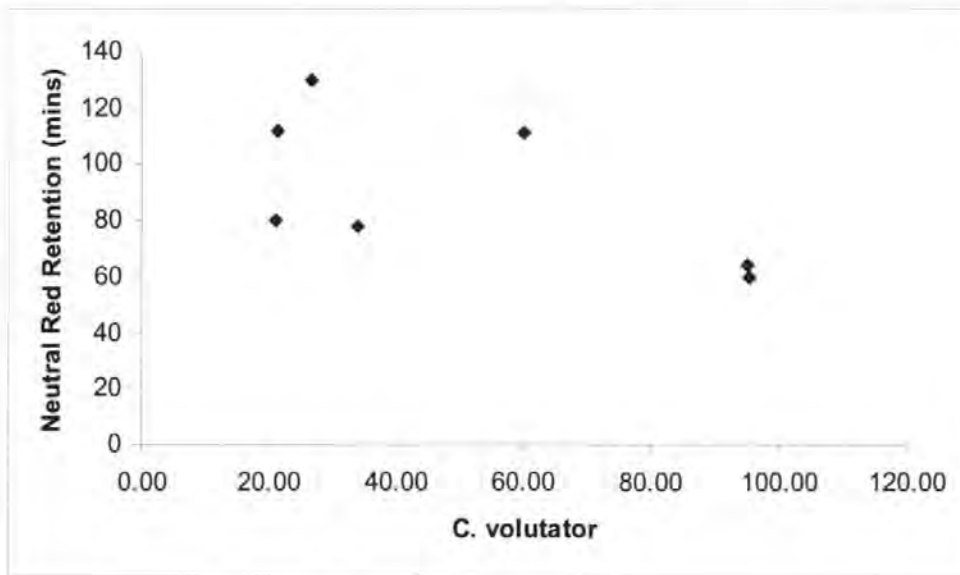


Figure 43. Scatter plot showing the relationship between the neutral retention in *Mytilus edulis* haemolymph with *Microtox* acute bioassay data for water samples taken at the time of mussel collection. ($R = 0.817$).

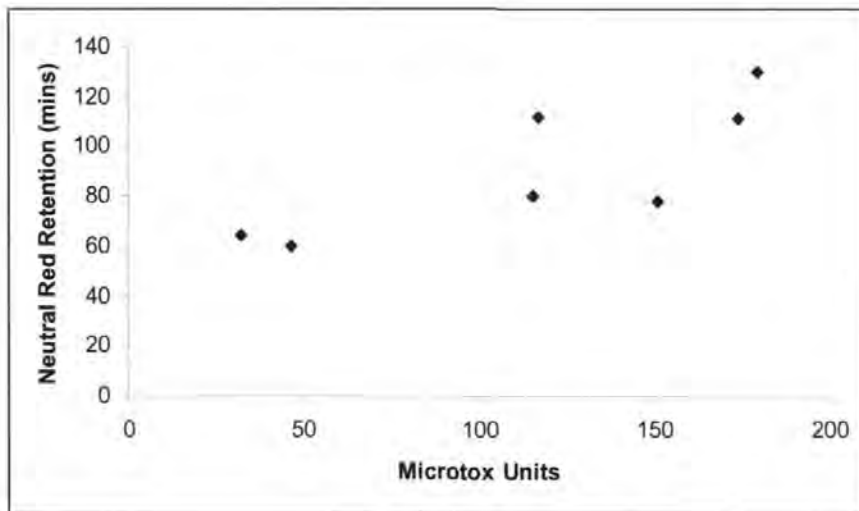


Figure 44. Scatter plot showing the relationship between the neutral retention in *Mytilus edulis* haemolymph with *Microtox* acute bioassay data for water samples taken at the time of mussel collection. ($R = 0.817$).

The neutral red test and the cardiac monitoring were not shown to strongly correlate with the Tees estuary data set and there was no correlation between these data sets for the Mersey estuary. There was no overall correlation between the neutral red assay and the biological survey data. There was, however, some correlation between the neutral red data and several key indicator species, of environmental quality, included in the study. There is no correlation between the neutral red data and the toxicity data for the Mersey. However, the heart rate data for the Mersey was shown to compare well with the toxicity data for the Mersey.

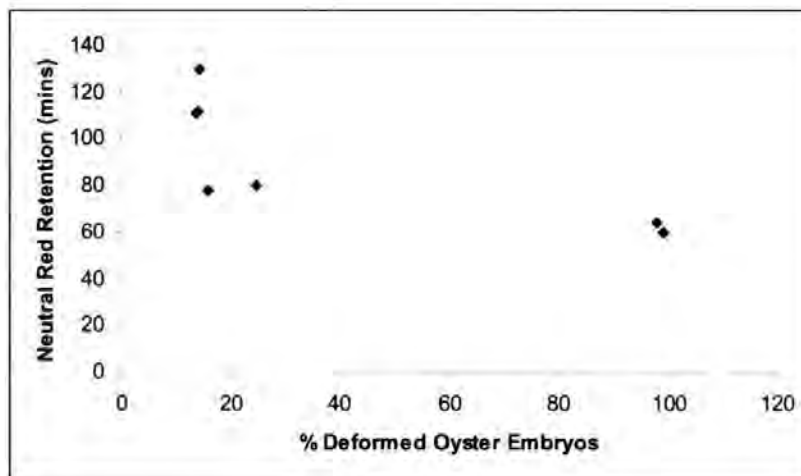


Figure 45. Scatter plot showing the relationship between the neutral retention in *Mytilus edulis* haemolymph with oyster embryo bioassay data for water samples taken at the time of mussel collection. ($R = 0.76$).

7.4 Discussion

MT concentrations in mussels deployed at sites 2, 3, 5, and 6 on the Mersey estuary were within the range found for other mussel populations obtained from clean sites (up to $80\mu\text{g}/\text{g}^{-1}$; Cheung, unpublished). Mussels from the remaining sites on the Mersey, and from all the sites on the Tees, exhibited elevated levels of MT ($>86.6\mu\text{g}/\text{g}^{-1}$). No clear trends could be observed, nor was there any meaningful correlation between the sites or other techniques being utilised. Petrovi *et al* (2001) and Astley *et al* (1999) both demonstrated that the

examination of metallothionein levels was less sensitive than an examination of lysosomal fragility, when used together.

The elevated concentrations of MT present at some of the sites could indicate an adaptive response to metal contamination. However, in future it may be necessary to measure metal concentrations and relative proportion of metals bound to MT in order to gain a more integrated view. Differences may also be due to the temporal difference between the deployment of the mussels used for MT analysis and those transplanted for neutral red and heart rate.

Commonly, in an uncontaminated site, neutral red retention values for molluscan haemocytes have been recorded as being in the range 100-150 min. Lysosomal retention times were <100 min on both the estuaries. Lysosomal retention was shown to be substantially depressed at sites 4, 5, and 6 on the Tees estuary (<55 minutes). The mean retention values for the Mersey (44 min) were lower than the Tees (66 min) but there was no significant difference between the individual sites. The low values recorded are in accordance with values obtained in other studies from contaminated coastal sites. Lowe et al (1993) found that the retention of neutral red was significantly reduced in haemocytic lysosomes in mussels collected from contaminated sites in the Venice lagoon. Moncheva (pers. comm.) recorded retention times in the range of 15-40 minutes for a severely impacted harbour site on the Black sea coastline. Wedderburn (unpublished) recorded values in the range 60-70 minutes for a moderately contaminated site in Southwest England.

The non-invasive cardiac monitoring system used in this study proved to be reliable for recording heart rates from groups of 16 mussels, simultaneously, for

7 hours. There is no reason why recordings could not have been continued for several days or weeks. The absolute heart rates reported here are within the range found for other populations of mussels occupying estuarine habitats (mussels 4-5 cm length, 20-28 beats per minute at 15°C; Bayne *et al.*, 1976). The reduced heart rates observed at site 8 on the Mersey and site 5 on the Tees are typical of bivalve molluscs in poor condition (Coleman, 1974). The fall in heart rate at these sites may be indicative of pollutant toxicity. For instance, Grace & Gainey (1987) reported decreased heart rates for mussels after 4 days exposure to five concentrations of copper (between 0.05 - 0.4 ppm). In addition, Sabourin and Tullis (1981) demonstrated that high concentrations of three aromatic hydrocarbons effected significant decreases in heart activity for the mussel *Mytilus californianus*.

On the Tees, the neutral red and cardiac data suggests that site 5 is of lower environmental quality than the other stations on this estuary. On the Mersey, the heart rate data indicated that site 8 was impacted. The significance of the close association of the toxicity data and the neutral red results has important ramifications. If the aim is to provide useful information for *in situ* assessment then the suite of techniques employed here has performed well. A possible criticism of the toxicity testing of sediment or water in the laboratory is that it is examining biological response in a test organism that may not respond in a realistic manner. Conditions in the laboratory may not adequately correspond to the complex interplay of environmental factors which exists in the real world. By using a test organism *in situ*, this problem is addressed.

The differences in the neutral red responses between the Mersey and Tees have been shown, to a certain extent, to reflect the differing conditions

experienced at each of the study sites. These results are corroborated by the findings of the toxicity tests and the biological data. However, the retention values for the Mersey are, on average, lower than the values for the Tees. This conflicts with the toxicity tests that tend to suggest that the sediment and water, from the Mersey study sites is less toxic than the Tees. There is very little discrimination in the neutral red data between the various sites on the Mersey and this does not correlate with the other toxicity or biomarker data. The difference in the retention times, between the Mersey and the Tees, could be the result of a lack of sensitivity in the technique with levels of toxicity on the Mersey being beneath the detection level of the method. However, the neutral red test has been shown to remain sensitive to low levels of contaminants in the environment. Further, the Tees data set shows a neutral red response in association with similar levels of toxicity, to those found on the Mersey.

Spawning of mussels from the Mersey, indicated by the presence of gametes in the mussel haemolymph, may have affected the results of the neutral red assay. Bayne *et al.*, (1988) showed the disruptive effect of spawning on lysosomal latency and suggested that the perturbing effect of reproductive state has to be considered in any study of this nature. If the translocated mussels were in a reduced physiological condition, as a result of pre/post spawning activity, this could suppress the retention times to the levels exhibited. However, a number of the toxicity tests indicate that site 8 on the Mersey is significantly impacted compared to the other sites. This is also indicated by the physiological data that shows a significant reduction in heart rate activity. If the mussels had been spawning then it is likely that any additional stress would have caused an augmented response, in conjunction with any environmental contaminants, with a resulting reduction in retention time. This is not the case.

However, the majority of the toxicity tests show reduced toxicity at the most stressed of the Mersey sites compared with the Tees. It is possible that the effect of spawning could be to reduce the retention times to a level at which any site related differences in retention time are lost. This type of problem would have been greatly reduced if replicate samples, of translocated mussels, had been utilised. The apparent correlation between the neutral red retention and cardiac response is significant as it demonstrates a whole animal effect in conjunction with indications of cellular pathology. This suggests that the biomarkers are demonstrating the initial biological responses that may contribute to ecological change. Further study would be required to investigate this fully but the implications are encouraging for the possibility of a mechanistic link between pathological events at the subcellular level and whole animal.

