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Metallothioneins as Biomarkers of Metal Pollution in Estuaries on the South Coast of England

by

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Abstract

Contamination of aquatic environments via anthropogenic release of metals is an increasing global environmental concern. The greatest concerns exist within estuarine and harbour environments, where point and non-point metal sources are prevalent. Historically, monitoring programs were initially (and largely) based on chemical analyses; however, the identification of a number of shortcomings in this approach resulted in a shift towards the complementary use of biological monitoring (biomonitoring).

The aim of this study is to investigate and compare the suitability of selected aquatic invertebrate taxa biomarker responses (by production of metallothionein- MT) for assessment of aquatic environmental conditions in three coastal estuaries in Southern England: Poole Harbour, the Fal estuary and the Solent. This was delivered through a critical review of the current status of metal contamination in biota, water and sediment within these estuaries; by investigation of the suitability of selected species' metallothioneins to act as biomarkers by assessing their correlation with metal pollution; by assessment of the effectiveness of these organisms as bioindicators in biomonitoring studies; and though investigation of the potential use of MT concentrations in these species as biomarkers of metal exposure in monitoring programmes.

Legislation has been established to facilitate the protection, and enhance the quality, of all water bodies, including coastal waters and estuaries to prevent their deterioration and to ensure they achieve 'good' ecological health; but examination of water and sediment samples showed that historical metal pollution has not been dispersed and still affects environmental quality. In Poole Harbour, metal contamination was detected in all environmental compartments. The enclosed nature of the harbour and its secondary embayments make it vulnerable to the effects of these metal sources which disperse extremely slowly due to restricted tidal exchange; and a significant part of the pollution load remains in coastal areas close to land based contamination sources. In Poole Harbour, water was polluted with As (ranged from 29.6 to 212.8 µg/l) and Hg (ranged from 0.368 µg/l to 11.06 µg/l) which are above the Environmental Quality Standard (EQS) set by the European Union dangerous

substances directive (EUDSD). Sediment metals were mostly within “the possible effect range” at which adverse effects occasionally occur, according to the Canadian Sediment Quality Guidelines (CSQG). Although cockles (*Cerastoderma edule*) had higher tissue concentrations of Ni, Ag and Hg in areas close to pollution sources, and sponges (*Haliclona oculata*) had accumulated Cu and Zn with a very high magnitude, the absence of EQS for metals in living organisms’ tissues makes it difficult to specify whether the metal concentrations reached a dangerous level or not in these organisms. The results confirmed the presence of a marked metal pollution gradient in the Fal Estuary between the ‘clean’ sites in the north and east of the estuary and the ‘polluted’ sites in the west of the estuary which were historically polluted by water from mines. Moreover, the concentrations of metals in almost all of the contaminated sites in the Fal and Solent estuaries have not changed significantly over the past few decades.

This study investigated the potential of MT as biomarker of metal pollution in the following wild species: two well-studied bioindicators- common cockles (*C. edule*) from Poole Harbour, and mussels (*Mytilus edulis*) from the Fal estuary and the Solent; and tested the following novel bioindicators: sponges (*H. oculata*) from Poole Harbour, ragworms (*Hediste diversicolor*) From the Fal estuary, and limpets (*Patella vulgata*) from the Solent. The spectrophotometric method was used to measure MT in these species as it has been reported to be a sensitive, time saving, and low-cost technique able to detect MT content in the tissues of aquatic organisms. The results showed that MT concentration in these estuarine wild invertebrates vary between sites; however, these variations were not exclusively associated with metal concentrations and that other environmental factors may explain some of the MTs variability. The many uncertainties surrounding MT in wild organisms indicate that it may have limited potential as a biomarker in estuarine environments. It is also apparent that the importance of biotic and abiotic factors at polluted sites may limit its application.

In this study, transplanted mussels (*M.edulis*) and Manila clams (*Tapes philippinarum*) were used as active biomonitoring tools in Poole Harbour. It was found that under extreme conditions of metal pollution, the relationship between metal concentrations and MT deviates from linearity: a threshold appears to be reached beyond which the physiological capacity of organisms introduced to polluted areas is exceeded. In the above transplanted animals, MT

induction increased to peak levels with increased metal concentration, and then declined with further increases in the concentration.

Keywords

Metal pollution; biomonitoring ; Poole Harbour; the Fal estuary; the Solent; metallothionein; *Cerastoderma edule*; *Mytilus edulis*; *Haliclona oculata*; *Hediste diversicolor*; *Patella vulgata*; *Tapes philippinarum*

Declaration of Authorship

I, Walid Mohammed Taher Elsawy Aly declare that the thesis entitled “Metallothioneins as Biomarkers of Metal Pollution in Estuaries on the South Coast of England” and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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- parts of Chapters 4 & 5 incorporate work submitted as dissertations for Master of science degree for the following students I co-supervised: Robert James Barker, Laura Humphries and Suisheng Li

Signed: ……………………………………………………………………..

Date:………………………………………………………………………….

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I would like to dedicate this dissertation to my daughters Lena and Samma for the betterment of their lives and in the hope that it may make a positive contribution to their future, to my wife, Dalia who stood beside me through the difficult times of study, and to the memory of my mother, who died a few months ago.

Above all I would like to thank God for the blessing of the opportunity given me as well as the strength He gave me to complete it.

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Definitions and abbreviations

AAS	Atomic Absorption Spectrometry
CCME	Canadian Council of Ministers of the Environment
CSO	Combined Sewer Outfall
CSQG	the Canadian Sediment Quality Guidelines
DPP	Differential Pulse Polarography
EA	the Environment Agency in the UK
EQSs	Environmental Quality Standards
EUDSD	the European Union dangerous substances directive
GSH	reduced Glutathione
HMW	High Molecular Weight
HPLC-AAS	High performance liquid chromatography coupled with atomic absorption spectroscopy
HPLC-ICP-AES	High performance liquid chromatography coupled with inductively coupled plasma source atomic emission spectroscopy
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	Hydride Generation Inductively Coupled Plasma Optical Emission Spectrometry
LMW	Low Molecular Weight
MED POL	the programme for the assessment and control of pollution in the Mediterranean region
MTLPs	Metallothionein-like proteins
MTs	Metallothioneins
PEL	the Probable Effect Level
PMSF	phenylmethanesulfonylfluoride
PTE	Potentially Toxic Element
Ramsar site	Wetland of International Importance
SPA	European Special Protected Area

SSSI	United Kingdom Site of Special Scientific Interest
STWs	Sewage Treatment Works
TEL	the Threshold Effect Level
UKAS	the United Kingdom Accreditation Service
UNEP	the United Nations Environment Programme

Chapter 1: Introduction

1.1. Pollution in aquatic Environment

The term "Pollution" has many definitions, one of them is "the presence of a substance in the environment that because of its chemical composition or quantity prevents the functioning of natural processes and produces undesirable environmental and health effects" (United States Environmental Protection Agency, 2008). Under the USA Clean Water Act (1972), the term has been defined as "the man-made or man-induced alteration of the physical, biological, chemical, and radiological integrity of water and other media"(United States Environmental Protection Agency, 2008).

Throughout the world, coastal ecosystems are at risk. More than half of the coastline in the world suffers from severe pressures of development based on growth of human populations and the propensity of these populations to be concentrated in coastal areas. With the rapid urbanization coastal industrial and commercial development that has increased the pressure on habitats of coastal wetlands, including estuaries, mudflats and salt marshes (World Resources Institute, 1995).

1.2. A definition problem: Are they heavy, trace, toxic or potentially toxic metals (or elements)?

“Heavy metals” was the term used to be applied to a large group of elements which are both industrially and biologically important. Although not completely satisfactory from a chemical point of view, 'heavy metals' was until recently the most widely recognised and used term for these elements based on their atomic density. Duffus (2002) reported 13 different research works that used lower limits on the density of a “heavy” metal ranging from 3.5 to 7 g cm. The author stated that the threshold varied depending upon the evidence available to the authors and their interpretation of it, and he stated that “it is impossible to come up with a consensus”. Moreover, he concluded that “any idea of defining “heavy metals” on the basis of density must be abandoned as yielding nothing but confusion”.

Furthermore, the term 'heavy metals' is often used disparagingly with emphasis on the pollution and toxicity aspects rather than as a collective name for a group of elements of considerable economic and environmental importance.

Another term for this group of elements is 'toxic metals' which is even less appropriate because all trace elements are toxic to living organisms when present in excess. However, some such as Cobalt (Co), Chromium (Cr), Copper (Cu), Manganese (Mn), Molybdenum (Mo) and Zinc (Zn) are essential in small but critical concentrations for the normal healthy growth of either plants, animals or both, although they are toxic at high concentrations.

Another term “trace metals” is gaining some acceptance, however, not all ‘trace metals’ are metals in the first place; arsenic and antimony, for example, are usually classified as ‘trace metals’, regardless of the fact that they are metalloids (semi-metals) (Hubner et al., 2010a), and the term “trace elements” is very broad expression which emphasis on quantity rather than specific properties and could include any element. A newer term which is being increasingly used with reference to the harmful properties of these metals is 'potentially toxic element' (or PTE) and this seems to be gaining acceptance. In this study, the elements determined were collectively called “metals” in this text although it contains arsenic, which is a metalloid as stated above.

1.3. Metal pollution in aquatic Environment

The aquatic environment receives waste products from a wide range of anthropogenic sources, including mining, foresty, industrial and domestic waste disposal, and fuel combustion, Figure 1.1. As a result of such activities, aquatic environments are becoming contaminated with metals. While the massive dilution of contaminants in the open sea makes it difficult to prove that metals are having a significant effect on the biota, estuaries are a different situation. Many estuaries are grossly polluted; particularly those fed by rivers that pass through heavily industrialized regions or regions of mining activity (Walker et al., 2006). When polluted fresh water reaches the sea, the flow rate slows down, suspended sediments settle on the bottom, and dissolved metals are precipitated. Even if the discharges to rivers are cleaned up, the estuaries they feed may continue to be affected for many years because of remobilization of past sediment contamination (Walker et al., 2006). Estuaries are regions of high productivity, important in the life cycles of many birds, fish and invertebrates including commercially important coastal species. Metals like Cd, Cu and Zn feature in any list of potentially toxic contaminants, particularly in the estuaries of the rivers draining regions with a history of mining or heavy industry (e.g. oil industry, shipping) (Geffard et al., 2005b).

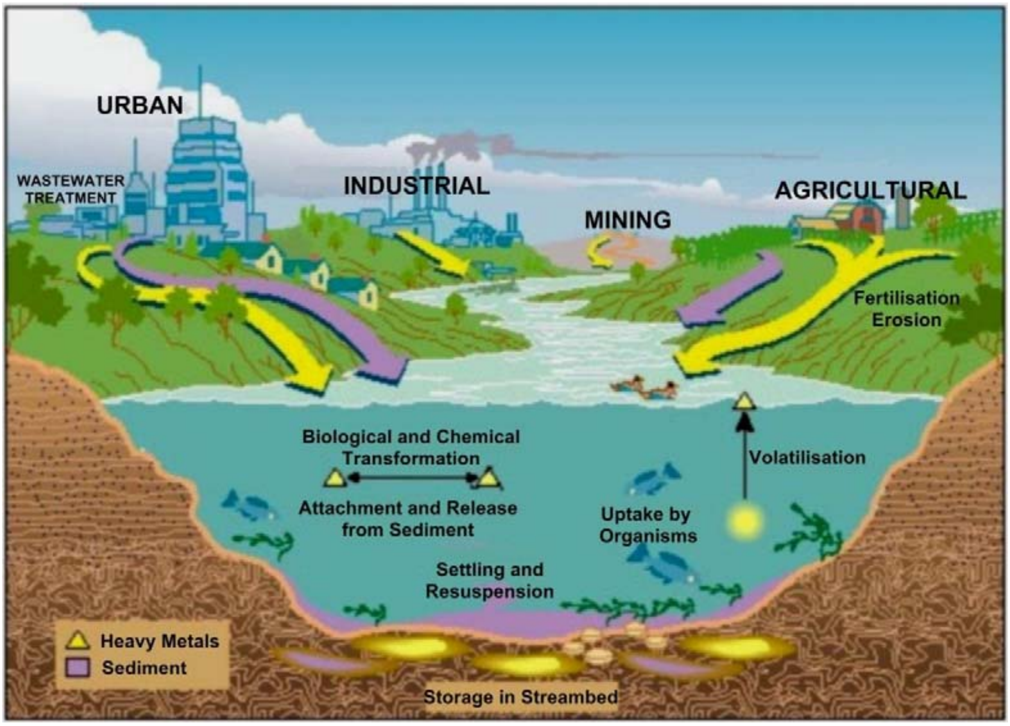


Figure 1-1: Sources and sinks of metals in aquatic environment (Meade, 1995).