The demonstration of a strong correlation between the neutral red assay, indicating perturbation of the lysosomal membrane, and cardiac activity, a result of physiological responses is extremely encouraging. This type of mechanistic link, through the various levels of organisation within an organism is an important demonstration of the consequences of pollution exposure. Evidence of laboratory studies does indicate that environmental stress may effect fecundity directly (Bayne *et al.*, 1981, 1975). If events at the whole organism level can be extrapolated up to adaptive changes at the population level then greater information will be gained as to the impact of a polluted environment, both in the present and into the future. Furthermore, Bayne *et al* (1979) showed a direct correlation between scope for growth and lysosomal destabilisation which form part of a general stress response, this which could be associated to impaired reproductive ability.

The results here must be treated with caution when statements are made concerning the relationship between the biomarkers and the biological data, given the relative paucity of the available data, but some important conclusions can be drawn. The demonstration of a biomarker response, supported by laboratory toxicity testing, in an *in situ*, biologically relevant species, clearly supports the use of this type of approach. Further, the demonstration of a similar response, or at the very least a trend, in population studies conducted at the same sites sends an encouraging message regarding both the predictive capacity of biomarkers and their inherent sensitivity. This is a direct result of using indigenous species to integrate the biological effects of pollutant exposure.

Rather than criticising the effectiveness of laboratory toxicity testing it has been shown that using several techniques in tandem improves the robustness of the data set and allows greater understanding of the particular environment studied.

In a study of this size, there is a fundamental compromise which has to be made between logistical concerns and the robustness of the experiment. Hence, the lack, in this study, of replicate organisms to carry out reciprocal testing on, and therefore, further validate the biomarker response. Future work should allow for this type of eventuality and the inclusion of replicate translocated groups will provide for this. Control animals, maintained both in 'pristine' sites and laboratory conditions will further improve the design of such a study. Finally, if studies of this nature are to have any real value in terms of the monitoring and possible rehabilitation of polluted environments then it is necessary to move away from the use of 'one off' spot testing but to a more constant monitoring regime. It should be noted that the small sample sizes of the animal ($n=10$) for each study do not necessarily represent an inherent

weakness in experimental design. Bayne *et al.* (1981) demonstrated that, with the mussel *Mytilus edulis*, a sample size of 10-15 individuals was sufficient to detect pollution effects. The biomarkers demonstrated here clearly have the potential for testing of this nature. Constant cardiac monitoring could be easily accomplished on a permanent *in situ* basis and the non-destructive mode of the neutral red test will also permit regular testing. Forbes and Forbes (1994) recommended the use of, at least, dual biomarker techniques. The addition of toxicological testing to this scenario would allow the biomarker results to be ratified and further, exploratory, investigation to take place. This could be accomplished at a very low cost, both in logistics and man hours, but could provide vital information on environmental quality.

8.0 The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stages and adult histopathology.

8.1 Abstract

Indigenous and deployed mussels, *Mytilus edulis*, were examined at sites on the Tees estuary, an industrialised region of Northeast England. Four sites were sampled for water and biota, two of which had been recently exposed to an acute effluent discharge. Mussels were deployed for 24 h before return to the laboratory. Tests for lysosomal stability were conducted on the haemolymph; both heart rate activity was measured and tissue was preserved for histopathology.. Bioassays were conducted on larvae from indigenous and deployed mussels. Clean site (Whitsand Bay, Southwest England) larvae were also exposed to the sampled water. Lysosomal stability, heart rate activity, larval bioassays, and histopathology showed significant differences between sites and correlations between these data were derived. There is a need for biomarkers that can rapidly provide sensitive environmental data on marine organisms in a cost-effective manner. Consequently, these results are encouraging for the continued application of these biomarkers as initial indicators of environmental quality.

8.2 Introduction/Aims

There is a need for biomarkers of environmental quality to be tested in field situations if they are to become accepted, outside of the academic environment, and implemented as part of monitoring programmes. This study was an attempt to examine the responses of a suite of simple biomarkers in the early and adult life stages of the mussel *Mytilus edulis* in an industrialised

estuary, the Tees, in Northeast England. The objective was to discern the environmental quality of the different field locations using simple, rapid, techniques which were neither costly nor time consuming. An emphasis was placed on techniques which could be employed as the first stage of a risk assessment exercise, which would then allow the focusing of other, more costly techniques, such as chemical analysis.

Deployed and indigenous adult animals were sampled, where possible, and sites were chosen with particular reference to an area that had been recently exposed to an acute polluting incident (Environment Agency, *pers com*). This had involved the accidental release of a large quantity of acidified water into the estuary. Four biomarker techniques were utilised: the lysosomal integrity of blood cells, heart rate activity, tissue histopathology and embryo bioassay.

Several physiological responses of the mussel, *Mytilus edulis*, have been tested under laboratory conditions for their suitability to detect environmental stressors. Those that offer potential as physiological biomarkers include respiration and filtration rates, pumping rates, valve activity and heart beat (Baldwin & Kramer, 1994). Heart-rate data are useful in that they can provide an indication of the physiological status of *Mytilus edulis*. High levels of heart rate are associated with periods of activity as indicated by valve gape, high rates of respiration, and filtering/pumping activity, whereas periods of inactivity are associated with low heart rate (Coleman, 1974). Heart rate data can thus provide useful information on the physiological status of the mussel (Baldwin and Kramer, 1994). Cardiac activity in *Mytilus edulis* has typically been recorded using impedance cardiography (Trueman, 1967), which in itself can give rise to some degree of physiological disturbance. More recently, however, technological developments have permitted simultaneous recordings of cardiac

activity from several invertebrates, for indefinite periods, without imposing undue stress (Depledge & Andersen, 1990). Physiological measurement of this type has been largely restricted to the study of crustaceans such as *Carcinus maenas* (Bamber and Depledge, 1997). The use of invertebrate heart rate response in conjunction with additional biomarkers has been addressed by a small number of authors (Depledge and Lundebye, 1996; Astley *et al.*, 1999).

Histopathological examination of bivalve digestive gland has shown it to be a sensitive target for the injurious action of many pollutants (Lowe *et al.*, 1981; Moore *et al.*, 1987; Lowe, 1988; Syasina *et al.*, 1997). Pathological alterations are a reflection of disturbances at the molecular level (Moore and Simpson, 1992) and identification of these disturbances can aid in the understanding of the whole animal consequences of early cell response to pollutant exposure.

Water quality bioassays, utilising the pelagic stages of marine invertebrates, have been routinely used to monitor the integrated biological effects of contaminants and the variables that influence their toxicity in sea water (Stebbing, 1980; His and Beiras, 1995). Mussel offspring follow a clear pattern of development, 12 h after fertilisation the larvae are recognisable as a swimming embryo (the trochophore); and approximately 42-60 h later this is superseded by the D shell (or prodissoconch) larva which secrete a rudimentary shell. The formation of distinct life stages over a very short period of time enables the identification of abnormalities, perhaps due to poor ambient environmental quality, to be quickly quantified. Invertebrate larval abnormality has been used in a number of studies and has been shown to be a useful determinant of environmental quality (McFadzen and Cleary, 1994; His and Beiras, 1995; Conroy *et al.*, 1996).

This study considers biological responses in mussel larvae (developmental stages, growth and mortality) and relates these to other biomarker techniques

in the adult mussels. Not only can this allow a greater potential for the determination of site related effects but will also allow an examination of the relative sensitivities of the chosen biomarkers.

8.3 Methods

8.3.1 Experimental Animals

Mussels, *Mytilus edulis*, (3.5 - 4.0cm) were collected from Whitsand Bay, an open coast reference site (Dixon, 1982; Lowe and Pipe, 1994; Widdows *et al.*, 1995; Lowe *et al.*, 1995b; Minier and Moore, 1996) in Southwest England. Mussels were stored under constant conditions in the laboratory (filtered seawater, 15°C) before transportation. During transportation the samples were placed in plastic zip lock bags, in cool boxes, for approximately 8 h and then deployed on the rising tide. Cages were constructed from plastic mesh (1cm) and tied shut, to prevent predators gaining access and to stop the accidental release of non-indigenous animals. Fifty mussels were deployed on each site for a 24-hour period, encompassing a high and low tide. Indigenous mussels were collected on the low tide and stored in cool boxes for transportation. On return to the laboratory the mussels were kept at 15°C, in the water from their sampling site for 7 h, to allow recovery and equilibration from possible transportation stress.

8.3.2 Sampling Sites

The site where the discharge had been previously released was investigated (Old pipe) as was the site of the New discharge (New Pipe). The Old Pipe site had been discharging for approximately 5 days and the New Pipe for 2 days, but discharged effluent was from the same source in both cases. These were

approximately twenty metres apart and located on a rocky embankment, in a highly industrialised area. Mussels were deployed and indigenous populations sampled at both these sites. Two reference sites were utilised: these were a large sandy beach near the mouth of the Tees (Hartlepool Beach); and a Breakwater at North Gare, at the estuary mouth, located at the top of the beach. Indigenous mussels were collected from the Breakwater and Whitsand mussels deployed at the beach (see Fig. 46). Sediment samples were taken on a transect, between the two impacted sites, for an examination of benthic diversity. Samples were stored in zip locked bags and examined within 24 h on return to the laboratory.

8.3.3 Techniques

On return to the laboratory four techniques were utilised. The neutral red lysosomal stability test, heart rate monitoring, larval bioassay and histopathology were conducted. The neutral red retention and histopathology were conducted on the same individual animals. Neutral red retention and heart rate monitoring were started within 24 h of return to the laboratory. Mussels were sacrificed for histopathology after haemolymph had been withdrawn for the neutral red assay.

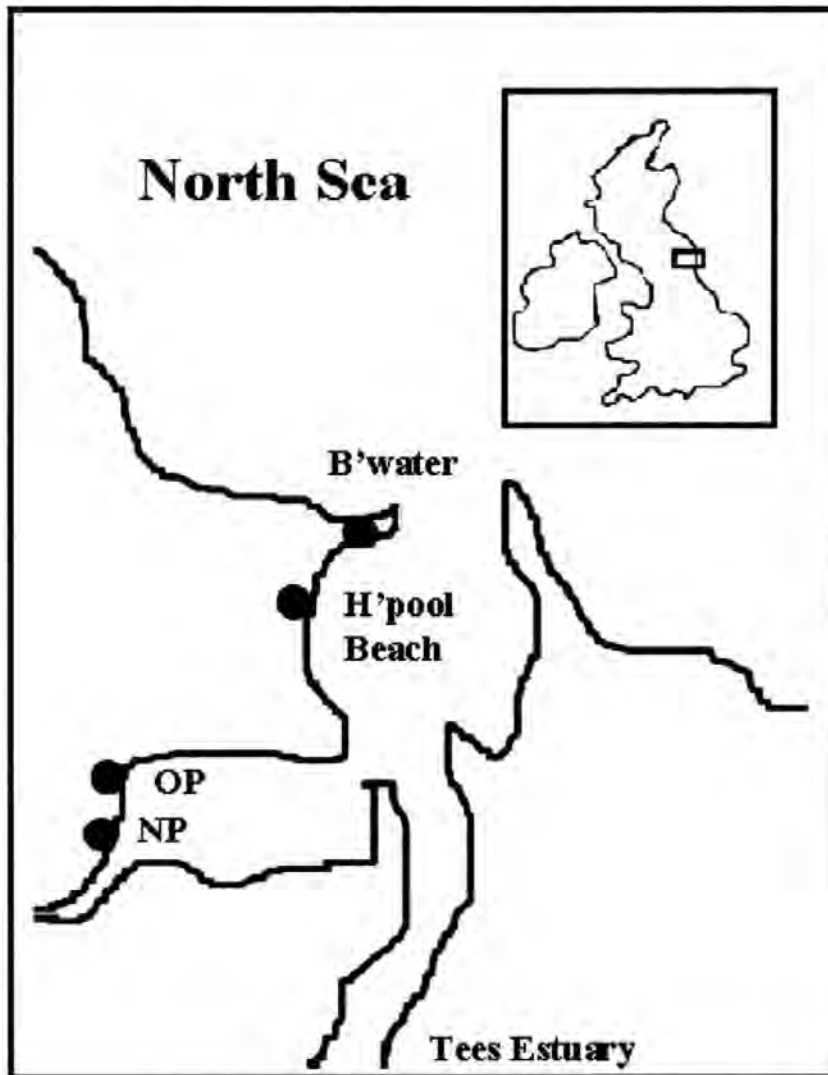


Fig. 46. Map showing location of Tees Estuary, sampling sites (indicated by black spots) were located close to the estuary mouth, on the Northern side. Inset box shows position of the Tees in the UK.