Metals are non-biodegradable and do not break down in the environment. Once metals get into soils or sediments, they typically have long residence times before they are transferred to another environmental compartment (e.g. surface layers of soil will remain contaminated with lead for several hundreds of years) (Walker et al., 2006). Although measurements of metals in sediment and water are a useful guide to evaluate environmental contamination, ultimately it is the impact on biota which is of most concern (Langston et al., 2003). Metals can accumulate in the marine food web up to concentrations which are toxic to marine organisms, particularly predators, and they may also represent a health risk for humans (HELCOM, 2007). Metals are assimilated by some organisms to a great extent which is reflected in the bioaccumulation of these metals in their tissues. High bioconcentration (e.g. concentration of Zn in mussel's tissue could reach 12355 times that of seawater (Kwon and Lee, 2001)) may be a result of the substance in question being more bioavailable (Walker et al., 2006).

Some species of invertebrates are known to be efficient accumulators of trace elements for example mussels e.g. Common mussel *(Mytilus edulis)*; oysters e.g. Pacific oyster (*Crassostrea gigas)* (Amiard et al., 2007); polychaete e.g. ragworm *(Nereis (Hediste) diversicolor)* (Ng et al., 2008) and *sponges e.g.* Moon Sponge *(Cinachyra spp)* (Gomes et al., 2006). Generally, metal accumulation by such organisms is based on efficient detoxification mechanisms, such as intracellular compartmentalization, or metal inactivation by binding to certain protein molecules (Demuynck et al., 2004).

Metal toxicity is often postulated to arise from reactions occurring in the cytosol (intracellular fluid found inside cells), through non-specific binding of the metal to non-thionein ligands, which are physiologically important molecules (e.g. metalloenzymes or small peptides such as glutathione) and are inactivated by metal binding (Mason and Jenkins, 1995). Control of intracellular metal toxicity and detoxification of accumulated trace metals is generally achieved via the production of metal-binding ligands that sequester metals. These cellular ligands can be found in the particulate fraction of the cells (e.g. mineral deposits, granules or lysosomes (George, 1990; Mason and Jenkins, 1995)) and in the cytosol (e.g. metallothioneins (Engel and Roesijadi, 1987; Viarengo and Nott, 1993; Demuynck et al., 2004)); Figure 1.2.

1.4. Biomarkers and Metal Pollution Monitoring

Historically, it was common practice in aquatic monitoring to record the total (and not necessarily bioavailable) concentrations of a small range of pollutants in different matrices in a given environment. A more modern and meaningful means of expressing the "health status" of a specific environment is to use a suite of biomarkers to measure an integrated response index. Chemical analysis shows only the concentrations of single pollutants in the environment, while an integrated biomarker index also demonstrates their bioavailabilities and interactive and combined effects with regard to numerous environmental factors characterizing the location (Amiard et al., 2006). Biomarkers are defined as "any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status" (Walker et al., 2006). Thus biochemical, physiological, histological, morphological, and behavioural measurements are to be considered as biomarkers. Biomarkers could be divided into biomarkers of exposure, which indicate exposure, not the adverse effect pollutants cause, and biomarkers of effect, which demonstrate the adverse effect on the organism (Walker et al., 2006). Examples of exposure and effect biomarkers included in the Natural England suite of assays of marine environment are shown in Tables 1.1 and 1.2. Biomarkers are regarded as early warning signals because the changes at molecular level occur at a threshold that is less toxic than the levels that can be detected by monitoring change in a species, population, or community (Won et al., 2008).

1.5. Aquatic organisms as indicators of metal pollution

Organisms which bio-accumulate metals have often been used as indicators of environmental metal pollution. This means that, ideally, metal concentrations in the organism's body reflect quantitatively or semi-quantitatively environmental pollution (Dallinger, 1994). In reality, however, many factors, such as the animal's weight and age, can disturb such quantitative relationships. These factors have, therefore, to be considered carefully before an invertebrate is utilized as a bio-accumulative indicator for metal pollution (Brown and Depledge, 1998).

Many surveys of trace metal concentrations in marine organisms have been conducted; However, the vast majority of information available in literature focuses on metal concentrations in whole organisms and tissues in molluscs (Baudrimont et al., 1997; Rousse et al., 1998; Amiard et al., 2008), and crustaceans (Engel and Brouwer, 1993; Pedersen et al., 1997; Astley et al., 1999; Legras et al., 2000; Galloway et al., 2004); although there are now substantial databases for annelids (*polychaete*) (Geffard et al., 2005b; Amiard et al., 2007; Ng et al., 2008), *colenterates* and *echinoderms* (Amiard et al., 2006), and to lesser extent marine algae (Campanella et al., 2001), and more recently sponges (*porifera*) (Philp, 1999; Gomes et al., 2006).

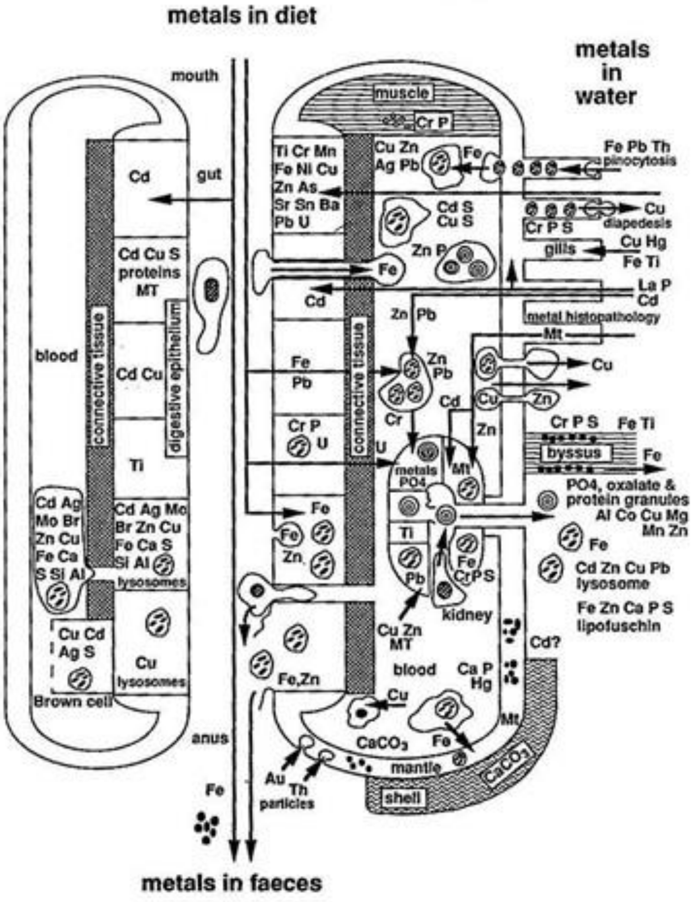


Figure 1-2 : Mechanisms for metal detoxification in bivalves (Viarengo and Nott, 1993).

Table1-1: Biomarkers of exposure included in the Natural England suite of assays. (modified after (Galloway et al., 2008)).

Biomarkers of exposure	Issues Addressed	Biological significance
AChE inhibition	Organophosphates and carbamates or similar molecules. Possibly algal toxins	Measures exposure to organophosphate and carbamate pesticides
Metallothionein Induction and metal partitioning	Measures induction of metallothionein protein by Certain metals (e.g., Zn, Cu, Cd, Hg)	Measures exposure and disturbance of copper and zinc metabolism
Intersex (also a biomarker of effect)	Specific to reproductive effects of organotins	Reproductive interference in coastal (littoral) waters
PAH urine metabolites	PAHs	Measures exposure to and metabolism of PAHs

Table 1-2: Biomarkers of effect included in the Natural England suite of assays (modified after (Galloway et al., 2008)).

Biomarkers of effect	Issues Addressed	Biological significance
On-line monitoring (including heart rate)	Not contaminant-specific; will respond to a wide range of environmental contaminants.	Measures the effects of chemicals on heart rate using a simple and inexpensive remote biosensor. Gives an integrated response
Scope for growth (incorporates clearance rate, oxygen measure of consumption etc)	Responds to a wide variety of contaminants	Integrative response; a sensitive sub-lethal measure of energy available for growth
Micronuclei	Not contaminant- specific, although agent would be genotoxic	A measure of DNA agent damage which may have higher consequences due to loss of DNA material
Lysosomal stability (including NRR)	Not contaminant- specific, but responds to a wide variety of xenobiotic contaminants and metals	Measure cellular damage and is a good predictor of pathology. Provides a link between exposure and pathological endpoints. Possibly, a tool for immunosuppression studies in white blood cells.
Immunocompetence (including phagocytosis)	Not contaminant- specific; will respond to a wide range of environmental contaminants.	Measures factors that influence susceptibility to disease

1.6. Metallothioneins as biomarkers of metal pollution

Apart from bioaccumulation, many invertebrates exposed to elevated metal concentrations respond to this stress by metal-induced synthesis of certain protein molecules called metallothioneins (Dallinger, 1994). Metallothioneins (MTs) are non-enzymatic proteins with a low molecular weight, high cysteine content, no aromatic amino acids and heat stability. The thiol groups (-SH) of cysteine residues enable MTs to bind particular Metals (Amiard et al., 2006).

Metallothionein-like proteins (MTLPs) are represented in many branches of the phylogenetic tree, from microorganisms to humans, and consequently are regarded as a central constituent of metal metabolism (Langston et al., 1998). MT was first reported more than 50 years ago as the Cd, Cu, and Zn binding protein responsible for metal accumulation in the horse kidney (Margoshes and Vallee, 1957). The high cysteine content (30%), low molecular weight, heat stability and non-enzymatic nature, together with a strong affinity to bind many metal cations such as Ag, Cd, Cu, Hg and Zn, enable MT to be differentiated from most other proteins. Though occurring mainly in cytoplasm, MT has also been detected in the nucleus and lysosomes. Several studies on the chemistry (Geret and Cosson, 2002; Ivankovic et al., 2002), molecular biology (Tanguy and Moraga, 2001; Tanguy et al., 2003; Knapen et al., 2004), cellular physiology (Chong and Wang, 2001; Demuynck et al., 2004; Geffard et al., 2005b; Ng et al., 2008) and toxicology (Amiard et al., 2007; Abbas Alkarkhi et al., 2008; Amiard et al., 2008) of MT in aquatic organisms have been published. The occurrence of MTs or MTLPs has been reported for some 50 different aquatic invertebrates from five phyla, three quarters of which are molluscs or crustaceans (Langston et al., 1998). The functions of MT include: the control of intracellular availability of essential metals to satisfy the requirements of metal-dependent components (metalloenzymes, respiratory pigments, nucleic acids and membranes); and gene regulation (Roesijadi, 1994). Functions also include detoxification: faced with an excess of essential or pollutant metal, binding to MT offers protection against toxicity by limiting metal availability at inappropriate sites (Langston et al., 1998).

MT in metal-loaded organisms can be present in different isoforms that are specifically synthesized in response to different metals. These characteristics make MT a potential biomarker for metal stress in invertebrates. One possibility may be to assess parameters of MT synthesis at the molecular or biochemical level. Moreover, MT isoform patterns could provide information on different isoforms synthesized in response to different metals or chemicals (Dallinger, 1994). One of the objectives in the use of biomarkers in ecotoxicology is to assess exposure or toxicity to specific contaminants from an environment characterized by multiple anthropogenic and natural stressors. The existence of metal-specific isoforms of MT represents such a potential, and it would be of value as biomarkers in hazard assessment (Roesijadi, 2003). Studies have showed that MT exhibits such properties, e.g. a cadmium-specific form in an earthworm *Lumbricus rubellus* (Sturzenbaum et al., 2001). MT isoforms with similar promise have been reported for Pacific oyster *Crassostrea gigas* (Tanguy and Moraga, 2001) and the mussel *Mytilus edulis* (Geret and Cosson, 2002). These might have utility where the environmental effects of the specific metals are of interest. In any case, however, care must be taken to consider intrinsic physiological parameters, such as nutritional or developmental factors, which could also interfere with MT synthesis (Dallinger, 1994).

Although all the studies which have demonstrated the clear MT induction by metal in some aquatic species (e.g. (Bebianno and Machado, 1997; Amiard et al., 2006; Figueira et al., 2012)), some other studies (e.g. (Amiard-Triquet et al., 1998; Mouneyrac et al., 1998; Legras et al., 2000; Strogyloudi et al., 2012)) present many confounding factors which can interfere with the estimation of concentration change induced by anthropogenic sources and limit the possibility of using MT concentration as a biomarker of metal exposure. Biotic and abiotic factors seem to have a significant influence on MT levels in some organisms. The effect of such factors on MT concentrations may lead to confound analysis of toxicologically related responses in the natural environment. Any factor is able to influence protein metabolism and/or metal uptake and accumulation will be able to influence indirectly variation in MT concentration. Our current understanding of MT in the different species inhabiting natural environments does not allow universal generalisation regarding its utility as a biomarker applicable to all species and/or in all types of environments (Ladhar-Chaabouni et al., 2012).

1.7. Metallothioneins classification and structure

Fowler et al. (1987) divided MTs into three classes:

- Class I: comprises all proteinaceous MTs with locations of cysteine closely related to those in mammals. Some molluscan and crustacean MTs belong to this class, such as those characterized in mussels, oysters, crabs and lobsters.
- Class II: includes proteinaceous MTs which lack this close similarity to mammalian MTs
- Class III: consists of non-proteinaceous MTs also known as phytochelatins.

More recently, (Binz and Kägi, 1999) have proposed a new classification of MTs taking into account phylogenetic features as an additional classification criterion. Class I and Class II MTs identified to date have been differentiated into 15 families of evolutionary related sequences. Such families are characterized by a unique sequence pattern, a taxonomic range, a set of common sequence characteristics obtained from both amino acid and polynucleotide sequences and a multiple sequence alignment or a phylogenetic tree. Some of the MT families allow a further subdivision into a number of phylogenetically separable subfamilies with different structural features or other genetic properties. In some particular cases, based on phylogenetic separation, the subfamilies can be further subdivided into subgroups and isolated isoforms and alleles. According to (Binz and Kägi, 1999)'s system, molluscan MTs belong to family 2 whereas crustacean MTs belong to family 3. (Binz and Kägi, 1999) did not allocate annelid or sponges MTs to a family.

Mammalian MTs take the form of single-chain polypeptides which are formed of 61 to 68 amino acid residues. The number and position of the cysteine residues is highly conserved in all mammalian species and form cys-x-cys, cys-x-y-cys and cys-cys sequences, where x and y are non-cysteine amino acids, Figure 1.3. Divalent metals are bound by sulphur atoms in thiolate clusters with a tetrahedral geometry, where no free thiol groups are exist. MT has two subunits: the more stable α-domain (C-terminal), which incorporates four divalent metal atoms, and the more reactive β-domain (N-terminal), which contains only three, Figure 1.4. The tertiary structure of MT is dynamic, and metal ions exchange rapidly within the β-domain, more slowly in the α-domain, and may also exchange with other ions bound to intracellular ligands (Coyle et al., 2002).

In contrast to mammals, where the existence of two major isoforms of MT appears to be the rule, some fish species appear to possess only a single MT isoform. However, tetraploidy fish, such as salmonids (salmon and trout) and cyprinids (common carp and goldfish), appear to have two isoforms of MT (Chan, 1995). These two MT isoforms are formed of 60 amino acids contained 20 cysteine residues, mostly in classical Cys-x-Cys and Cys-Cys-x-x-Cys-Cys motifs (Knapen et al., 2005).

Studies on molluscan MTs showed some differences, mostly in the cysteine content, between them and mammalian MTs. Oyster and mussel MT sequences have been confirmed to belong to class I MTs. The oyster's MTs show a greater similarity to vertebrate MTs than to other invertebrate MTs. All cysteines in the first 27 residues of the oyster MTs align with those in the mammalian forms. Moreover, 21 cysteine residues were arranged in the form of 9 Cys-X-Cys motifs, 5 Cys-Lys-Lys and a single Cys-XX-Cys. The two, oyster and mussel, chromalographilcally isolated MTs were virtually identical in amino acid composition. (29% cysteine. 16% glycine, and 13% lysine) (Carpene, 1993).

Crustacean MTs have a peptide chain length of 58 to 59 amino acids, with 18 Cys per MT. The alignment of crustacean MT sequences illustrates their high sequence similarity with very few exceptions where, remarkably, MTs contain two Cys triplets and show almost identical primary structures, which differ significantly from those of other crustacean MTs (Serra-Batiste et al., 2010).

Sponge MTLP is, in spite of the certainly close relationship to other MTs, different from the other known metazoan MTs in 2 aspects. Firstly, the sponge MTLP is larger, 20 kDa, in comparison with MTs from *Metazoa*, which are characterized by a molecular mass of 6 to 7 kDa. Secondly, while sponge MTL represents a tandem duplicated polypeptide composed of 2 parts which are highly related to metazoan MTs, some studies revealed that the domain composition and the degree of domain duplication in the sponge genome is unusually complex in comparison to other metazoan phyla (Schroeder et al., 2000).

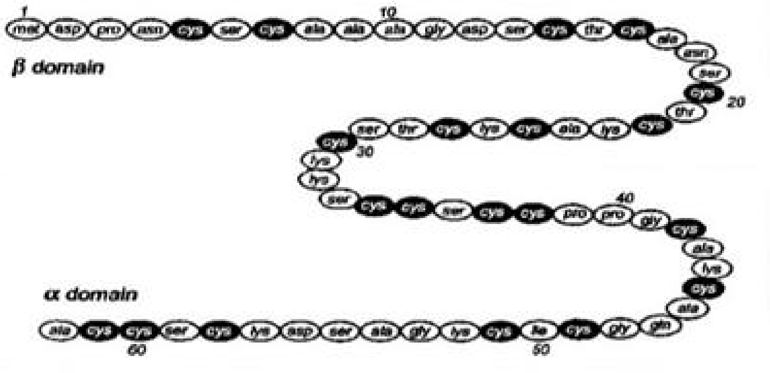


Figure 1-3: Amino acid sequence of rabbit liver metallothionein isoform 2a. The diagram shows the distribution of the nine cysteines in the β domain and the 11 cysteines in the α domain (modified after (Stillman, 1995)).

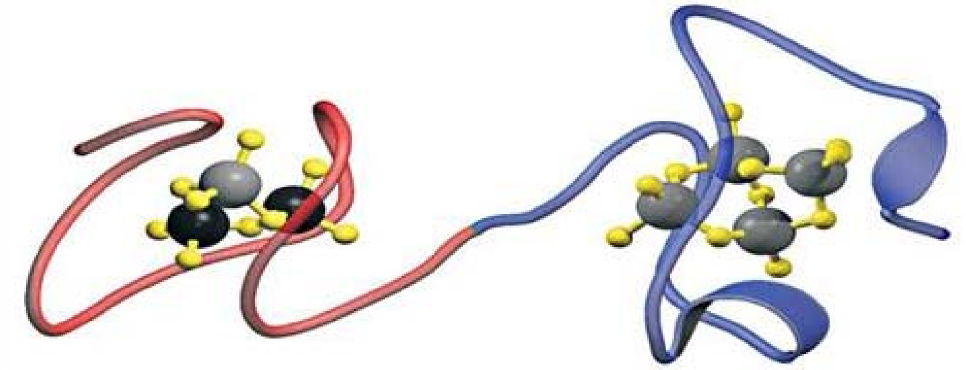


Figure 1-4: Ribbon diagram of the X-ray structure of rat liver MT2 showing the metal-sulphur clusters with zinc atoms drawn as black and cadmium atoms as gray spheres (adopted from (Messerschmidt et al., 2004)).

1.8. Metallothioneins as biomarkers in different aquatic species

The literature on MT synthesis in different marine species is vast; most of the knowledge available on this topic has been reviewed by (Amiard et al., 2006). The majority of the studies emphasized, besides fish, three major invertebrate taxa: the molluscs, the crustaceans and the annelids, although some data exist for other invertebrate phyla (e.g. echinoderms, sponges and coelenterates). This may be because that these three taxa are among most ecologically important of the aquatic invertebrates and contribute greatly to estuarine and coastal ecosystems, in terms of biomass, and ecosystem structure and functioning (Amiard et al., 2006).

1.8.1. Fish

Metallothionein has been identified as playing a role in metal homeostasis in several marine and freshwater fish species, including salmoniformes (rainbow trout), pleuronectiformes (flounder and plaice), cypriniformes (stone loach and goldfish) and gadiformes (cod) (Langston et al., 2002). Evidence is largely based on laboratory exposures (e.g. to Cd, Cu, Hg, Zn), which confirm that, generally, MT levels and associated metals increase as a function of the administered dose (George and Olsson, 1994). The fact that both fish and invertebrates have a complex response to metal stress, which is based on several ligands detoxification mechanisms, makes fish as good as invertebrates to be candidates for monitoring based on MT determination (Amiard et al., 2006). However, most native fish populations are considered less suitable candidates as bioindicators of environmental contamination gradients than many invertebrates, because of their mobility which means that fish do not fulfil the absolute criterion of a good indicator, the representativeness of the ecosystem (Langston et al., 2002). There are also a variety of other conditions, besides metals, that, potentially, could result in MT induction in fish. It has been suggested, for example, that basal concentrations of MT in some fish species vary with time of year, reproductive state, water temperature and developmental state (Duquesne, 1992; Hamzachaffai et al., 1995).

1.8.2. Crustaceans

Many studies involving MT in different crustacean groups have been carried out e.g. crabs (Pedersen et al., 1997; Astley et al., 1999; Legras et al., 2000), shrimps (Moksnes et al., 1995; Pourang et al., 2004) and lobster (Brouwer et al., 1986; Canli et al., 1997). Although convincing evidence of clear relationships between MT and metal concentrations in the field has been reported (Pedersen et al., 1997; Galloway et al., 2004), the use of crustaceans for monitoring based on MT determination raises different questions. A question occurs surrounding the representativeness of their site of collection, as they are not strictly sedentary; some species engage in limited migrations towards deeper waters in winter, and are not easily available all the year round (Amiard et al., 2006). Other potential confounding factors include the presence of the respiratory pigment (haemocyanin), and the deep physiological changes including copper metabolism, which occur during moulting in crabs (Engel and Brouwer, 1993). In fact, fluctuations in crabs' MT concentrations are more closely correlated to variations in general protein metabolism in response to salinity changes, than to variations in accumulated metal concentrations (Legras et al., 2000).

1.8.3. Molluscs

Since the 1970s, bivalves have been used as bioaccumulators of chemical compounds in so-called “Mussel Watch” biomonitoring programmes. The characteristics that have led them to be considered as the best choice for these programmes are generally the same as those needed to choose candidate species for the assessment of environmental quality with biomarkers. This is one of the reasons why bivalves have been intensively studied for the determination of MT (Amiard et al., 2006). Another reason is that the MT in mussels and oysters has been characterized as a Class I MT, sharing most of the characteristics of mammalian MT (Langston et al., 1998). However, it must be underlined that, despite the priority given to mussels and oysters, other molluscan species have also been intensively studied e.g. winkles: Common periwinkle (*Littorina littorea*) (Langston et al., 2003); cockles: Blood cockle (*Anadara granosa*) (Abbas Alkarkhi et al., 2008); and clams: Asian clam (*Corbicula fluminea*) (Baudrimont et al., 1997), Baltic macoma (*Macoma balthica*) (Bordin et al., 1997) and Manila clam (*Tapes philippinarum*) (Bocchetti et al., 2008).

1.8.4. Annelids

Annelids might offer interesting candidates in the identification of a biological model representative for sediment quality assessment with biomarkers. However, in the oligochaete (*Limnodrilus hoffmeisteri*) and in the *polychaete* (*Hediste diversicolor*), both the importance of insoluble detoxification processes and the existence of metal-tolerant populations at impacted sites limit the relevance of these species as candidates for the use of MT as a biomarker (Amiard et al., 2006). In *H. diversicolor*, for example, detoxificatory systems include metal-containing extracellular granules present in the epicuticle, mineralized lysosomes in epidermal cells, as well as the spherocrystals and mineralized lysosomes in the gut walls (Mouneyrac et al., 2003; Poirier et al., 2006). Thus it is likely that the concentration of MTs is not necessarily in itself a good measure of their involvement in metal detoxification. Moreover, there was no significant increase of MT concentrations in *H. diversicolor* upon laboratory or field exposure of relevant metals (Ng et al., 2008).

1.8.5. Sponges

Sponges are sedentary filter-feeding invertebrates in which flagellated cells, choanocytes, organized as a single layer, pump unidirectional water current through the body. This current enters through a system of small pores (ostia), and exits by way of larger apertures (oscules). A system of canals conducts the water current between inhalant and exhalant apertures (Bergquist, 2001).

Sponges have some of the fundamental characteristics of good biomonitors because they are sessile filter feeders, constitute the dominant phylum found in the marine hard substrates, are characterized by a sophisticated aquiferous system, a strong pumping activity and the ability to absorb a wide array of microscopic particles and dissolved material and have high lipid content that accumulates some very persistent xenobiotics (Perez et al., 2003). They are tolerant of physical-chemical fluctuations and may be abundant in conditions of extreme pollution. Their feeding habits make sponges particularly vulnerable to seawater quality. The concentration of pollutants in these organisms is mainly dependent on the quality of the environment. Nevertheless, part of metal concentration may be resulted from amplification phenomena as sponges feed on a wide array of material from the surrounding water column such as bacteria, phyto- and zoo-plankton (Burns and Bingham, 2002; Ramoino et al., 2011) and some of these organisms were reported to accumulate metals (Vranken and Heip, 1986; Langston and Bebianno, 1998; Rejomon et al., 2008; Rossi and Jamet, 2008). Moreover sponges have limited ability to contaminate higher trophic levels since not many organisms feed on them, and spongivory is largely restricted to a few species of fish, dorid nudibranchs, gastropterids and green and hawksbill turtles (Burns and Ilan, 2003; Malaquias et al., 2009; Burkepile and Hay, 2011). The detection of large quantities of metals in sponges suggests the existence of detoxification systems and indeed, the presence of MTLPs has been reported in different species of sponges, *Suberites domuncula* (Schroeder et al., 2000), Red beard sponge (*Clathria (Microciona) prolifera*) (Philp, 1999), Burnt sponge (*Spongia officinalis*) (Berthet et al., 2005) and Moon sponges (*Cinachyra* spp) (Gomes et al., 2006).