8.3.4 Neutral Red Test

The neutral red test, for lysosomal stability, was conducted as described in section 2.1.1. Twelve mussels were examined from each site; and the order in which the sites were examined was randomly determined and the assays were all conducted within 12 h.

8.3.5 Cardiac Activity

The mean resting heart rate of the mussels was measured over 24 h using the technique described in detail in section 2.2. Mussels were all monitored within

48 h of collection. Mussels were submerged in UV-treated natural seawater, for 24 h, at 15 (+/-1) °C with a 12-hour light/dark cycle during the heart rate measurement.

8.3.6 Histopathology

Following the extraction of haemolymph, for the neutral red test, each mussel was uniquely identified, shell length measured, dissected and tissue preserved in 4°C Baker's formol calcium fixative. Mussels were dissected to provide a mid-cross section through the digestive gland, gill, gonad and kidney. The foot and associated musculature was removed prior to fixation. After 24 h fixation, tissues were dehydrated through an alcohol series before embedding in paraffin wax. Wax sections (7µm thick) were cut and stained in Papanicolaou. Prior to microscopic analysis the slides were coded to disguise the identity of each site sampled. Interpretation of all the slides was therefore conducted 'blind'. Relative pathologies were determined using a semi-quantitative method where specific abnormalities (gonad maturity, presence of adipo-granular cells, oocyte atresia, granulocytoma, granulocyte number; digestive tubule vacuolation, luminal integrity, duct epithelium, phasic activity, and the presence of brown cells) were scored on a scale of 1 – 5, lower numbers indicating the increased presence or relative severity of the particular pathology. The described method was carried out by M. Hornsby (Technical support, Plymouth Marine Laboratory).

8.3.7 Larval Bioassays

Effect of Parental Exposure

Adult mussels (deployed and indigenous) were induced to spawn by potassium chloride (0.75M, 0.5 ml per mussel) injection into the posterior adductor muscle in conjunction with transferral to filtered sea water at 25°C. Mussels were fed unicellular algae to encourage filtration. Spawning mussels were observed to release gametes within an hour. Mussels from the Old Pipe indigenous group produced male gametes but no females, and thus could not be included. Gametes were collected, eggs were sieved with a 200 micron mesh to remove debris and then with a 30µm mesh for collection. Eggs were then mixed with sperm in filtered seawater, 10-100 sperms per egg, at 15°C. Gametes were left for 1 hour to fertilise and then examined, by light microscopy, for fertilisation success. This was in the range of 74.5%-99%. Excess sperm was washed off through a 30µm mesh with filtered sea water. Developing larvae were then stored in constant conditions (15°C) in 250ml filtered seawater at 20 embryos ml⁻¹ for two days. Embryos were then strained on a 30µm mesh, collected by pipette, and fixed in 100µl formalin in a 2ml multiwell plate.

Water quality and direct effects on embryogenesis

Mussels from Whitsand Bay, SE Cornwall were spawned as described above. Fertilised eggs were cultured in clean filtered seawater at a concentration of 50 eggs ml⁻¹. The spawning and culture of embryos was conducted in the Plymouth laboratory, at the same time as field sampling took place. Pooled gametes from 3 males and 3 females were used. Upon return to the laboratory, water from each site was filtered (1µm GFC filter) and stored in 250ml glass vials at 15°C. Trochophore larvae (32 h post-fertilisation) were then

concentrated on a 30µm mesh and an estimate of the numbers made from a 500µl sub-sample. Trochophore larvae were then added to each test water sample to a final concentration of approximately 20ml⁻¹. Larvae were then allowed to develop for a further 60 h prior to fixation, as described above.

Larvae were analysed at random (per 500) for soft tissue damage and shell deformity in a modification of the oyster embryo bioassay (Thain, 1991). Developmental success was measured by counting numbers of normal 'D' shells, dead 'D' shells, severe/slight deformities, abnormal velums and normal/abnormal trochophores. The lengths of the first 30 D-shell larvae encountered in each treatment were also measured using a filar eye piece micrometer.

Analysis of Experimental Data

Data were tested for normality by comparison with a normal distribution plot and shown to be not normally distributed. They were examined by non-parametric analysis; and were compared by Kruskal-Wallis median comparison and shown as box and whisker plots. The box is divided at the median and encompasses 50% of the data points. Notches that do not overlap indicate that the data are significantly different.

8.4 Results

8.4.1 Neutral Red Retention

Retention times were in the range 0 – 105 minutes. The deployed animals were shown to have greater retention times than the indigenous species, indicating greater lysosomal stability. No significant differences could be found between the Old Pipe/New Pipe indigenous or deployed animals ($p>0.5$, $n=12$; see Fig.

47). Deployed mussels exhibited greater retention times at these sites than the indigenous. Indigenous breakwater animals were shown to have suppressed retention times but no significant differences could be found between these and the indigenous mussels at the discharge sites. Significant differences were found between the Hartlepool Beach deployed mussels and those deployed at the old/new pipe sites ($p < 0.05$, $n = 12$; see Fig. 47).

8.4.2 Heart rate monitoring

Heart rates were in the range 18-27 BPM (beats per minute). Significant differences were observed between the deployed Hartlepool Beach animals and the other deployed animals ($p < 0.001$, $n = 6$; see Fig. 48). Significant differences were also observed between the indigenous breakwater animals and those from the Old Pipe site ($p < 0.001$, $n = 6$; see Fig. 48). Old Pipe deployed and indigenous animals were shown to exhibit higher heart rate activity than those from the New Pipe site ($p < 0.001$, $n = 6$; see Fig. 48).

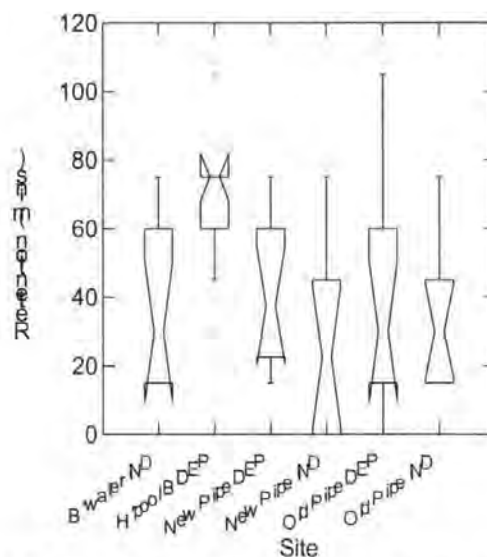


Fig. 47. Box and whisker plot showing neutral red retention (minutes) in haemocytes extracted from indigenous (IND) and deployed (DEP) mussels, *Mytilus edulis*. Old pipe and new pipe are the effluent discharge points. Hartlepool beach (H'pool B) and the Breakwater (B'water) are the 'reference' sites. Where notches do not overlap $p < 0.05$.

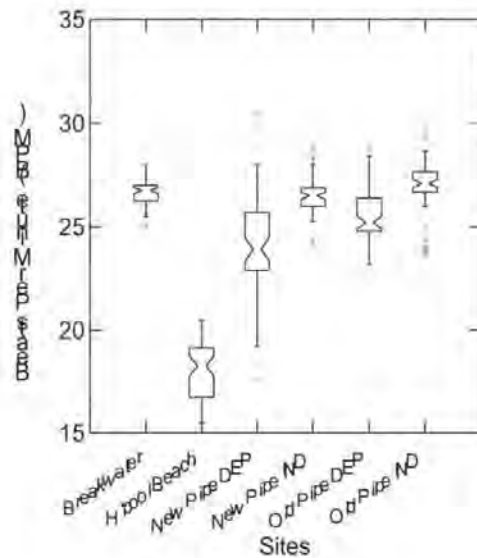


Fig. 48. Box and whisker plot showing heart rate activity of indigenous and deployed mussels. Heart rate was measured by the non-invasive CAPMON technique.

8.4.3 Larval Bioassay

Parental Exposure

Of the deployed animals those with larvae exhibiting the highest occurrence of deformity were from the Old Pipe site (see Figs. 49,50, 51). Those with the lowest were from the Hartlepool Beach site (see Figs. 49,50,51,54). Deployed animals at the impacted sites had significantly smaller D shell lengths than those from the Hartlepool Beach site ($p < 0.001$, $n=30$; see Fig. 53).

Indigenous mussels collected from the New Pipe site showed a greater degree of deformity than the Breakwater site (see Figs. 54,55). There was no significant difference, in D shell length, between the indigenous population from the New Pipe site and those from the Breakwater ($p > 0.05$, $n=30$).

Water Quality

Larvae raised in New Pipe water showed the greatest degree of trochophore abnormality (see Fig. 52) with poor developmental success to the prodissoconch stage. Larvae exposed to Old Pipe water showed deformity though some D shells were observed. Larvae exposed to Hartlepool Beach water had a reduced occurrence of deformity.

Larvae exposed to the Old Pipe water had a significantly smaller D shell length ($p < 0.05$, $n = 30$) than those exposed to Hartlepool Beach water.

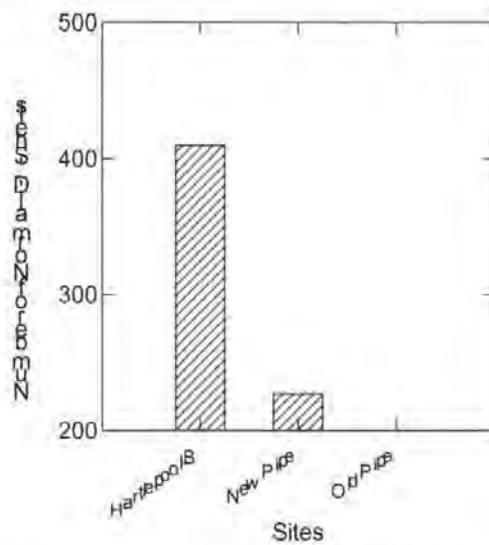


Fig. 49. Bar chart showing numbers of normal 'D' shells per 500 larvae from deployed mussels. Mussels from Hartlepool Beach produced the most viable larvae, mussels from the Old Pipe the least.

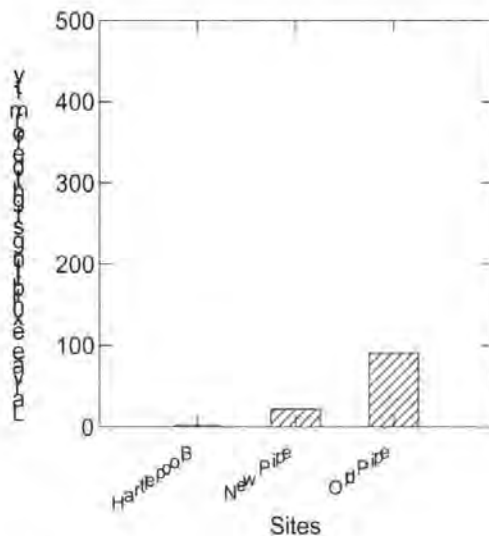


Fig. 50. Bar chart showing occurrence of slight deformity in larvae from deployed mussels. Mussels from Hartlepool beach produced larvae with the least amount of deformities, mussels from the Old Pipe the most.

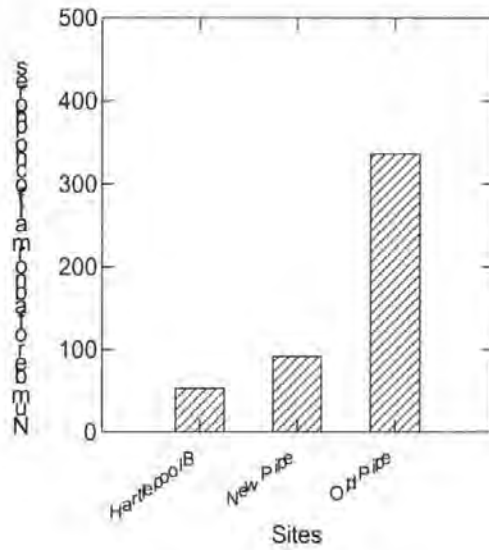


Fig. 51. Bar chart showing occurrence of abnormal trochophores in larvae from deployed mussels.

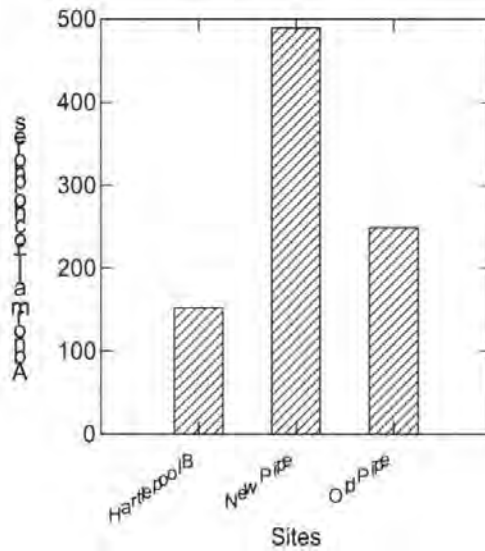


Fig. 52. Bar chart showing incidence of abnormal trochophores in larvae exposed to water from sample sites. Larvae from clean site mussels (Whitsand Bay, South West England) showed the greatest number of abnormal trochophores when exposed to New Pipe water, in contrast to the larvae produced by *in situ* mussels.

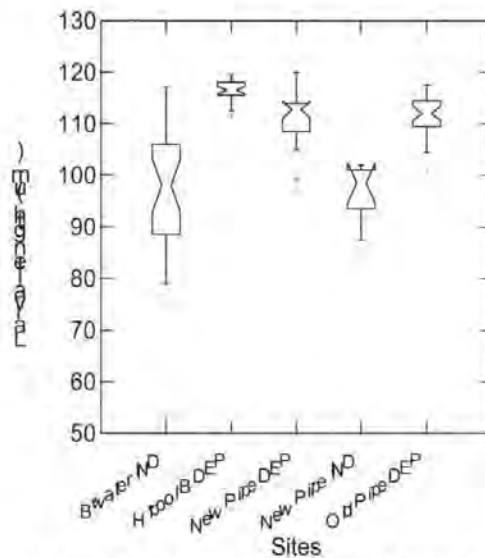


Fig. 53. Box and whisker plot showing larval length in indigenous and deployed animals.

8.4.4 Histopathology

Deployed mussels

Pathological alteration of the digestive gland (changes in connective tissue, alterations in tubule phasic activity and thinning of the tubule epithelium) occurred slightly at the impacted sites compared to Hartlepool Beach but was not statistically significant. Granulocyte number was significantly increased in the digestive gland at the Old pipe site compared to the reference site ($p < 0.05$, $n = 12$, see Table 3). This trend, for granulocyte number, was reflected in the gonad ($p < 0.05$, $n = 12$, see Table 6).

Indigenous mussels

Pathological alterations at the Old Pipe site were observed compared to the reference site. These included, increases in digestive gland granulocytes ($p < 0.05$, $n = 12$), increases in vacuolation ($p = 0.001$, $n = 12$), increases in perturbed connective tissue ($p < 0.05$, $n = 12$), degeneration of the duct epithelium ($p < 0.05$, $n = 12$) and changes in phasic activity ($p < 0.001$, $n = 12$). The New Pipe site showed significant pathological alteration in increased vacuolation ($p < 0.001$, $n = 12$) and perturbed phasic activity ($p = 0.001$), compared to the reference site. Granulocyte number in the gonad was significantly increased at the New Pipe site compared to the Old Pipe site ($p < 0.05$, $n = 12$, see Table 6).

8.4.5 Benthic Diversity

A rapid qualitative assessment of the benthic invertebrates was made. The fauna were strongly dominated by the oligochaete *Tubificoides benedi* but small numbers of polychaetes (e.g. *Streblospio shrubsolii*) and bivalves (e.g. *Macoma balthica*) were also observed. The meiofauna was dominated by nematodes

and ostracods. No differences in invertebrate species distribution or number were found between the discharge sites. The assessment was carried out by Mike Kendall and Richard Warwick at Plymouth Marine Laboratory.

PATHOLOGY	Old Pipe DEP	New Pipe DEP	Hartlepool Beach DEP	Old Pipe IND	New Pipe IND	Breakwater IND
Gonad-Granulocytes	38	40	47	23	30	35
Gut-Vacuolation	25	29	33	16	13	31
Gut-Connective tissue	25	29	33	27	31	33
Gut-Phasic Activity	26	23	23	19	17	34
Gut-Duct epithelium	39	34	37	33	29	42
Gonad-Maturity	14	15	8	27	15	22
Gonad-Atresia (mean)	2.8	3.75	2	3.8	2.14	3.6

Table 6. Table showing pathology scores in indigenous and deployed mussels. Scores are summed values of observations on sectioned digestive and gonadal tissue from 12 mussels per group. Tissue was scored blind on a relative 1-5 scale per slide, 1 representing extreme pathological alteration/proliferation, 5 representing healthy tissue. Decreasing values therefore represent increased levels of abnormal pathology.

8.4.6 Environmental Data

No chemical sampling took place during the time of sampling though some historical water and sediment data, from the UK Environment Agency, is shown in Tables 8 and 9. This data shows no overall correlation with the biological data though there is some relationship with individual contaminants. Complete data on other metals and organics, in water and sediment, was not available for the sites under test.

Sampling Site	Hg	Cd	Pb	Zn	Cu	CHCl ₃	C ₃ H ₆ O	pp DDT
Old Pipe/New Pipe discharge site	0.02-0.06	2.0-3.02	2.25-4.30	5.0-9.0	2.00-3.02	<0.1-0.28	----	----
Breakwater	0.02-0.04	0.08-0.17	4.18-5.17	5.0-21.0	1.64-3.16	<0.1-0.23	300-600	<0.001- <0.004
Hartlepool Beach	0.01-0.05	0.14-0.24	2.75-6.70	7-52.4	1.78-1.96	<0.1-0.59	----	----

Table 8. Table showing data from water quality samples taken by the UK Environment Agency between 1995-1998. The concentrations are the maximum and the minimum detected during the sampling period in $\mu\text{g l}^{-1}$. ----: no data available

Sampling Site	Cu	Cd	As	C ₆ HCl ₅ C	pp-DDT	C ₆ Cl ₆
Old Pipe/New Pipe discharge site	11.2-245	0.1-1.51	10.4-23	<20-40µg/kg	<1-27µg/kg	<1-53µg/kg
Breakwater	44	0.1	-----	<5-40µg/kg	<1-2µg/kg	<1µg/kg
Hartlepool Beach	-----	-----	-----	-----	---	-----

Table 9. Table showing data from sediment samples taken by the UK Environment Agency between 1995-1998. The concentrations are the maximum and minimum detected during the sampling period in mg/kg, except where stated. -----: no data available

8.4.7 Correlation Analysis

Data were examined in the context of their relationship to each other. Pearson correlations were conducted and Bonferroni tests used to test probability. The leverage of the individual data sets on the correlation as a whole was examined using regression analysis. Significant relationships were shown between neutral red and heart rate (Pearson correlation coefficient -0.967, Bonferroni probability 0.002), neutral red and the number of normal 'D' shells (Pearson correlation coefficient 0.920, Bonferroni probability 0.009), and neutral red and number of normal trochophores (Pearson correlation coefficient 0.950, Bonferroni probability 0.004). Relationships were also shown between heart rate and number of normal 'D' shells (Pearson correlation coefficient -0.966, Bonferroni probability 0.002) and number of normal trochophores (Pearson correlation coefficient -0.943, Bonferroni probability 0.005). However, the techniques were not shown to significantly correlate when considering the two discharge sites in isolation. As neutral red values fell, pathological alteration increased. Notably, neutral red and digestive gland granulocyte proliferation (Pearson correlation coefficient 0.882, Bonferroni probability 0.022), neutral red and gonad granulocyte proliferation (Pearson correlation coefficient 0.821,

Bonferroni probability 0.048) and to a lesser extent neutral red and gut vacuolation (Pearson correlation coefficient 0.743, Bonferroni probability 0.094) had an inverse relationship.

8.5 Discussion

If biomarkers are to become established as part of monitoring and policy-making procedures, there is a need for techniques which are rapidly implemented, easily learned, simple to use and relatively low in cost. Techniques which enable the detailed examination of very discrete responses are not without value but are beyond the scope of many monitoring agencies and may not be wholly appropriate for their needs. As a result there is a strong argument for the use of simple biomarkers which can demonstrate biological effect and therefore provide the impetus for examining causality in a more detailed and structured fashion.

In this work a suite of simple biomarkers was applied, in tandem, using deployed and indigenous mussels from a number of different field sites. Van der Oost *et al.* (1997) emphasised the importance of biological effects monitoring by demonstrating the greater ability of a suite of biomarkers to distinguish between moderately and heavily contaminated sites compared to sediment analysis. Förlin *et al.* (1996) demonstrated the value of using a suite of biomarkers to determine environmental quality through the identification of polluted sites in the Skagerrak area. Moreover, Moore and Simpson (1992) showed that pathological studies which included cellular biomarkers have the potential to reveal biologically significant differences between organisms from contaminated and reference sites.

The aim of these studies was not only to determine the environmental quality of the field sites through the biomarker responses but also to examine these

biomarker responses in relation to each other. Using a suite of biomarkers rather than one or a few undoubtedly strengthens a field study but a greater understanding needs to be obtained on how different biomarkers will perform under the same circumstances. Further, examining biomarkers of whole animal perturbation in conjunction with an examination of larval aberration allows an examination of the implications that parental exposure may have for the population. Bayne *et al* (1979) demonstrated a decline in fecundity in adult mussels which could be linked to pollutant exposure and which represented deterioration in ecological fitness.

Finally, the period of time during which animals were deployed was very low (24 h), allowing the sensitivity of the tests and their potential for rapid environmental quality assessment to be examined.