1.9. Quantitative determination of MT

Despite the great demand for MT determination, no standard protocol has yet been identified and investigators must select a method among those available according to their experimental systems (Santovito et al., 2008).

A number of techniques have been used to estimate MT concentrations in tissue samples. MT concentrations in the tissues can be measured by quantifying the amount of metal cations bound to these proteins by High performance liquid chromatography coupled with atomic absorption spectroscopy (HPLC-AAS)(Lehman and Klaassen, 1986) or high performance liquid chromatography coupled with inductively coupled plasma source atomic emission spectroscopy (HPLC-ICP-AES)(Suzuki, 1991). Radioimmunological procedures (Shaikh, 1991) and metal substitution assays (Scheuhammer and Cherian, 1991; Klein et al., 2002) are also utilized for the evaluation of MT concentrations. However, these procedures often prove to be too sophisticated and/or expensive to be used in the routine analyses required in environmental monitoring studies (Viarengo et al., 1997). On the other hand, a number of methods based on the determination of sulphydryl levels such as employing electroanalytical techniques (e.g. differential pulse polarography (DPP))(Olafson and Sim, 1979; Thompson and Cosson, 1984; Bebianno and Langston, 1989) and spectrophotometric method using Ellman's reagent, (Viarengo et al., 1997; UNEP/RAMOGE, 1999; Linde and Garcia-Vazquez, 2006) seem to be the most promising techniques for routine evaluation of MTs in marine species (Ivankovic et al., 2002).

1.9.1. Spectrophotometric determination of MTs

The spectrophotometric method, as mentioned above, is based on the estimation of the sulfhydryl content of MT proteins. Metallothioneins are characterized by an extremely high cysteine content (about 20-30%) when compared to other proteins eventually present in the ethanolic extracts, and therefore the metallothionein determination based on the SH detection allows a more selective evaluation of these metalloproteins. This method has been reported to be a sensitive, time saving, and low-cost technique able to detect metallothionein content in the tissues of marine organisms and has been intercalibrated and standardized by a number of laboratories (UNEP/RAMOGE, 1999; Zorita et al., 2005). Several modifications (UNEP/RAMOGE, 1999; Galloway et al., 2004; Linde and Garcia-Vazquez, 2006) have been done to the original protocol introduced by (Viarengo et al., 1997). The main modifications of the spectrophotometric method and protocols used by (Berthet et al., 2005) and (Gomes et al., 2006) to purify and concentrate sponges' MTLPs are illustrated in Figures 1.5 and 1.6.

In the few papers that have been published on the use of MTLPs as biomarker in some animal groups e.g. sponges, limpets and worms, the methods used to evaluate MTLPs were mainly the Differential Pulse Polarography (Berthet et al., 2005; Gomes et al., 2006), and silver-hem assay (Philp, 1999).

1.10. Summary

Metal contamination in aquatic environments is a substantial environmental concern and its monitoring is a very important aspect in order to control this type of contamination. The increased focus on the impact of metal contamination on biota recently become of most concern which led to shifting towards biomonitoring. The new emphasis on detecting the biological effects of pollutants has emerged strongly in UK and Europe in recent years, however, studies specifically designed to evaluate the issue of bioavailability in UK, using appropriate bioindicators, are still few (Depledge, 2009).

MTs are considered highly specific biomarkers of metal contamination that are recognized by international monitoring programmes. The majority of studies of of MTs as biomarkers emphasized fish, molluscs, and crustaceans. Whilst sponges, limpets and ragworms have the fundamental characteristics of (potentially) good biomonitors, there have been very few studies of MTLPs as biomarkers in these species. Studying these species' MTLPs as biomarker and comparing them with bivalves in establishing the relation between metal pollution and MTLPs concentration is the gap that this research aimed to cover. To include novel species on the list of species that could be used as bioindicators of metal pollution will broaden the limit of available species to choose between, besides applying the spectrophotometric method which has been reported to be a sensitive, time saving, and low-cost technique to detect metallothionein content in these species tissues for the first time.

1.11. Research aims and objects

The primary aim of this study is to investigate and compare the ability of the selected aquatic invertebrate taxa biomarker (metallothionein) responses to assess aquatic health. The specific objectives of the study are to:

- Identify and quantify any relationship between metal and metallotionein concentrations in selected species' tissue, and to evaluate the effect of abiotic local conditions on any resulting relationship.

- Investigate the suitability of different species' metallothioneins, to act as biomarkers by assessing their correlation with metal pollution.
- Assess the effectiveness of these organisms as bioindicators in biomonitoring studies and investigate the potential use of MT concentrations in novel species as a biomarker of metal exposure in monitoring programmes.
- Critically review the current status of metal contamination in biota, water and sediment within study area.
- Identify and quantify any spatial and temporal variations in metal pollution within the study area using biomarker responses of target species.
- Assess the utility of a spectrophotometric method, introduced by (Viarengo et al., 1997), as a cost-effective and practical methodology to determinate metallothioneins in different aquatic species.

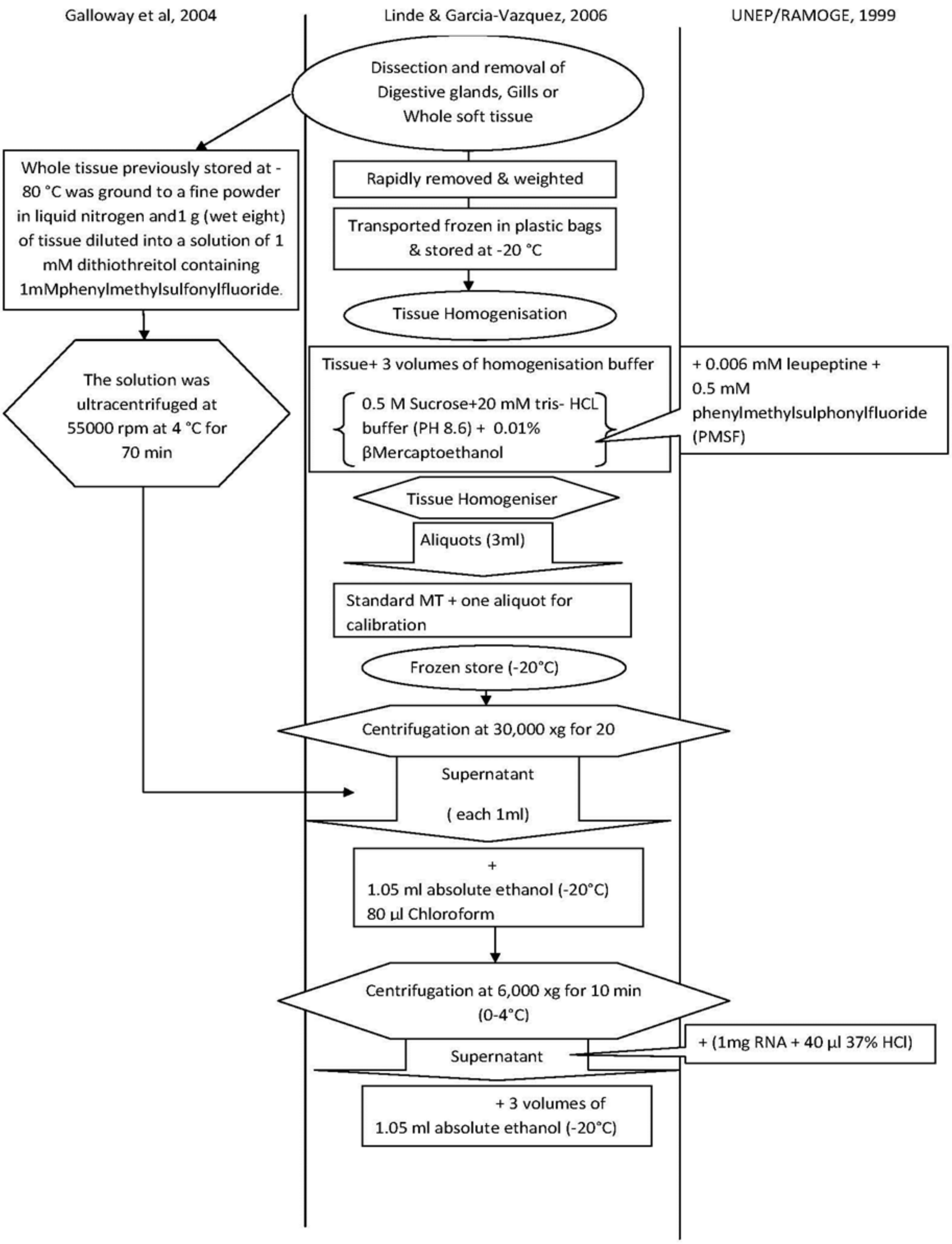


Figure 1-5: The main modifications to spectrophotometric method used to determine MTs

Figure 1.5(*cont.*): The main modifications to spectrophotometric method used to determine MTs

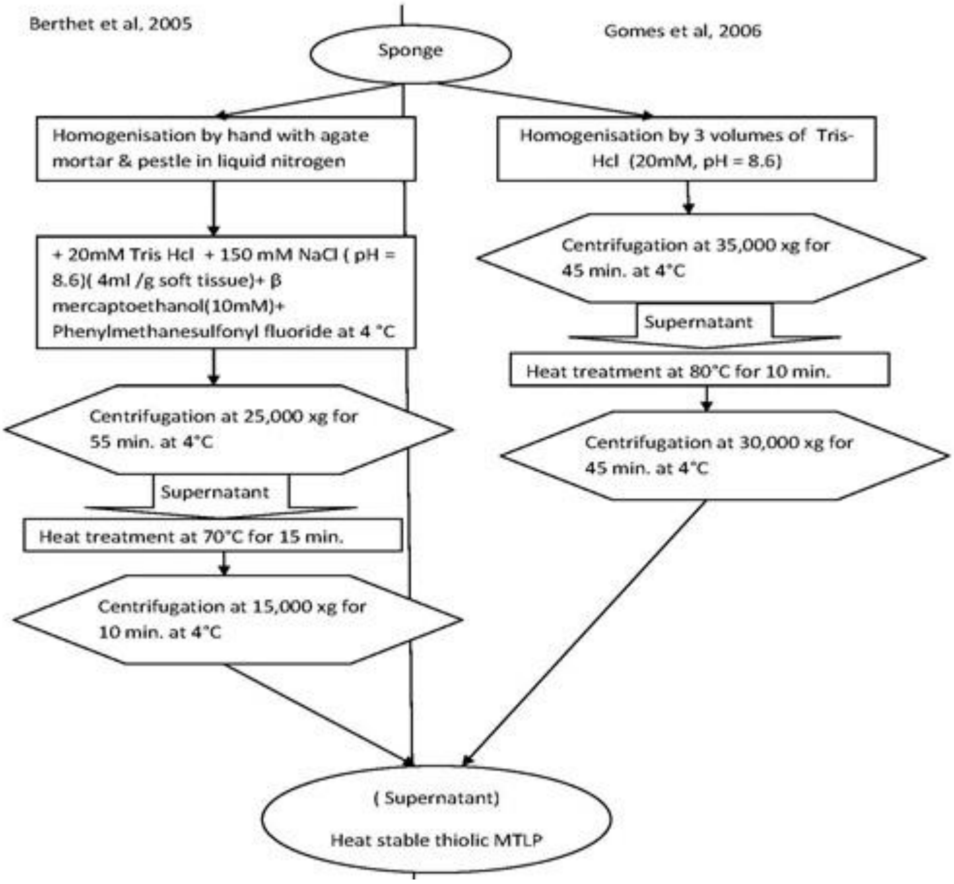


Figure 1-6: Protocols used to purify and concentrate sponges' MTLPs

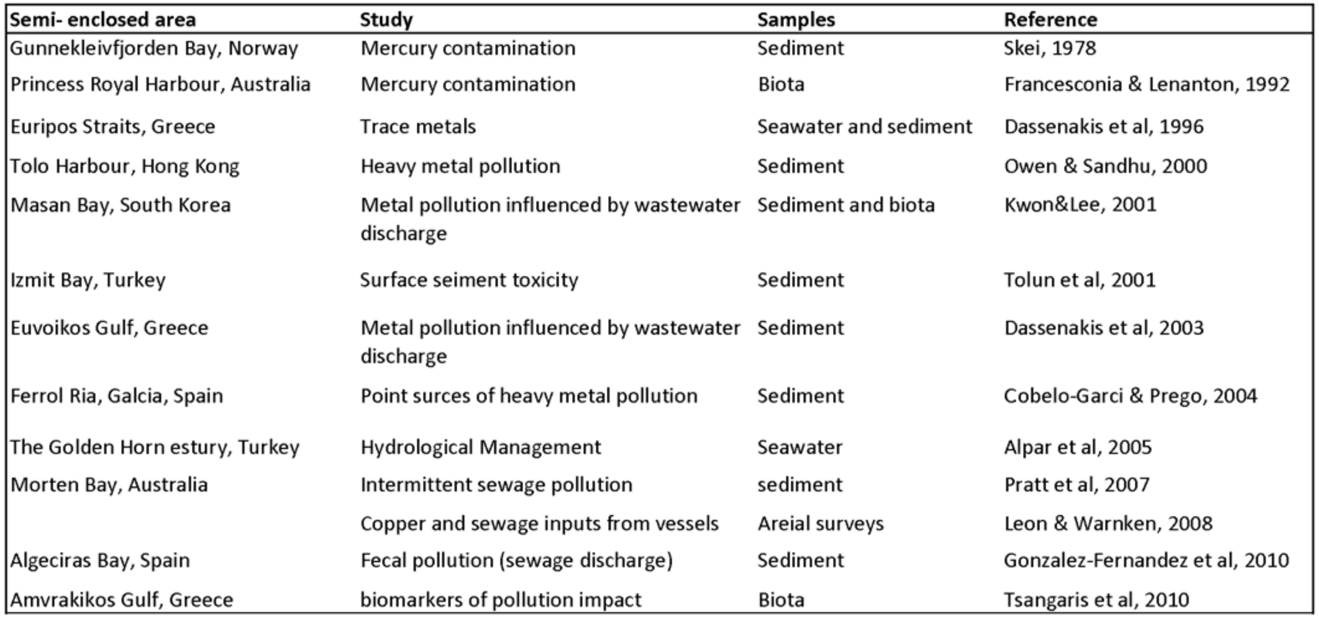
Chapter 2: Metal contamination in water, sediment, and biota from a semi-enclosed coastal area