The low dye retention times, indicating poor membrane integrity, shown by lysosomes from mussels sampled from the industrialised sites is consistent with the findings of other studies on lysosomal integrity in indigenous, chronically exposed, mussel populations (Krishnakumar *et al.*, 1994; Lowe *et al.*, 1995a; Cheung *et al.*, 1998). In the deployed mussels, heart rate was significantly lower at the Hartlepool Beach site when compared to the Old and New Pipe sites. Astley *et al.* (1999) reported increases in the heart rate of deployed mussels relocated to contaminated sites compared to less impacted sites.

The apparent association between alterations in heart rate and perturbation of the lysosomal system may indicate the important role that the lysosomal system plays in maintaining cellular homeostasis. Lysosomal damage has been shown to be a prognostic biomarker for pathology and reduced fitness (Hawkins, 1980; Moore, 1990; Lowe *et al.*, 1992; Depledge *et al.*, 1993; Moore,

2002). However, the changes in heart rate may simply be the response of chemotaxis and an attempt by the mussel to purge itself of contaminated water and sediment. Given the short exposure time utilised in this study, responses such as the rise in heart rate may represent the first stage of a more complex adaptive response. The cost of such an adaptation may be represented by the reduced biomarker responses exhibited by the indigenous mussels.

Pathological alteration of mussel tissue, as a result of pollutant exposure, has been reported in a number of studies (Lowe *et al.*, 1981; Moore *et al.*, 1987; Lowe *et al.*, 1988). Increased numbers of granulocytes were observed in the gonad and digestive gland in mussels from the impacted sites. Granulocytes are phagocytic in nature (Moore and Lowe, 1977; Carballal *et al.*, 1997); their increased presence in mussels may be indicative of enhanced scavenging of damaged tissue. Increased digestive tubule vacuolation was observed at the impacted sites, and was particularly severe in the indigenous mussels. Vacuolation of bivalve digestive gland has been previously demonstrated after pollutant exposure (Lowe, 1988; Moore, 1988; Sarasquete *et al.*, 1992; Kela and Bowen, 1995). Connective tissue was shown to be reduced at the impacted sites, and Gold-Bouchot *et al.* (1995) showed a decline in connective tissue in *Crassostrea virginica* after exposure to cadmium. Indigenous mussels showed a reduction in digestive tubule phasic activity and thinning of the duct epithelium at the impacted sites. Bivalve epithelial thinning after pollutant exposure has been shown in a number of studies (Lowe *et al.*, 1981; Moore *et al.*, 1987). This suggests a possible reduction in the ability of these mussels to digest food material adequately. Deployed mussels show some loss of phasic activity and some epithelial thinning but do not differentiate between sites. This may be a function of the short period of time that the deployed mussels were exposed to the sampling sites.

The incidence of deformity and aberrant larvae from deployed mussels not only reinforces the trend suggested by the heart rate, pathology, and neutral red data but also indicates a differing response between the two discharge sites. Larvae from the mussels deployed at the Old pipe site show increased numbers of abnormalities and aberrant development.

Bioassays conducted on the larvae of indigenous mussels did not reflect such a clear trend. The Breakwater mussels had a greater incidence of deformity than those deployed at Hartlepool Beach, while animals from the Old Pipe site did not yield any larvae. However, this lack of larvae may in itself be meaningful, and the poor quality of larvae from the indigenous animals may represent the consequence of long term exposure to an impacted environment.

The measurement of shell length in larvae from deployed animals exposed to the discharge sites showed a decrease in larval length compared to those animals deployed at Hartlepool Beach. Stenalt *et al.* (1998) showed a significant reduction in mussel larval shell length after exposure to environmentally realistic levels of TBT. However, there was no discrimination between the discharge sites in this study. There was no significant difference in shell length between the indigenous populations; however, larval length in the indigenous animals was significantly smaller than those which were deployed. Reduction of larval shell length, in bivalves, has previously been demonstrated in the field (Boltovskoy *et al.*, 1997) and in laboratory studies (Ruiz *et al.*, 1995). Gametes from clean site mussels (collected from Whitsand Bay, Cornwall) were also exposed to the sampled water. When these larvae were exposed to the New Pipe water, they expressed a greater degree of deformity than those exposed to water from the Old Pipe or the Beach, which displayed the least. This does not follow the pattern of effect indicated by the larval bioassays conducted on the deployed mussels. However, the bioassay response is a

result of the interaction between exposure, toxicokinetics, and toxicodynamics (McCarty and Mackay, 1993) which will differ according to the nature of exposure. For example, prolonged exposure of the adult to the whole environment will involve the integration of the exposure, detoxication, bioaccumulation and response. However, larval exposure solely to the water, removes many of the potential factors, present in adults, which could complicate and effect both the exposure and the toxicological response.

These data suggest that conditions at the Pipe sites are generally not of a quality conducive to the production of large numbers of viable larvae. Larval deformity and increased larval mortality have been shown to be indicators of increased contaminant impact (Ringwood, 1992; McFadzen and Cleary, 1994; His and Beiras, 1995; Stenalt *et al.*, 1998). Thus, the increase of aberrant larvae at the Old Pipe site suggests a greater toxicological effect at this site. In the mussel a decline in fecundity after stress imposed on the adult is caused in part by the resorption of eggs as a result of the release of lysosomal enzymes into the cytoplasm of the developing oocytes (Bayne *et al.*, 1979). Release of lysosomal enzymes in eggs and embryos could lead to chromosomal damage resulting in death or developmental abnormalities and a reduced capability for survival (Moore, 1982). The consequences that the production of less viable offspring may have for the continued success of the population is difficult to assess due to the high mortality larvae suffer naturally, though the implication may be that the population will be less capable of surviving further stressors. However, recruitment probably occurs mainly from the pool of larvae in this region of the North Sea and not necessarily from the indigenous mussels.

The benthic diversity data suggests a reasonably rich population of invertebrates (Kendall, *pers comm*) and showed no difference between the impacted sites. However, the expression of a number of biomarkers, in mussels

indigenous to the local ecosystem, which are known to be associated with a sub-lethal response to poor environmental quality, may reflect the difference in the speed of response between the individual and the population.

The limited chemical data set available does not allow adequate comparison with the biological indices utilised here. However, if the biological responses identified are a result of chemical stress then it is unlikely that one particular contaminant is the cause. The assessment of the combined effect of chemicals has been recognised as a problem for ecotoxicological assessment (Murphy, 1980) and in a heavily industrialised estuary, such as the Tees, the potential for contaminant interactivity could be high.

These data are of particular interest as they allow the comparative sensitivity of these techniques to be addressed. All of the techniques were able to distinguish between the Pipe sites and the Beach sites in terms of the deployed animals. Only the larval bioassays seem to have distinguished between the Pipe sites but this data is confounded by the differing response of the larval bioassay to the collected water.

This study demonstrated a significant difference in biological response between different field sites using a suite of biomarkers that are known to respond to the presence of chemical stressors. Indigenous mussels were not shown to differentiate between sites as clearly as the deployed mussels; but were, however, generally shown to exhibit a greater biomarker response known to be indicative of pollutant exposure.

The sensitivity of the biomarkers, in the deployed mussels, was particularly encouraging in the context of the very short exposure times to the sample sites.

This has implications for the construction of future monitoring studies given that logistics are usually the limiting factor. However, the addition of more

detailed chemical analysis on sediment and mussel tissue would aid in establishing causality and future studies will adopt this more integrated approach. Biological data of the type presented here could be used to focus further, more detailed, studies. Salazar and Salazar (1997) suggested that the use of caged bivalves to estimate chemical and biological effects represents a cost-effective approach that could reduce the uncertainties associated with more traditional approaches.

Given the ease with which these generally non-destructive techniques can be implemented, their application to long term monitoring programmes, particularly with reference to the remediation of impacted areas, is promising.

9.0 The Field Application of the Neutral Red Test in Conjunction with an Assessment of Land Based Inputs of Marine Contaminants in the Black Sea

9.1 Abstract

The Black Sea is under increasing stress as a result of inputs of contaminants and eutrophication discharges. This study was an attempt to implement a 'mussel watch' programme (supported by UNESCO/IOC, International Mussel Watch) to assess the health of mussel populations in the Black Sea. By utilising mussels for biological monitoring those areas suffering poor environmental quality can be identified. A non-destructive biological marker (lysosomal integrity) of pollutant effect was investigated. Analysis of mussel tissue burden was also conducted. This data was supplemented with the results of a rapid source inventory of land based discharges into the Black Sea. Lysosomal integrity indicated significant differences ($p < 0.05$) between sites and correlated with the results of the rapid source inventory. There was an association of high tissue burden with reduced lysosomal integrity at a few sites but no overall direct relationship. This combination of the biomarker approach with the Mussel Watch framework represents a novel method for rapidly obtaining biological data on the status of marine ecosystems.

9.2 Introduction/Aims

The identification of areas that are being impacted has involved the use of 'biological' monitoring, where species from within the impacted ecosystem are used as tools to determine the nature and extent of contamination. Certain marine organisms, particularly sedentary filter feeders such as bivalve

molluscs, accumulate chemical contaminants directly from the seawater or via particulate food filtered from the water column. The use of molluscs as monitors of anthropogenic pollution in the coastal environment is becoming increasingly established (Phillips, 1981, 1988).

The application of mussels as integrators of marine pollutants in their tissue and shells (Goldberg, 1986) is the underlying concept of the International Mussel Watch Project. This formed in the 1970's with the goals of assessing the extent and severity of contamination of the coastal waters of the world with respect to selected contaminants (Tripp *et al.*, 1992).

The residues of contaminants within the organisms are identified by means of analytical chemistry. However, although this indicates what contaminants are present in the environment it does not provide information on their biological availability once within the organism, nor does it demonstrate any resultant toxicological effect.

Investigation of the endangered state of marine ecosystems has more recently involved the development of monitoring techniques utilising indices of biological response. These are known as biomarkers, which can identify the biological reaction occurring in an organism, as a result of contaminant exposure, and could act as early warnings of adverse ecological change (Moore, 1985). Biomarkers can demonstrate that chemicals have entered an organism, that they have reached sites of toxic action (Depledge *et al.* 1993) and that they are instigating a pathological response.

This work was an attempt to conduct a 'Mussel Watch' monitoring programme in the Black Sea. The Black Sea, a partially enclosed sea in Eastern Europe, has suffered catastrophic ecological damage as a result of pollution, principally from land based sources (Mee, 1992). This has resulted in eutrophication and contamination by pathogenic microbes and toxic chemicals (Mee, 1992).

This work attempts to not only integrate the biomarker approach and the chemical analysis of mussel tissue, but also, to supplement this data with the results from a rapid source inventory of land based inputs to the Black Sea (Sarikaya *et al.*, 1997). This inventory provided information on amounts of oil, suspended sediment, nitrogen, phosphorus, and the biochemical oxygen demand (BOD) of the inputs into the Black Sea. A high BOD indicates the increased activity of heterotrophic organisms and thus heavy pollution (Lawrence, 1995).

The importance of including biological measurements in 'mussel watch' programs has been previously emphasised (Gray, 1992). The rapid source inventory was conducted under a protocol obtained from the World Health Organisation by the Black Sea Environment Programme (Sarikaya *et al.*, 1997). The information, obtained from these techniques, was used to assess the status of the mussel populations of the Black Sea littoral states. This represented a novel adaptation of the 'Mussel Watch' concept and allowed the effective implementation of this study in laboratories located in the six littoral countries bordering the Black Sea. The lysosomal results obtained, by means of the neutral red assay, were used to examine evidence of linkages between the xenobiotic exposure of the mussel populations and subsequent cellular effects. The overall aim of the work was to rapidly generate a meaningful data set that could aid the management and remediation of the polluted areas of the Black Sea coastline.

9.3 Method

The neutral red test, for lysosomal stability, was conducted as described in section 2.1.1. The rapid source inventory technique was conducted as described in section 2.5 and the tissue analysis conducted as described in 2.6.

9.4 Results

Lysosomal retention times were recorded from all six participating Black Sea countries. The rapid inventory assessment data was obtained for all the countries, with the exception of Georgia. Analytical chemical analysis of mussel tissue was conducted at the Bulgarian and Ukrainian sites for tissue burden of hydrocarbons and pesticides (See Tables 9 and 10).

COUNTRY/SITE.	Mean lysosomal retention time (mins)	BOD (t/y^{-1})	Oil (t/y^{-1})	TSS (t/y^{-1})	Mussel tissue burden. PAH (ng/g^{-1})
BULGARIA					
Port Varna	12±6	1740	2323	3170	254
Shabla	28±5.5	-----	-----	-----	60
Bourgas	31±8.5	636	1533	1533	217
Kavarna	74±12.5	57.9	23.4	125.4	157
Skorpilotsyvyky	84±18	42	68.1	75.6	-----
ROMANIA					
Navodari	5±3.6	38840	62700	50730	-----
IRCM	12±3.0	1000	3196	-----	-----
Costinesti	46±4.5	1000	3196	-----	-----

Table 9. *Mytilus galloprovincialis* lysosomal retention times (measured using the neutral red technique and (mean ± 95% CI) BOD and oil inputs obtained by the rapid source inventory technique, and mussel tissue burden, in selected sites from the Black Sea littoral states of Bulgaria and Romania ----- indicates that this data could not be obtained.

COUNTRY/SITE	Mean lysosomal Retention time (mins)	BOD (t/y ¹)	Oil (t/y ¹)	TSS (t/y ¹)	Mussel tissue burden: PAH (ng/g)
UKRAINE					
Arkadia	15±6.25	-----	-----	-----	-----
Odessa	17.5±8.5	29858	62700	13047	6882.6
Liman Sukchoy	33±10.2	13300	650	890	2663.8
Liman Grygorevsky	61.5±20.3	-----	-----	-----	-----
UkrSCES	71±17.5	-----	-----	-----	-----
Koblevo	150±20.2	-----	-----	-----	1049
RUSSIA					
Bzugo	28.5±10.4	110.9	0.11	115.4	-----
Sochi-Port	31.5±15.2	548.9	1.19	557.7	-----
Dagomis	40.5±18.0	98.15	0.09	99.2	-----
Tuapse-Port	49.5±8.9	113.3	16.5	144.8	-----
Adler	52.5±12.4	123.5	0.57	142.2	-----
Lazarevck	108±17.5	1.05	1.08	1.3	-----
Lao	114±17.1	-----	-----	-----	-----
GEORGIA					
Poti-Port	31±8.2	-----	-----	-----	-----
Kobuletre	117±13.1	-----	-----	-----	-----
Poti-Rec	120±17.3	-----	-----	-----	-----
TURKEY					
East Samsun	28±8.9	-----	-----	-----	-----
Samsun	63±21.4	-----	-----	-----	-----
West Samsun	82±23.2	-----	-----	-----	-----

Table 10. *Mytilus galloprovincialis* lysosomal retention times (measured using the neutral red technique and (mean ± 95% CI) BOD and oil inputs obtained by the rapid source inventory technique, and mussel tissue burden, in selected sites from the Black Sea littoral states of the Ukraine, Russia, Georgia, and Turkey ----- indicates that this data could not be obtained.

Differences in lysosomal retention time were shown in the haemolymph of *Mytilus galloprovincialis* collected from the various sites. The rapid inventory assessment indicated considerable differences in levels of biochemical oxygen demand, suspended sediment, as well as nitrogen and phosphorous at the surveyed sites. The residue data indicated some sites where the indigenous mussels had an increased body burden of hydrocarbons or pesticides.

9.4.1 Lysosomal Data

Mean lysosomal retention times are shown in figures 54 and 55, for each individual country. The Romanian data set contained the lowest retention values, with results in the range of 0 to 60 minutes. Lysosomal membrane destabilisation was most pronounced in mussels collected from the Romanian sites of Navodari and IRCM, as shown by the low retention times of 5.4 and 12 minutes, respectively. These are exceptionally low values, when compared with

the results presented here and data from other field studies (Lowe *et al.*, 1995) which utilised *M. galloprovincialis*.

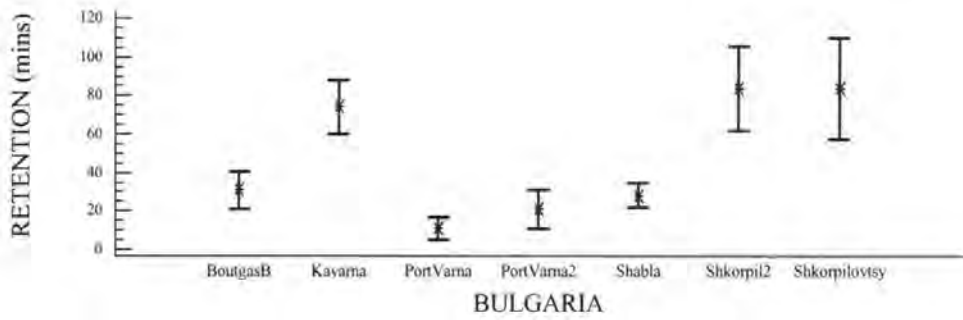


Figure 54. *Mytilus galloprovincialis*. Mean plots, with 95% confidence intervals, of heamocyte lysosomal retention time of the neutral red probe. BourgasB=Bourgas Bay, Shkor.=Shkorpilotsyvyky, L-Grig=Liman Grygorevsky, L-Sukc.=Liman Sukchoy. '2' indicates repeat sampling conducted.



Fig 55. *Mytilus galloprovincialis*. Mean plots, with 95% confidence intervals, of heamocyte lysosomal retention time of the neutral red probe. Laz.=Lazarevck. Poti Pt=Poti Port. Poti Rec=Poti Recreational Beach.

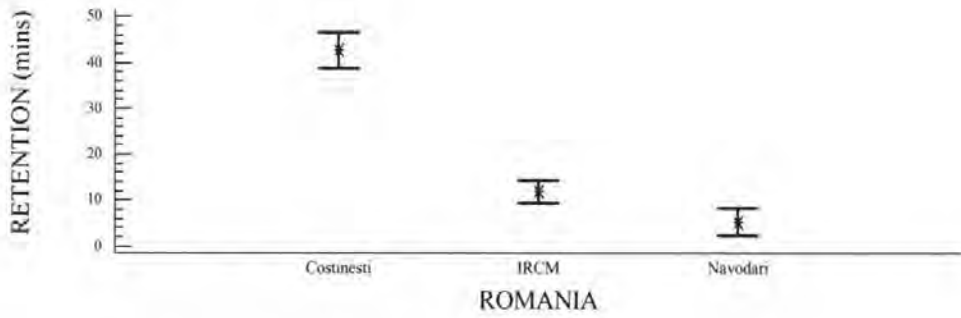


Fig. 56. *Mytilus galloprovincialis*. Mean plots, with 95% confidence intervals, of haemocytes lysosomal retention time of the neutral red probe.



Fig. 57. *Mytilus galloprovincialis*. Mean plots, with 95% confidence intervals, of haemocytes lysosomal retention time of the neutral red probe.

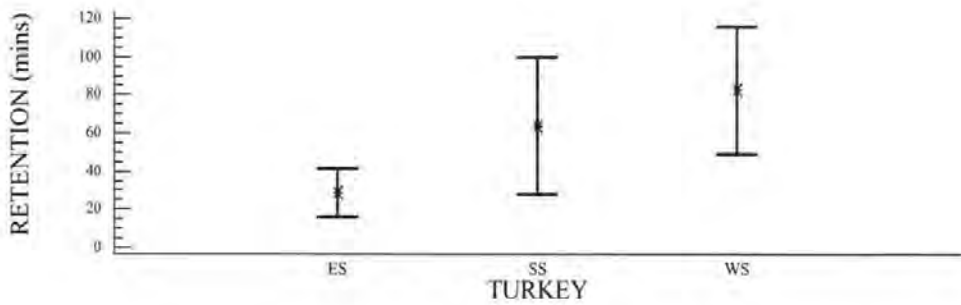


Fig. 58. *Mytilus galloprovincialis*. Mean plots, with 95% confidence intervals, of haemocytes lysosomal retention time of the neutral red probe.

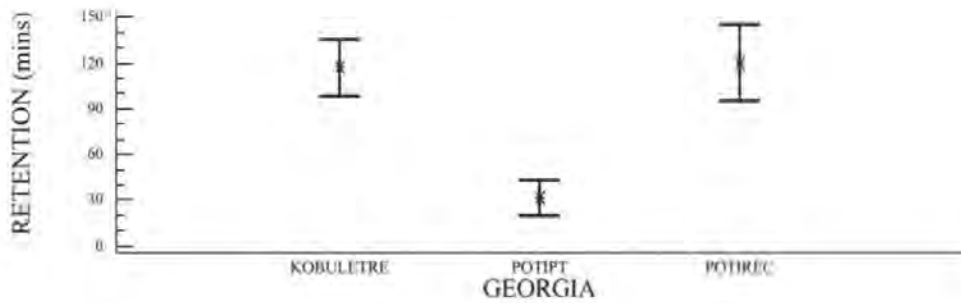


Fig. 59. *Mytilus galloprovincialis*. Mean plots, with 95% confidence intervals, of haemocytes lysosomal retention time of the neutral red probe.

Mussels sampled from the Ukrainian port of Odessa had a similarly depressed lysosomal retention time of 17.5 minutes. The Bulgarian site of Port Varna had mussels with a low retention time of 11 minutes. Mussels from the Bulgarian site of Shkorpilovtsy had a greater retention time of 84 minutes. The highest retention times were recorded in mussels from the Russian site of Lazarevck, 108 minutes, the Georgian sites of Kobultre, 117 minutes, and Poti Recreational site, 120 minutes, and the Ukrainian site of Koblevo, 150 minutes (Fig. 60 illustrates retention times around the Black Sea coastline in a schematic form).