2.1. Introduction

Semi-enclosed coastal areas (harbours and lagoons with narrow opening to open water) are more sensitive to anthropogenic impacts because of their lower ability to flush out contaminants than coastal areas with open boundaries with the sea (Owen and Sandhu 2000). Contaminants released to semi-enclosed systems tend to remain close to their sources as it is difficult to disperse the contaminants out to the open sea (Dassenakis et al., 2003). A significant part of the pollution load remains in coastal areas close to their land based contamination sources, alongside polluting remote sites with the small polluted particles which are transported, deposited and accumulated (Tolun et al., 2001).

Examples of previous studies that have focused on pollution in semi-closed coastal areas are shown in Table 2.1. Their main findings can be summarised as follow: the sediments of semi-enclosed areas may act as a source of metal pollution a long time after the primary source is removed and may influence the levels of this metal in water, biota and sediments of the entire system (Skei, 1978); although the extent and degree of contamination in the semi-enclosed areas may be lower than in other areas, the effects of bioaccumulation along food chains are more pronounced than in open systems (Francesconi and Lenanton, 1992; Tolun et al., 2001; Dassenakis et al., 2003); in semi-enclosed areas, the tidal current affects the distributions of metals, which have their highest concentrations at the near shore zone (Dassenakis et al., 1996; Cobelo-Garcia and Prego, 2004; Gonzalez-Fernandez et al., 2010); the poor sediment flushing conditions, and accumulation of sediments suitable for adsorption lead the semi-enclosed areas to be sensitive system to pollution (Owen and Sandhu 2000; Pratt et al., 2007) ; semi-enclosed areas need a site specific management to control pollution and require continuous and careful evaluations of the current changes (Kwon and Lee, 2001; Dassenakis et al., 2003; Alpar et al., 2005; Leon and Warnken, 2008; Gonzalez-Fernandez et al., 2010) ; it is possible to detect contaminant impacts and differentiate the type of contamination in coastal environments using a combination of bioenergetics and biochemical biomarkers of exposure in biota (Tsangaris et al., 2010a).

Table 2-1: Examples of studies that have focused on pollution in semi-closed coastal areas



Semi- enclosed area	Study	Samples	Reference
Gunnekleivfjorden Bay, Norway	Mercury contamination	Sediment	Skei, 1978
Princess Royal Harbour, Australia	Mercury contamination	Biota	Francesconia & Lenanton, 1992
Euripos Straits, Greece	Trace metals	Seawater and sediment	Dassenakis et al, 1996
Tolo Harbour, Hong Kong	Heavy metal pollution	Sediment	Owen & Sandhu, 2000
Masan Bay, South Korea	Metal pollution influenced by wastewater discharge	Sediment and biota	Kwon&Lee, 2001
Izmit Bay, Turkey	Surface seiment toxicity	Sediment	Tolun et al, 2001
Euvoikos Gulf, Greece	Metal pollution influenced by wastewater discharge	Sediment	Dassenakis et al, 2003
Ferrol Ria, Galcia, Spain	Point surces of heavy metal pollution	Sediment	Cobelo-Garci & Prego, 2004
The Golden Horn estury, Turkey	Hydrological Management	Seawater	Alpar et al, 2005
Morten Bay, Australia	Intermittent sewage pollution	sediment	Pratt et al, 2007
	Copper and sewage inputs from vessels	Areial surveys	Leon & Warnken, 2008
Algeciras Bay, Spain	Fecal pollution (sewage discharge)	Sediment	Gonzalez-Fernandez et al, 2010
Amvrakikos Gulf, Greece	biomarkers of pollution impact	Biota	Tsangaris et al, 2010

Many bivalves species exist in area of study (Poole Harbour) and they have proved to be valuable bioindicators (Langston et al., 2003). An important example is the common cockle (*Cerastoderma edule*) which is one of the few species that occur throughout the harbour and both its recorded numerical and biomass densities were high during the most recent survey of macro-invertebrate species in Poole Harbour (Caldow et al., 2005). Many studies have been carried out utilising bioaccumulation in *C. edule* as biomarker of metal pollution. (Baudrimont et al., 2005; Blinda et al., 2005; Jung et al., 2006).

The literature dealing with sponges in Poole Harbour is very limited. The most recent study was carried out by (Dyrynda, 2005) involved the sponge species in the harbour . One of these species is the Mermaid's glove sponge (*Haliclona oculata*) which was found in most of the marinas in the north part of the harbour, the mid-stream channel sections and upon submerged dock walls within the channel leading to Holes Bay (Dyrynda, 2005) and it is easily identified by eye alone (Ackers et al., 2007).

The Environment Agency in the UK defines pollution by dangerous substances as exceedance of Environmental Quality Standards (EQSs). They adopt the water quality standards laid down by The European Union dangerous substances directive (EUDSD) 2008/105/EC (European Commission, 2008), while the EQSs proposed by the Canadian Sediment Quality Guidelines (CSQG) are used for sediment (Hubner et al., 2009). The Environment Agency has not adopted any EQSs for metals in living organisms' tissues. This study was carried out to identify and quantify the spatial variations of metal contamination in biota, water and sediment within a heavily anthropogenically impacted semi-enclosed estuarine-coastal area with a very low ability to disperse and flush contaminants that is affected by significant point and non-point sources of metal contamination.

2.2. Materials and Methods

2.2.1. Study area

Poole Harbour is one of the largest examples of an estuary with an enclosed lagoon characteristic in Europe. The area of the harbour's water at high water spring tides is about36 km2. It has a long, indented shoreline that exceeds 100 km, five main islands, and a single entrance which is more than 370 m wide (Humphreys and May, 2005) (Figure 2.1). Most of the harbour is shallow with depths varying between 0.5 m below and 2.5 m above chart datum. The tidal range (mean springs) is of the order of 1.7 m, which is low for the south of England.
There are two major freshwater sources to Poole Harbour, the Rivers Frome and the Piddle, along with much smaller inputs from the River Sherford, Corfe River and a number of smaller tributaries such as Holton Heath stream. Much of the natural shoreline is marked by a low bluff (commonly less than 5 m in height) and eroding cliffs, but the northern shoreline is mainly artificial with walls, embankments, marinas and wharves (Humphreys and May, 2005).
There are three main channel networks in the harbour: South Deep drains the southern lowland heaths; the Wych Channel drains the Corfe River whose catchment is mainly on the Wealden sands and clays of the Isle of Purbeck; and Wareham channel which receives the outlets of the River Piddle and the River Frome. The northern harbour forms the estuary of the Rivers Frome and Piddle (with a combined catchment of over 770 km^2) and two smaller embayments with restricted mouths; Lytchett Bay (the Sherford River estuary) and Holes Bay (draining the heathlands around Creekmoor and urban and industrial areas). Holes Bay has a narrow entrance (approximately 60 m) compounding restrictions to the flow and the tidal prism here is about 40% greater than in the outer harbour. Consequently, a sheltered nature and reduced currents encourage siltation, particularly in the upper bay, within which water flow is also restricted by a railway embankment with narrow culverts connecting it to the southern part (Langston et al., 2003).

Poole Harbour and its environs have long been recognised as being of high biological importance (Drake and Bennett, 2011) . As a whole, the harbour supports important numbers of waders in winter (over 20,000 wildfowl and waders of around 60 different species) and is also an important breeding site for terns and gulls, hence its designation as a United Kingdom Site of Special Scientific Interest (SSSI), a European Special Protected Area (SPA) and a Wetland of International Importance (Ramsar site).

2.2.2. Samples collection and storage

Sampling locations (Figure 2.1) were chosen to cover the whole Poole Harbour area including rivers' estuaries and embayments and to reflect the various sources of metal contamination as well as the selection of an unpolluted, or less polluted sites (see Table 2.2). Spot samples (no replicates) were collected manually (water and *H. oculata* sponges) or via a trailed dredge (*C. edule* cockles and sediment) during November and December 2009. Samples of biota were collected when present at each sampling site, and were not present at all sites (Table 2.2). Water samples were collected from all sampling sites (designed as S01- S18 and showing in Figure 2.1). Sediment samples were collected from sites S1 -S13. Cockle samples were collected from sites S01-S13 except sites S06 and S07 (where they were not available). Five sponge samples were collected from the marinas in the north, north-east parts of the harbour (sites S14-S18). Water samples were kept in polyethylene bottles and sediment and biota samples in polyethylene bags. All samples were kept on ice in an isothermic container during transport and transferred to storage in laboratory at -20°C (Geffard et al., 2002) until analysis.

Cockles were not subject to any depuration period in the lab to empty their guts from any remaining sediment for the following reasons:

1- In standard protocols, it is not a standard practice to clear the gut of organisms before analysing their tissues for whole-body metal accumulation (UNEP/FAO/IAEA/ IOC, 1984; American Society for Testing and Materials, 2003).

2- Many studies on molluscs did not carry out depuration prior to the analysis of metals in soft tissues (Irato et al., 2003; Otchere, 2003; Gomes et al., 2004; Acker et al., 2005; Cravo and Bebianno, 2005; Ji et al., 2006; Smaoui-Damak et al., 2009; Van den Broeck et al., 2010; Machreki-Ajmi et al., 2011).

3- A recent laboratory study by (Yap and Cheng, 2010) showed that depuration of gut contents in the intertidal snail *Nerita lineata* is not necessary for the study of metal contamination and bioavailability.

4- The same organisms' tissues were used for both MTs and metal analysis and depuration period may cause underestimation of MT according to Baudrimont et al. (2003). They reported in their study of the role of MTs in Cd and Zn depuration processes in the freshwater bivalve *Corbicula fluminea* that MT concentrations decreased by 37% after transfer under unpolluted conditions, especially between 0 and 3days.