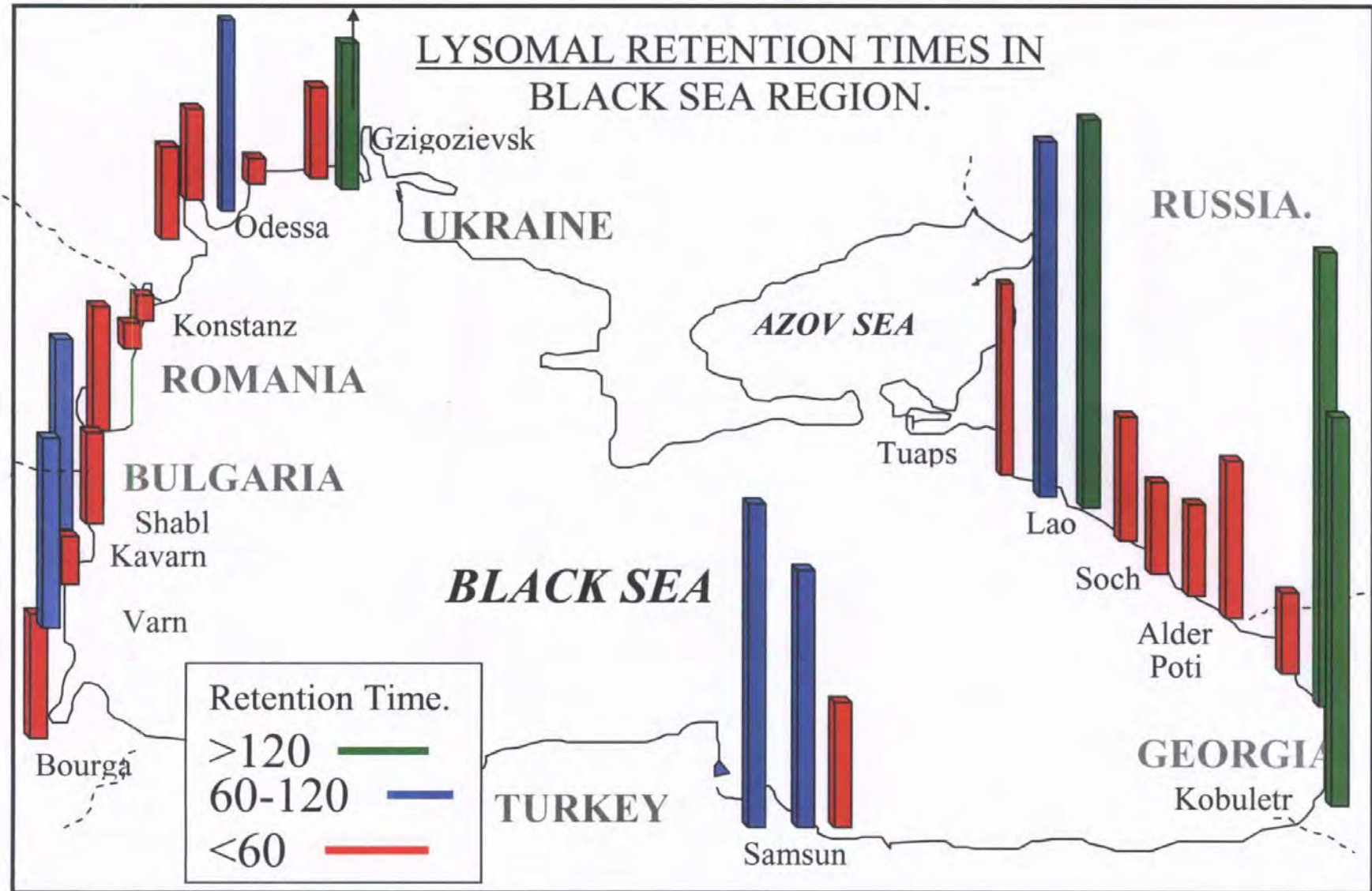


Fig. 60. Schematic map showing Black Sea and sample sites, by region. Colour and height of bars represent an approximation of neutral red values.

9.4.2 WHO Rapid inventory Data/Tissue Residue Data

The Romanian study sites had the greatest loading of BOD with an estimated input of 38,840 tonnes per year at the Navodari site alone. The Romanian land based sources also produced the largest inputs of phenol by discharging 1,254 tonnes per year into the Black Sea. The Ukrainian inputs had the largest amount of oil with 62,700 tonnes per year entering the Black sea from the Odessa port. The Odessa site also had the greatest inputs of total suspended solids (TSS), with 50,730 tonnes per year entering the Black Sea. The Romanian site of Navodari receives the greatest amount of total nitrogen (TN) input by discharging 50,730 tonnes per year. The Ukrainian and Romanian study sites were heavily influenced by the presence of the Danube, Dniester, and Dnieper rivers which are responsible for the greatest input of water, into the Black Sea, with elevated levels of oil, BOD, and phenol (Sarıkaya *et al.*, 1997). In total these rivers were responsible for 54,600 t/y of oil, 242 t/y of phenol, 1,493 t/y of copper, and 844,464 t/y of BOD into the Black Sea (Sarıkaya *et al.*, 1997). The Odessa site received a further 626.6 t/y of phenol from industrial sources.

Of the Bulgarian sample sites the Port Varna site received the highest loading of BOD, 1,740 t/y⁻¹, oil, 2,823 t/y⁻¹, and TSS 3,170 t/y⁻¹. The Bulgarian sample sites of Kavarna and Skorpilotsyvyky received the lowest amounts of land based discharges of the Bulgarian sites. The Russian site of Lazarevck had the lowest inputs of BOD, TSS, oil, TN, and TP with values all less than 1.5 tonnes per year (Figure 60 illustrates these findings in a schematic form).

The tissue burden analysis of the mussels indicated that those animals at the Ukrainian Odessa site had the greatest burden of poly-cyclic-aromatic hydrocarbons (PAHs), 6,882 ng/g⁻¹ and pesticides, 243.3 ng/g⁻¹ DDT. These animals also had high levels of PCBs, 700 ng/g⁻¹ in their tissue. Animals sacrificed

from the Port Varna population, Bulgaria, had a tissue residue of $1,565 \text{ ng/g}^{-1}$. The lowest concentrations of tissue residue for PAHs was at the Bulgarian site of Kavarna, with a measured residue of 63 ng/g .

9.4.3 Statistical Analysis

One way analysis of variance tests were conducted on the individual lysosomal integrity data sets from the participating countries to identify significant differences in lysosomal retention times. These data are presented in Figures 61 and 62 , significance being calculated to the 95% confidence interval.

Regression analysis of the individual components of the rapid assessment data was carried out to determine to what extent the BOD, oil, TSS, TN, and TP data could account for the variability of the lysosomal data. The results indicate that BOD (Fig. 61) and TSS (Fig. 62) could account for 70% ($p < 0.0002$) and 76% ($p < 0.0002$) respectively of the biological variability. TN and TP accounted for 65% ($p < 0.0009$) of the biological variability. The rapid assessment data for discharged oil only accounted for 46% of the variability in the lysosomal data and was not significant ($p > 0.08$).

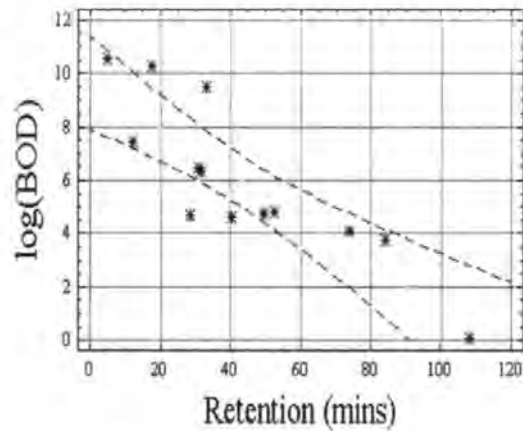


Fig 61. *Mytilus galloprovincialis*. Plot of fitted model for the regression analysis of land based sources of BOD versus molluscan haemocytic lysosomal retention time for the neutral red probe. Dotted lines show 95% confidence limits. ($p = 0.0001$, correlation coefficient = -0.84 , $R^2 = 70.54\%$)

The limited data sets for the detected tissue burden of either PAHs or pesticides did not produce a significant correlation with the lysosomal integrity ($p = 0.09$ and $p = 0.82$ respectively).

A regression analysis of the rapid source inventory components with PAH tissue burden in the mussels was carried out. The results indicated that TSS and BOD could account for 70% ($p > 0.10$, 90% confidence interval, correlation coefficient of 0.84) and 82% ($p < 0.10$, 90% confidence interval, correlation coefficient of 0.90) respectively of the variability in the PAH tissue burden.

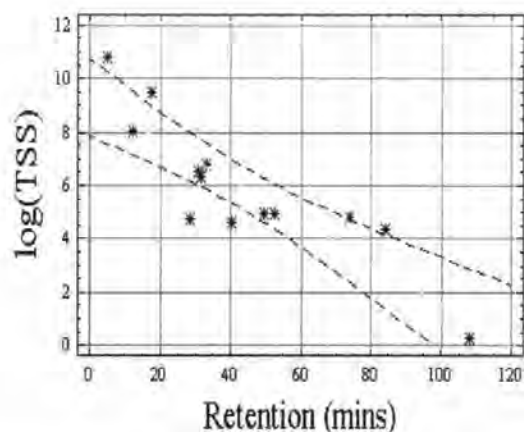


Fig 62. *Mytilus galloprovincialis*. Plot of fitted model for the regression analysis of land based sources of TSS versus molluscan haemocytic lysosomal retention time for the neutral red probe. Dotted lines show 95% confidence limits. ($p = 0.0001$, correlation coefficient = -0.88 , $R^2 = 76.90\%$)

9.5 Discussion

The low retention times shown by lysosomes from the blood cells of mussels sampled from the highly industrialised sites on the Black Sea coastal zone is consistent with the findings of other studies which have examined lysosomal integrity in indigenous, chronically exposed, mussel populations (Lowe *et al.*, 1995; Krishnakumur *et al.*, 1994). A strong relationship has been recorded in this study between reduced lysosomal retention time and elevated inputs of contaminants indicated by the rapid source inventory data. For example, the mussels from the Ukrainian sites of Odessa Port and Liman Sukchoy had greatly reduced lysosomal retention times in conjunction with massive inputs of organic micropollutants. This trend was repeated in mussels from the Romanian sites of Navodari and IRCM, and the Bulgarian site of Port Varna. These study areas were impacted by high quantities of industrial waste, in particular BOD and phenol (Sarıkaya *et al.*, 1997) and the lowest retention times for the entire study were recorded at these sites. Conversely, sites which had indigenous mussels

exhibiting higher retention times were also shown to have reduced inputs of organic and inorganic contaminants. For example, the Bulgarian sites of Kavarna and Skorpilotsyvyky showed increased retention times in conjunction with relatively reduced inputs of BOD and oil. The Russian site of Lazarevck also showed a similar trend.

The findings indicated an association of low retention times with high tissue residues of hydrocarbons at the Odessa Port and Port Varna sites. Mussels at the Odessa site were also shown to have extremely high levels of PCBs with a tissue burden of 700 ng/g^{-1} recorded. In contrast, mussels collected from the Ukrainian site of Koblevo, where higher retention times were observed, had a PCB tissue burden of 29 ng/g^{-1} . However, no significant relationship with retention could be obtained with the tissue residue values obtained at other sites. No studies have attempted to investigate the lysosomal biomarker on the ambitious scale shown here, nor with the use of such a rapid, simple means of assessment. This study has shown that the lysosomal biomarker, as investigated with the neutral red technique, is a rapid, simple means of determining the health of indigenous mussel populations. This represents a novel approach in environmental impact assessment as it is both simple and cheap, yet rapidly generates relevant and useful biological information by the indicating those mussel populations which are showing an adverse, contaminant-linked, biological response.

In this study, consistent pathological responses were observed in the haemocytic lysosomes of mussels collected from urban and industrial sites. A clear relationship was indicated, at certain sites, between the presence of hydrocarbons in the tissue and water, and low lysosomal retention times. Lysosomal retention times were shown to be particularly depressed in areas with a high input of

hydrocarbons, for example the Navodari and Varna sites in, respectively, Romania and Bulgaria. Laboratory studies have shown that PAHs, such as phenanthrene and anthracene, can induce dose-dependent lysosomal destabilisation in the digestive cells of bivalves (Pipe and Moore, 1986; Nott and Moore, 1987; Axiak *et al.*, 1988; Cajaraville *et al.*, 1989; Winston *et al.*, 1991; Variango *et al.*, 1992). However, in this study there was only an association of lysosomal response and hydrocarbon tissue burden in sites which had an extremely high level of background hydrocarbon contamination.