Table 2-2: Sampling locations descriptions and impacts

Site	Code	Coordinates	Description	Impacts	Samples collected
Holes Bay (NE)	S01	N 50° 43' 55.6" W 01° 59' 39.4"	The largest secondary embayment which has narrow entrance compounding restrictions to the flow.	Metals (historically), Trade discharges (now ceased) and STW have led to concentrations in sediments most in the upper, eastern part.	Water, Sediment, Cockle
Holes Bay (NW)	S02	N 50° 43' 53.2" W 02° 00' 25.2"			
Holes Bay (S)	S03	N 50° 43' 26.8" W 01° 59' 46.8"			
Lytchett Bay	S04	N 50° 43' 31.0" W 02° 02' 28.3"	Smaller than Holes Bay to the east, it is the Sherford River estuary which has become increasingly isolated.	Lytchett Bay receives inputs from Lytchett Minster STWs and is classified as prohibited area for fishing due to bacteriological contamination.	Water, Sediment, Cockle
Holton Heath	S05	N 50° 42' 26.4" W 02° 03' 43.7"	Muddy embayment, the northern edge of which is marked by the outfall channel from Lytchett Bay.	Chemical companies, engineering workshops and road transport hauliers. All sewage from the estate is treated at the local sewage works that discharges into Poole Harbour.	Water, Sediment, Cockle
Wareham Channel(W)	S06	N 50° 41' 41.1" W 02° 04' 30.9"	The area of water at the west of the main Harbour basin which extends south to the outlets of the River Piddle and the River Frome.	Receives inputs from Wareham STWs and the riverine inputs of the Frome and Piddle are the major influences on pollutants concentrations in the Channel.	Water, Sediment
Wareham Channel(E)	S07	N 50° 41' 26.9" W 02° 03' 34.1"			Sediment
Arne Bay	S08	N 50° 42' 01.1" W 02° 01' 34.4"	The eastern shore of Arne peninsula which formed of sheltered tree- lined marshes and mudflats.	The shorelines are generally remote from public access from the land, and form part of the Arne Nature Reserve of the Royal Society for the Protection of Birds.	Water, Sediment, Cockle
Wych Channel	S09	N 50° 41' 33.1" W 02° 01' 32.6"	Channels or streams, whose local name is ' Lakes', unite to the west of Round Island to form the Wych channel and, at low water ebb tides, flow northwards between Long Island and Shipstal Point.	Apart from the Corfe River, the Lakes drain local bogs, heathland and farmland, and there are many freshwater springs around the shoreline.	Water, Sediment, Cockle
Wych Lake	S10	N 50° 40' 49.1" W 02° 01' 23.7"			Water, Sediment, Cockle
Newton Bay	S11	N 50° 40' 03.6" W 01° 59' 39.7"	Located in the central part of the south shore which is a topographically and physiogeographically complex section of the Harbour.	The remoteness, complex topography, and diversity of feeding grounds make this section of shoreline particularly attractive to overwintering waders and wildfowl.	Water, Sediment, Cockle
Brands Bay	S12	N 50° 39' 49.4" W 01° 58' 14.0"	The most seaward inlet along the Harbour's south shore has a much-indented shoreline with several minor bays and promontories.	Easy access from the nearby roads makes the east shore popular with visitors, particularly in the north. This area is the only part of the south shore of the Harbour regularly dug for bait.	Water, Sediment, Cockle
Blue Lagoon	S13	N 50° 42' 35.5" W 01° 57' 07.2"	The shoreline largely marks the edge of private residences. West of the lagoon entrance these arc protected by concrete and stone walls.	Was reported to be particularly affected by abnormal proliferation of green algae.	Water, Sediment, Cockle
Royal Motor Yacht Club	S14	N 50° 41' 18.8" W 01° 56' 41.8"	A private members club situated on the Sandbanks Peninsula close to the Harbour entrance.	Marinas are sources of many things that can affect the environment. Materials such as paints, solvents, waste oil, fuel and solid waste can harm the environment and people, if they are not managed properly.	Water, Sponge
Parkstone Yacht Club	S15	N 50° 42' 35.4" W 01° 57' 32.3"	Located in Parkstone Bay		Water, Sponge
Fisherman's Haven	S16	N 50° 42' 39.9" W 01° 58' 43.6"	Situated at the east end of Poole Quay		Water, Sponge
Holes Bay Cobbs Quay	S17	N 50° 43' 16.3" W 02° 00' 03.1"	A large marina on the west side of Holes bay		Water, Sponge
Lake Yard marina	S18	N 50° 42' 41.2" W 02° 01' 07.0"	It is situated at Hamworthy, up the Wareham Channel in the NW corner of the Harbour.		Water, Sponge

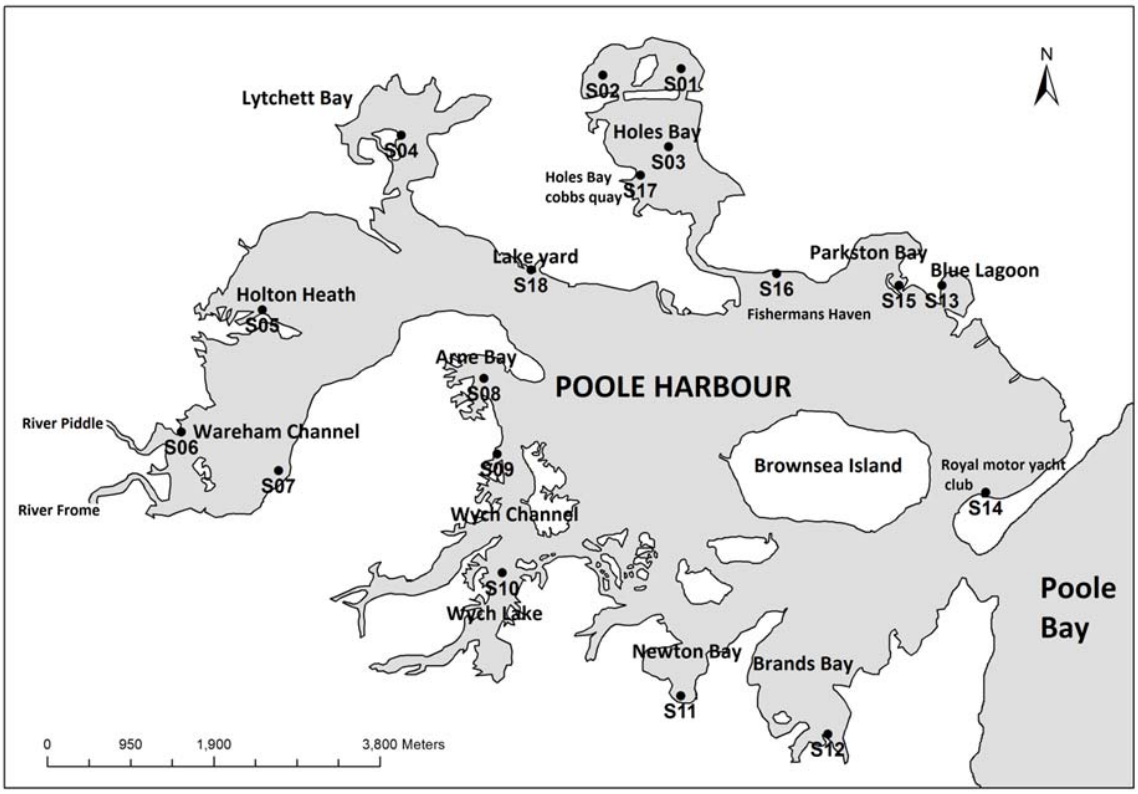


Figure 2-1: Map of the main features of Poole Harbour and location of the sampling sites.

2.2.3. Metal analysis

The elements determined were: As, Cd, Cr, Cu, Pb, Hg, Ni, Ag, Sn, and Zn. This group of elements will be collectively called “metals” in this text although it contains As, which is a metalloid.

A pool of whole soft tissues belonging to at least ten bivalves and whole tissue of sponges were physically homogenised using a pestle and mortar to produce one pool of homogenised tissue per site. Homogenised tissues were divided to two portions; one for MT analysis and the other was freeze-dried for 24 hours for metal analysis, whilst sediment samples were freeze-dried for 72 hours. Metal analysis of sediment and organisms tissues took place in Severn Trent laboratories in Coventry, UK. All measurements were covered by the United Kingdom Accreditation Service (UKAS). Metals were analysed according to the method by Standing Committee of Analysts (2006). A known weight of homogenised, dried, ground three subsample (to assess the accuracy of the analytical results) were digested in boiling *Aqua Regia* (a 3:1 v/v mixture of concentrated, 35%, hydrochloric acid and concentrated, 68%, nitric acid) in order to bring the metals into solution. The digested sample was filtered and diluted to a known volume before being analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) except for As and Hg which were analysed by Hydride Generation Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Quantification of the metal concentrations was achieved by comparison of the extract against multiple standard solutions containing the appropriate elements at known concentrations (Standing Committee of Analysts, 2006). Results are reported as mg/kg dry weight relative to the original sample weight taken.

Metal analysis of water samples took place in National Oceanography Centre, Southampton, UK. One cm^3 water samples were digested in 0.5 cm^3 of concentrated, 68%, HNO^3 and then diluted to 10 cm^3 with double distilled, deionised water prior to analysis using an X-Series 2 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Thermo Fisher Scientific, Bremen, Germany) which was set up in the standard configuration using an ASX-510 autosampler (Cetac, Omaha, Nebraska, USA). All samples, standards and blanks were spiked with internal standard elements Be, In and Re.

The data quality was monitored throughout the run by examination of the statistics produced after each analysis. Within-run reproducibility was typically better than 1% relative standard deviation for the four repeats. Data processing was completed using the Plasmalab software. Raw data were blank and internally corrected and then calibrated against five matrix matched synthetic standards.

2.2.4. Statistical Analysis

Cluster analysis is a broad suite of techniques designed to find groups of similar items within a data set. Hierarchical cluster methods produce a hierarchy of clusters from small clusters of very similar items to large clusters that include more dissimilar items (Kaufmann and Rousseeuw, 1990). In this study, cluster analysis was applied to metal data using Ward's linkage method, and Euclidean and correlation coefficient distance methods to test the relation between metal species in each site and in different sample types and how these sites related according to their metal composition. Cluster statistical analysis was performed using Minitab® 16.1.0 software. To verify the integrity of cluster results, an ANOVA was run using the cluster groupings as categorical values.

2.3. Results

2.3.1. Metals in water

The average metal concentrations in water were ranked (from largest to smallest) in the order As>Zn>Hg>Cu>Ni>Cr>Pb>Cd; both Ag and Sn were below their detection limits (Figure 2.2). The tested metals were within 2008/105/EC (European Commission, 2008) guidelines except for two metals: As and Hg. As concentrations in all samples were above the Environmental Quality Standard (EQS), which is (20 µg/l); they ranged from 29.6 µg/l at S10 (Wych Lake) to 212.8 µg/l at S16 (Fisherman’s Haven). For Hg, which is a List 1 (i.e. Priority) substance in the EUDSD 2008/105/EC directive (European Commission, 2008), concentrations ranged from 0.368 µg/l at S18 (Lake Yard) to 11.06 µg/l at S05 (Holton Heath). This latter value is very high when compared with the annual average EQS and the maximum allowable concentrations, which are 0.05 and 0.07 µg/l, respectively.

2.3.2. Metals in sediment

The concentrations of the metals extracted from sediment samples are shown in Figure 2.3. The average metal concentrations in sediment were ranked (from largest to smallest) in the order Zn>Pb>Cu>Cr>Ni>As>Cd>Sn>Hg>Ag. Hg and Sn concentrations were below their detection limits (0.35 mg/kg and 2.00 mg/kg, respectively) at all sites except for S01 and S02

(Holes Bay), where Hg concentrations were 0.99 and 0.80 mg/kg and Sn concentrations were 5.8 and 3.3 mg/kg at the two sites, respectively. Hg concentrations at these two sites exceed the Probable Effect Level (PEL) (0.7 mg/kg) proposed by the Canadian Sediment Quality Guidelines (CSQG) which is the standard used by the Environment Agency in the UK (Hubner et al., 2009); Sn is not covered by the CSQG. Ag exceeded the detection limit at one site only (S01, Holes Bay), with a concentration of 1.66 mg/kg, which is above the Threshold Effect Level (TEL) (0.73mg/kg) proposed by the CSQC but below the PEL (1.77mg/kg). For the other metals, all concentrations were below the PEL at all sites, although some sites were above the TEL (Figure 2.3).

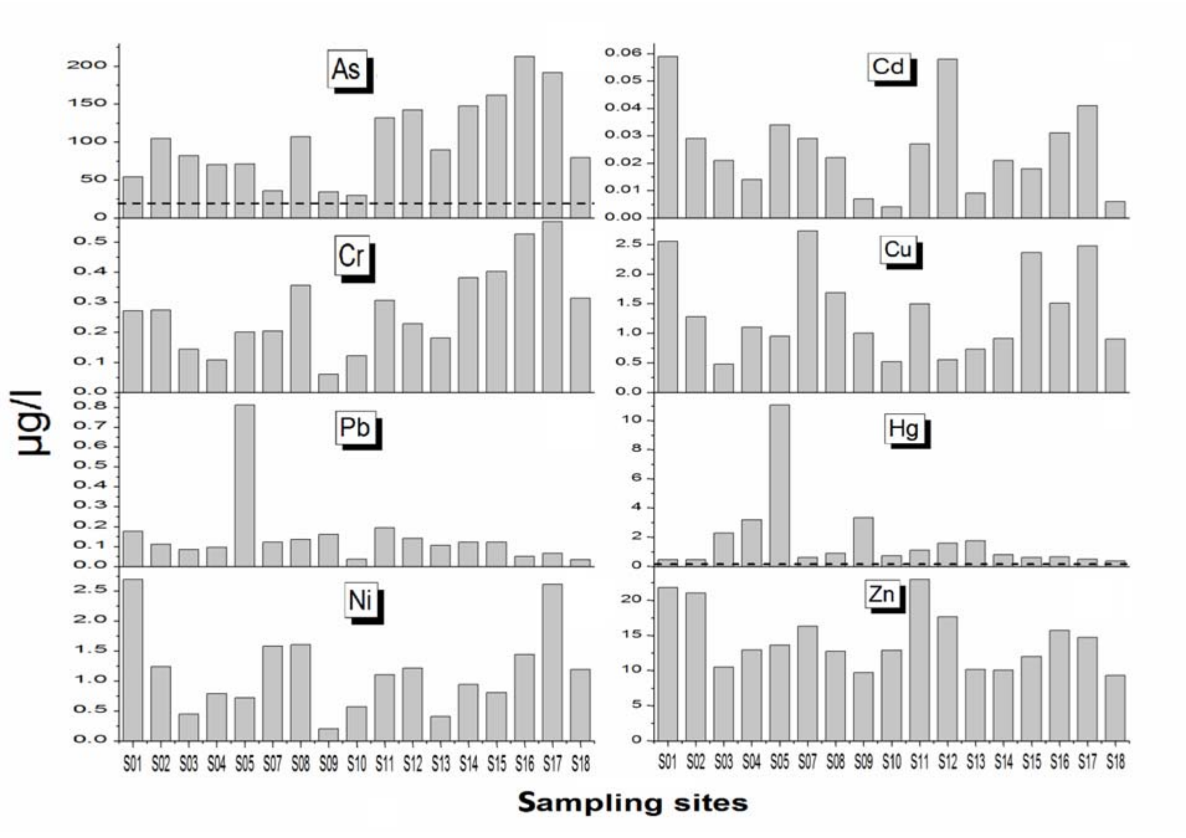


Figure 2-2: Metal concentrations in water samples (µg/l) from sites in Poole Harbour (Nov-Dec 2009), (dashed lines indicate the Environmental Quality Standard (EQS)according to the European Union water directive 2008/105/EC (European Commission, 2008)).

2.3.3. Metals in cockle tissues

Metal concentrations recorded above their detection limits in cockles' tissues from Poole Harbour are shown in Figure 2.4. The average metal concentrations in cockles were ranked (from largest to smallest) in the order Zn>Ni>Cu>Cr>As>Pb>Ag>Cd>Hg>Sn. Sn was the only metal that had concentrations below its detection limit (0.2 mg/kg) in all samples while the other metals all had at least one sample above their detection limits. Ag concentrations exceeded the detection limit (0.5mg/kg) in the sample collected from S01 where it was 5.1mg/kg, while Hg exceeded the detection limit (0.35 mg/kg) at S04 (Lytchett Bay), and S01 and S02 (Holes Bay) where its concentrations were 0.47, 0.43 and 0.46 mg/kg, respectively. Pb also exceeded the detection limit (2.0 mg/kg) at S04 (Lytchett Bay), and S03 and S01 (Holes Bay) where its concentrations were 2.2, 2.0 and 3.4 mg/kg, respectively. The other metals had their highest sample concentrations recorded at S01 (Holes Bay) while their lowest concentrations were recorded at three sites S13 (Blue Lagoon), S12 (Brands Bay) and S08 (Arne Bay) (Figure 2.4).

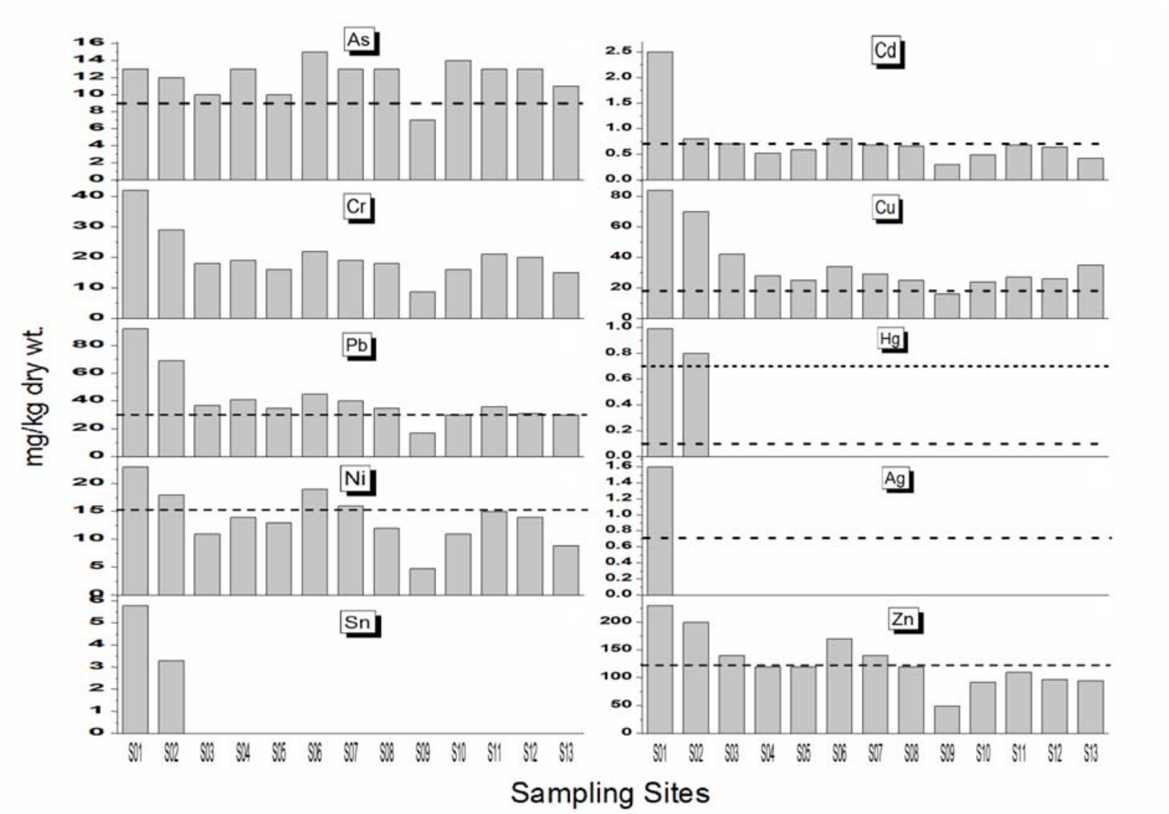


Figure 2-3: Metal concentrations in sediment samples (mg/kg dry wt.) from sites in Poole Harbour (Nov-Dec 2009), (dashed lines indicate the Threshold Effect Level (TEL) and dotted line indicates the Probable Effect Level (PEL) according to the Canadian Sediment Quality Guidelines (CSQG) (Hubner et al., 2009).

2.3.4. Metals in sponges

The average metal concentrations in sponge tissues were ranked (from largest to smallest) in the order Zn>Cu>Pb>Cr>As>Ni>Ag>Cd. Hg and Sn concentrations were below their detection limits (0.35 and 2.0 mg/kg respectively) at all five sampling sites, while Pb and Ni were below detection limits (2.0 and 1.0 mg/kg respectively) at S18 (Lake Yard) only and Ag (0.50 mg/kg) at S14 (Royal Motor Yacht Club) and S15 (Parkston Yacht Club)(Figure 2.5).

2.3.5. Hierarchical Cluster analysis

The resulting dendrograms of this analysis are shown in Figures 2.6 and 2.7. To verify the integrity of cluster results, an ANOVA was run using the cluster groupings as categorical values.

Cluster analysis of the composition of metal in each site showed that the relation between metals differs from one compartment to another:

- Water samples: metals can be classified into four groups (As, Cr), (Cd, Zn), (Cu, Ni) and (Pb, Hg). ANOVA statistical significance results for these clusters showed that all metals were significantly belonging to their group ($p<0.05$) except for Cd ($p= 0.055$)
- Sediment samples: (As) concentrations at different sites were not related to any other metals, while (Cd, Ag), (Cu, Hg, Sn) and (Cr, Pb, Zn, Ni) formed three groups. ANOVA statistical significance results for these clusters showed that all metals were significantly belonging to their group ($p<0.05$)

Metals in organisms' tissues showed different relationship patterns:

- Cockles: Cd was not related to other metals, while (As, Cr), (Cu, Hg, Ni, Zn) and (Pb, Ag) form three groups. ANOVA statistical significance results for these clusters showed that all metals were non-significantly belonging to their group ($p>0.05$) except Cd ($p=0.028$)
- Sponges' tissues: (As, Cr, Zn), (Pb, Ni), (Cd, Ag) and (Cu) were the four groups formed from the cluster analysis. ANOVA statistical significance results for these clusters showed that all metals were significantly belonging to their group ($p<0.05$)

Cluster analysis was also performed to test how different sites throughout Poole Harbour could be grouped according to their metals concentrations. The results showed that sediment and cockles samples from the north part of Holes Bay (S01 and S02) formed a distinctive group and ANOVA results showed that all metals directed this clustering ($p<0.05$) except sediment As ($p=0.771$) and cockles Cd ($p=0.162$) and Pb ($p=0.268$) .According to water samples, Poole Harbour could be divided to two main clusters: the eastern part including sites S11, S12, S14, S15, S16, and S17, and western part including the remaining sites (Figure 2.7). ANOVA results showed that As ($p=0.000$) and Cr ($p= 0.002$) were the only metals led to this clustering. Sponges samples showed that site S15 (Parkstone Bay) stand alone as separate cluster from all other sites and ANOVA results showed that Zn was the only metal led to this clustering ($p=0.012$) (Figure 2.7).

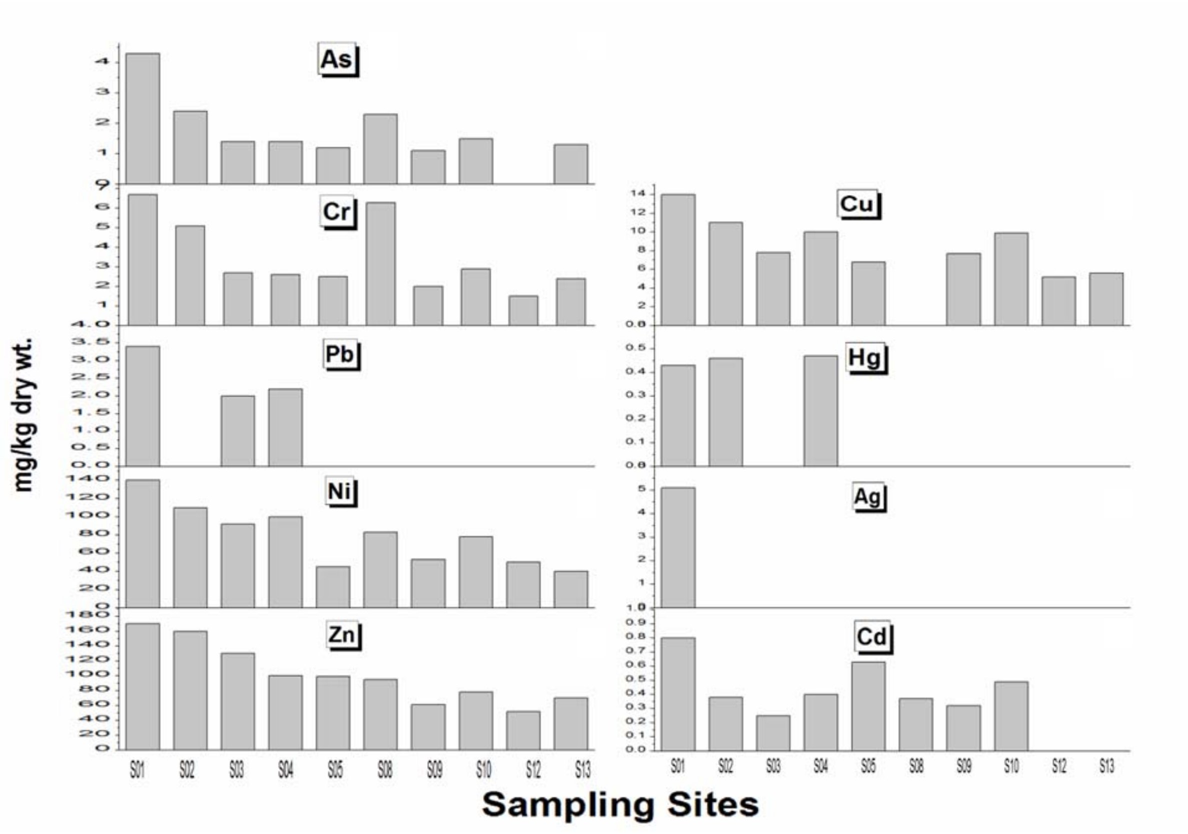


Figure 2-4: Metal concentrations in cockles' samples (mg/kg dry wt.) from sites in Poole Harbour (Nov-Dec 2009).