The strong association of lysosomal perturbation with the rapid assessment data indicated the impact that anoxia, as a result of elevated BOD, may be having on the mussel populations. Konsoulova (1993) indicated the susceptibility of the Black Sea zoobenthos to the lack of oxygen caused by anthropogenic eutrophication; and Tracy (1985) demonstrated a reduction in the growth of mussels in the presence of increasing eutrophication. A decline in the biomass of *M. galloprovincialis* has been indicated by the Romanian National Report (1995), which could be attributed to this increase in eutrophication. In the freshwater mussel (*Margaritifera margaritifera*) decreasing survival and establishment of juvenile mussels has been correlated with BOD (Bauer, 1988). Losovskaya *et al.* (1990) reported a decline in the population of *M. galloprovincialis* after significant discharges of eutrophic waters in the region between the Dnieper and Dniester rivers. Indigenous populations of mussels located near the Danube have also been reported to decrease in size during periods of heavy river flow (Losovskaya *et al.*, 1990). There may also be additive effects occurring between elevated levels of organic material and organic and inorganic pollutants. Gilek *et al.* (1996) reported an increase of PCB accumulation in the blue mussel (*M. edulis*) after

exposure in conjunction with high levels of algal enrichment; and Tracy (1990) reported a modification of copper uptake in *M. edulis* after exposure with varying levels of nutrient enrichment.

The lysosomal integrity of the mussels was strongly correlated with the presence of large inputs of suspended sediment, which may have associated particulate bound contaminants. These contaminants may only become available under certain conditions or after uptake by the biota. The input of suspended sediment, being largely composed of sediment derived from the watershed will no doubt include organic and inorganic contaminants. Suspension filter feeders, such as mussels, will respond to contaminants in both the dissolved and suspended phase (Rainbow, 1995) and higher levels of suspended sediment will presumably increase the potential for the uptake of greater amounts of contaminants. Nelson *et al.* (1987) found that scope for growth decreased in mussels exposed to increasing concentrations of suspended sediment from a contaminated harbour while Prins and Smaal (1987) reported a net carbon loss in molluscs exposed to high particle concentrations, possibly due to an increase in mucus production.

The evidence for the eutrophic nature of the northwestern shelf of the Black Sea is overwhelming (Mee, 1992). This part of the Black Sea is noted for the impact of the Danube, Dniester, and Dnieper rivers and their not inconsiderable contribution of contaminants (Sarıkaya *et al.*, 1997). The impact of the Danube river on BOD and the large inputs of oil from the Romanian coastline were almost certainly contributing to the severely reduced retention times observed in this area. For example, the Romanian oil refineries at Novadari were solely responsible for 25% of the total BOD to the Black Sea (Sarikiya *et al.*, 1997).

The demonstration here, in a major component of the zoobenthos, of a biological response that has previously been shown to be associated with toxicological stress demonstrates the sensitivity of the Black Sea's ecosystems to continual pollutant exposure. Indeed, in the north western shelf, the population of *M. galloprovincialis* has been shown to be reduced in numbers, and this has been attributed to the effects of eutrophication (Zaitsev and Mamaev, 1997).

Given the restricted nature of the available information, and the complex nature of many discharges, it is difficult to assign the pathological response observed in the mussel haemolymph to the toxic effect of a single polluting discharge, chemical, or group of chemicals. Depledge (1994) suggested that whilst biomarkers which indicated exposure to pollutants are useful, those which signify that organisms are experiencing adverse effects are more ecologically relevant. To function, in this manner, it is necessary for a biomarker to demonstrate a long-term response. Regoli (1992) demonstrated no recovery in lysosomal latency, in *M. galloprovincialis*, after a four-month depuration period, following metal exposure. This would indicate that the lysosomal response does not appear to be transient. Increased membrane fragility may have implications for the future growth and reproductive potential of mussels (Lowe *et al.*, 1995). Moore and Viarengo (1989) indicated that lysosomal membrane fragility in mussels is directly related to enhanced autophagic protein catabolism and, thus, a failure of lysosomal membranes to immediately recover. Furthermore, Krishnakumar *et al.* (1994) showed that mussels, sampled from urban associated sites in Puget Sound, exhibited decreased lysosomal stability and a corresponding reduction in size and somatic tissue weight. A number of studies have shown a functional relationship

between adverse alterations in lysosomal stability and scope for growth (Widdows *et al.*, 1982; Moore *et al.*, 1987), following contaminant exposure.

In conclusion, this study examined the contaminant impact on mussels sampled from indigenous populations around the Black Sea coastline, using a simple lysosomal integrity test as a biomarker of effects on haemocytes. In addition, using regression analysis, the relationship between the probe (neutral red) retention time and polluting discharges into the coastal zone was examined. In mussels from certain sites the hydrocarbon tissue burden was also examined. Konar and Stephenson (1995) suggested the tandem use of tissue contaminant data and bioassay tests as having promise for the evaluation of water quality criteria, rather than as stand alone techniques.

Mussels from some areas of the Black Sea coastline are clearly impacted, as indicated by the extreme perturbation of the lysosomal component within their blood cells. The presence of oil, but also significant inputs of suspended sediment and an elevated biochemical oxygen demand, appears to be a major contributing factor to the pathological response observed in the molluscan blood cells. This study indicates a biological response from a major component of the zoobenthos of the Black Sea ecosystem and the use of a simple approach to demonstrate contaminant induced effects. The results obtained in this study will add to the knowledge base of those individuals responsible for the management of the coastal and marine resources of the Black Sea. This work has also demonstrated the ease with which sensitive biological effects monitoring could in future be integrated with other chemistry orientated 'Mussel Watch' programmes.

10.0 CONCLUSION

There is now a considerable argument that for the biomarker approach to have a greater value in bio-monitoring programmes, there must be an increased understanding of the consequences of biological responses for the population and ecosystem level (Depledge *et al.*, 1993; Moore *et al.*, 1994; Goksøyr *et al.*, 1994; Parrett, 1998). The purpose of this work was to address the following aims:

- Further validate the use of the Neutral Red Test in *Mytilus edulis*
- Examine the use of the Neutral Red Test in *Carcinus maenas*
- Utilise the Neutral Red Test in conjunction with other biomarkers
- Explore any linkages between lysosomal perturbation and higher order effects
- Validate the use of the Neutral Red Test for environmental monitoring

The use of *M. edulis* for the purposes of determining lysosomal fragility with the neutral red test has been further validated. The laboratory exposures, described in section 3.0 and the examination of response in conjunction with histopathology in section 4.0 provide strong evidence that the haemolymph of *M. edulis* is suitable for examining the lysosomal biomarker.

Furthermore, the use of two novel species for determination of lysosomal response has been investigated. *C. maenas* and *A. cygnea* have shown similar lysosomal responses to those found in *M. edulis*, showing that the lysosomal response is highly conserved and that the neutral red test is a suitable method for other species. This work demonstrates that lysosomal membrane instability, which

is known to be associated with impaired scope for growth (Bayne *et al.*, 1979; Allen and Moore, 2004) occurs in association with a reduction in reproductive success. Furthermore, this study has shown lysosomal instability is relatively sensitive to pollutant exposure, is conserved between different species of marine and freshwater organisms and can be readily applied, in conjunction with other techniques, at a range of different scales.

Cardiac activity has been shown to be associated with lysosomal instability, and tissue pathologies, in this study. Physiological monitoring has been proposed as a measure of physiological competence (Depledge and Lundebye, 1996; Bamber and Depledge, 1997) and this work further supports this by demonstrating the induction of other pollutant responses (such as lysosomal dysfunction and tissue pathology) in conjunction with altered cardiac activity.

Measurement of metallothionein levels in both mussel and crab tissue did not provide consistent results and could not be correlated with other biomarker responses. Both Astley *et al* (1999) and Pedersen and Lundebye (1996) describe lower than expected metallothionein levels in organisms exposed to metal rich environments. Given the lack of metal analysis conducted in this study these low levels of metallothionein may actually be accurately reflecting metal exposure. Furthermore, the modification of metallothionein response to biotic factors such as age and sex, and environmental factors such as temperature and salinity is poorly understood (Serafim, 2002) and may have been contributing factors.

The demonstration of early life stage developmental abnormalities in association with sub-lethal biomarkers, such as lysosomal membrane fragility, is significant as

this further suggests that lysosomal response is not only a suitable biomarker for whole animal health but that it can be associated with higher order consequences. Krishnakumar *et al* (1994) demonstrated reduced growth and somatic tissue weight associated with increased lysosomal fragility and PAH body burden. Bayne *et al* (1978) described a reduction in mussel fecundity after stress as a result of the release of lysosomal enzymes and a reduction in the chances of survival for any gametes that do develop. This is supported here, particularly by the examination of sub-lethal, physiological, and reproductive responses within the same individuals.

On a practical level most of the techniques used here have been applied in a straight forward manner which is encouraging for their continued use in less developed regions. This supports the approach proposed by the Rapid Assessment of Marine Pollution (RAMP) methodology which encourages the use of focused diagnostic techniques to evaluate levels of marine pollution (Wells *et al.*, 2001). The Black Sea Mussel Watch Programme has been recognised as being a successful application of examining the relationship between environmental problems and social and economic pressures (Mee and Bloxham, 2002) through its utilisation of lysosomal fragility and rapid assessment data. Depledge *et al* (1993), Moore and Simpson (1992); Moore *et al* (1994) and Da Ros *et al* (2002) identified that biomarkers must be both rapid and robust if they are to be a suitable tool for environmental assessment. The evidence here is that the use of lysosomal dysfunction, in conjunction with other biomarkers such as pathological and reproductive measures of effect, provides both these characteristics.

There has been a failure to demonstrate any form of response that can be linked to a specific pollutant, or even pollutant class exposures. This is a limiting factor and has prevented greater interpretation of pollutant effect. Furthermore, laboratory results may be limited value or even misleading, if the aim is to extrapolate observed test results into the field since they do not consider factors such as salinity, temperature, light levels or the effects of other contaminants; all of which may alter pollutant effect. On the other hand, should we necessarily expect biomarkers of "health impairment" to be contaminant specific, since much of the actual toxicity is mediated by reactive nitrogen and oxygen species (RNOS) attacking membrane lipids and cellular proteins (Livingstone *et al.*, 2000).

Perhaps it is naïve to expect specificity when in fact much of the cell injury is generalised. Specificity can be delivered to some extent by exposure biomarkers for neurotoxins, genotoxins, and inducers of cytochromes P-450 and multidrug resistance transporters (Livingstone *et al.*, 2000; Viarengo *et al.*, 1990).

Nevertheless, the laboratory data presented here enabled some appreciation of the nature of responses to pollutant exposure and a greater confidence that observed field data were in fact responses to pollutant exposure. The introduction of more detailed chemical analysis, in the laboratory exposures and fieldwork, would have provided more information regarding true exposure concentrations.

This work has clearly shown the value of *in situ* field monitoring using a range of biomarkers in mussels, and crabs. Techniques have been applied in new species that will benefit the design of future monitoring studies. Important links have been demonstrated between different biological responses that will aid future monitoring programs examining the consequences of pollutant exposure and further support the application of the biomarker techniques utilised here.

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1999 Pollution Responses in Marine Organisms (PRIMO) 10 – Platform Presentation

Publications to be considered:

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