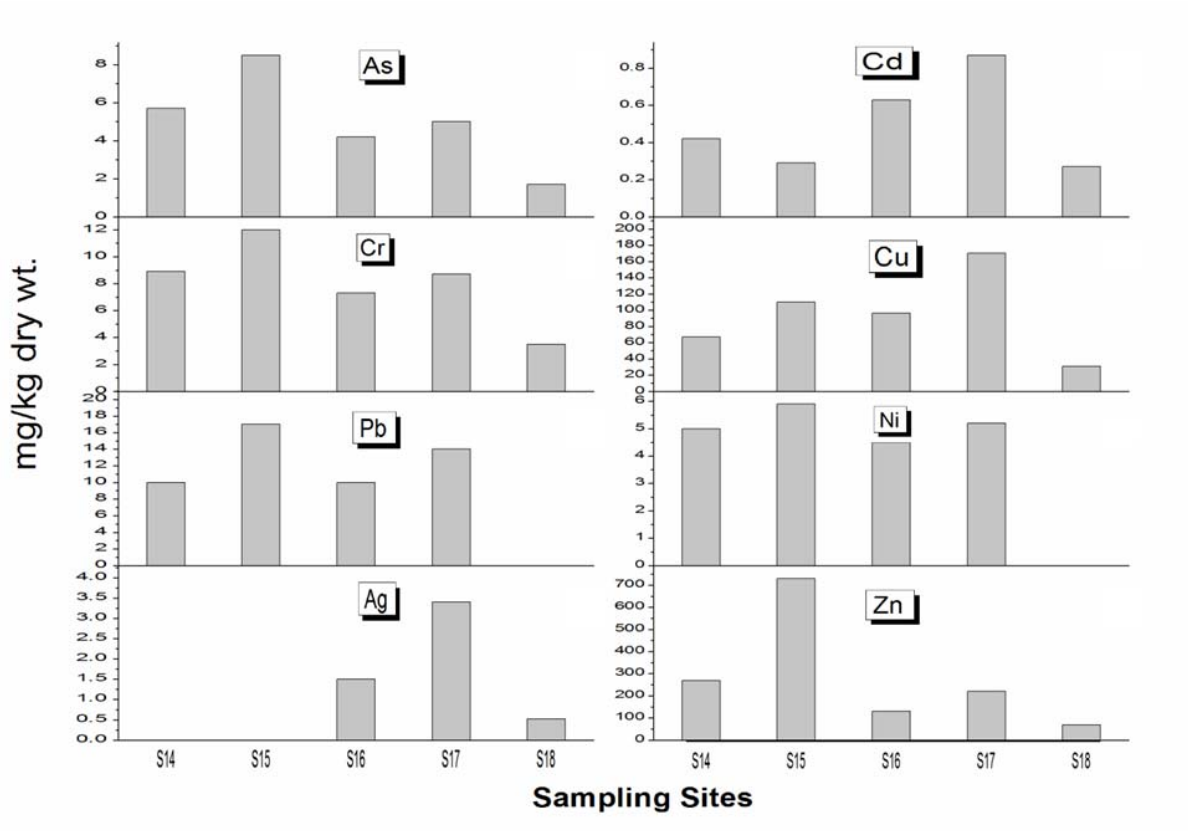


Figure 2-5: Metal concentrations in sponge samples (mg/kg dry wt.) from sites in Poole Harbour (Nov-Dec 2009).

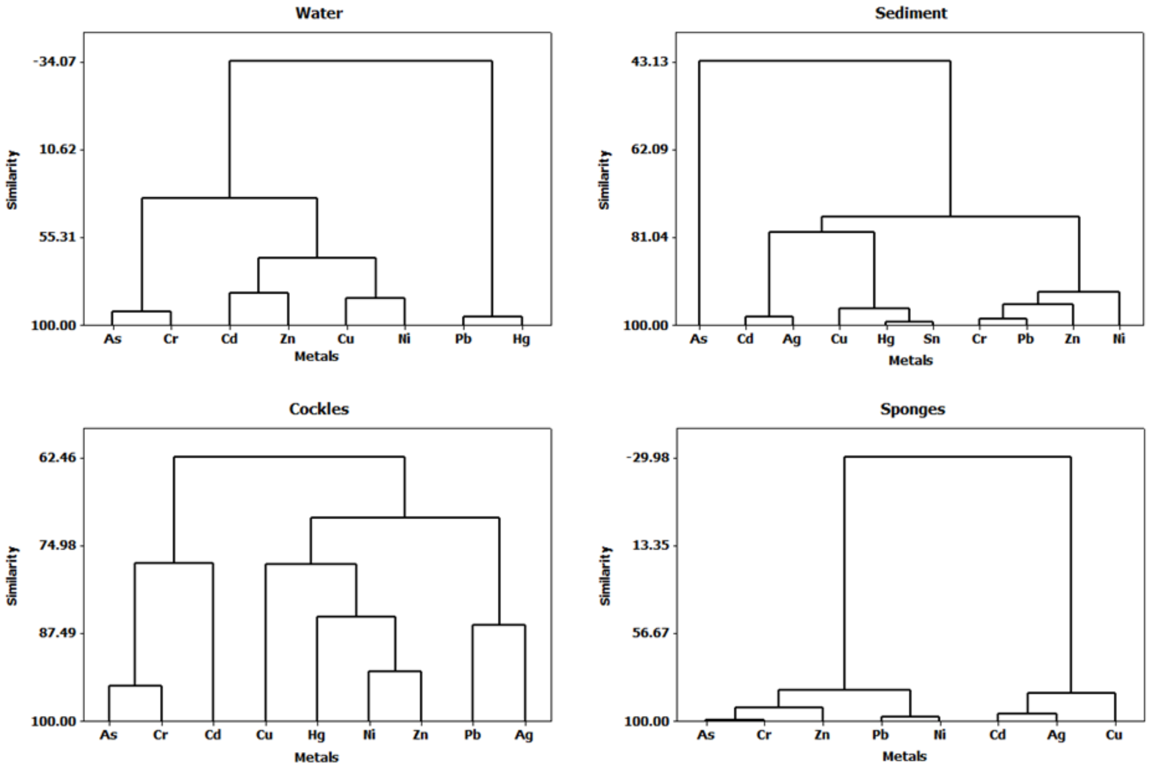


Figure 2-6: Dendrograms showing the relation between metals according to their spatial distribution. The dendrograms are drawn applying the ward linkage and correlation coefficient distance method.

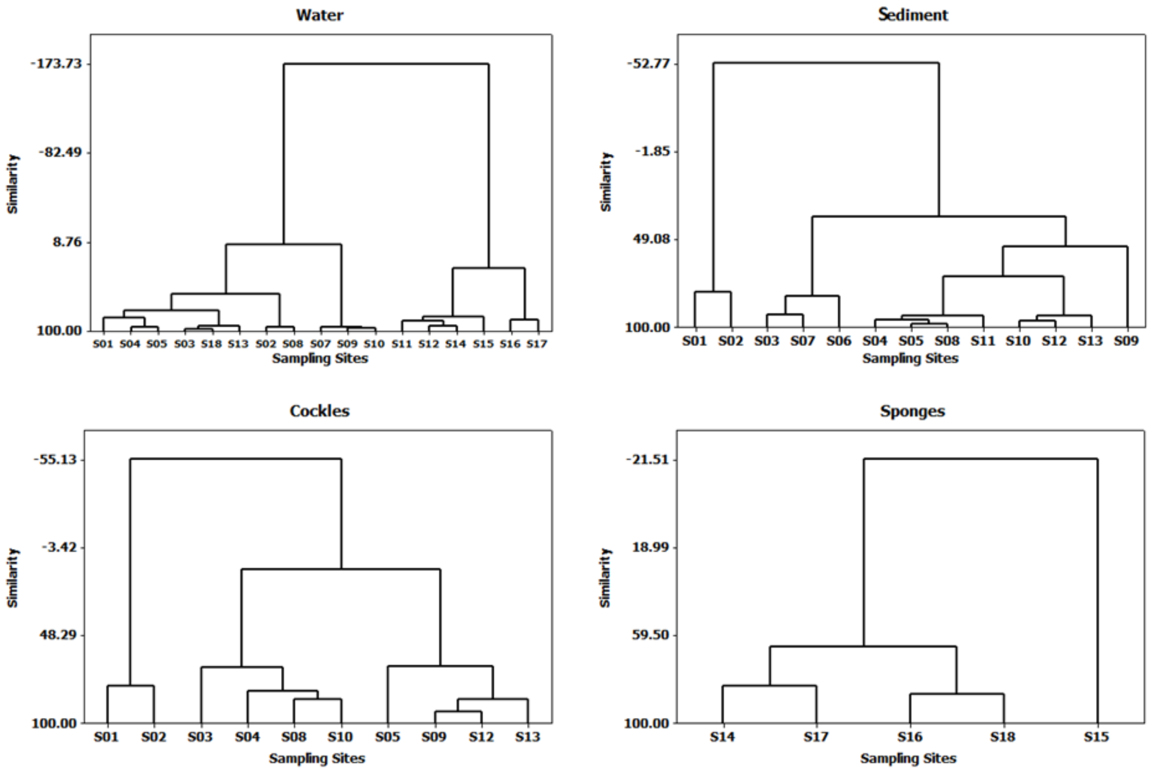


Figure 2-7: Dendrograms, showing how sampling sites were related according to their metal profile. The dendrograms are drawn applying the ward linkage and eucliden distance method.

2.4. Discussion

Sources of metal contamination in Poole Harbour include:

- Diffuse metals from historically polluting point sources e.g. copperas (iron (II) sulphate) production plant at Parkstone, a power station at Hamworthy (area between Holes Bay and Lytchett Bay), naval gun propellant and explosives factory at Holton Heath, and a chemicals factory at Holes Bay. These sources used to discharge to the harbour and have been eliminated or improved in quality (Wardlaw, 2005).
- Diffuse contamination from agricultural land runoff via the rivers that discharge into the harbour (Langston et al., 2003; Drake and Bennett, 2011). Hubner et al. (2010b) linked the die-back of the perennial cord grass *Spartina anglica* with the Cd concentrations in the sediments of the estuary. Sewage Treatment Works (STWs); the harbour receives treated sewage from three main STWs which serve the communities of Poole, Lytchett Minster and Wareham (Drake and Bennett, 2011).
- The harbour has currently eight yacht clubs and ten boatyards as well as marinas associated with residential developments (Humphreys and May, 2005), antifouling paint has been used in these areas causing contamination with Sn (Langston et al., 1987). Sn based antifouling paints have been banned for use on small vessels in many countries, and Cu based anti-foulants have been used as alternative (Omae, 2003).

The data provided by Environment Agency of metals in the waters of Poole Harbour were mostly for sites in the main harbour and did not cover the whole area, especially embayments, and they indicate that all the tested metals were below the European Union's EQSs (Witt, 2008).

2.4.1. Metals in water

Previous studies dealing with metal contamination in the harbour area, especially historical data from the 1970s and 1980s, suggested that Cd and Zn concentrations in water were elevated, particularly in Holes Bay, and were high enough to affect sensitive oyster larvae (Boyden, 1975). In the 1970s, dissolved metal concentrations in Holes Bay could exceed values considered typical for the English Channel by 30-40 fold for Cu, Ni and Zn, and more than 100-fold for Cd and Pb (Preston et al., 1972; Boyden, 1975). Also, in urbanised tributaries, enrichment with Ag, Cu, Cd, As, Zn and Pb has been observed downstream of industrial areas (Thornton et al., 1975). High levels of water contamination with Sn in areas closest to marinas and moorings was reordered during the 1980s (Langston et al., 1987). Higher values of Cr were recorded in 1993 in Holton Heath Stream, both upstream and downstream of the STW, and also downstream of the STW at Wareham than that of the riverine sources (Rivers Frome and Piddle) (Langston et al., 2003).

The results of this study showed that in Poole Harbour and its embayments water quality is generally considered to be good and achieves the statutory water quality standards laid down by the European Union (EUDSD 2008/105/EC)(European Commission, 2008) for all except two metals: As and Hg.

As concentrations in all samples were above the Environmental Quality Standard (EQS); however, they tended to be higher at sites towards the eastern part of the harbour (S11-S17) including two sites in the south region (S11, Newton Bay and S12, Brands Bay), and marinas and yacht clubs in the north-eastern part (S13- S17). These results suggest that such elevated values may be due to activity in this part of the harbour rather than from inputs via rivers and sewage pipes in the west and north embayments. In water, As usually exists as arsenate or arsenite, as well as the methylated arsenic compounds that result from biological activity (Sharma and Sohn, 2009). Generally, several possible sources may have been responsible for the As contamination including marinas that stripped arsenical paints from ships (Davis et al., 1997), which may explain the high As concentrations in areas of high vehicle activity (e.g. the eastern part of the harbour).

Hg, a List 1 (i.e. Priority) substance in the EUDSD 2008/105/EC Directive (European Commission, 2008), showed concentrations above both the annual average EQS (0.05 µg/l) and the maximum allowable concentrations (0.07 µg/l) in all sites, which indicates high contamination by this metal. The highest concentrations of Hg were recorded in the northern embayments of Holes Bay, Lytchett Bay and Holton Heath (sites S03, S04 and S05 respectively).

The enclosed nature of these areas and the history of severe local industrial pollution, which has accumulated pollutants in sediments, as well as receiving sewage discharge from sewage treatment works (STW) and combined sewer outfall (CSO) pipes (Langston et al., 2003) may lead to the concentration of Hg in water.

Holton Heath (site S05) shows the highest concentration of Hg (11.06 µg/l) and Pb (0.81 µg/l). This area used to be a centre for military activity; between 1915 and 1957 the Royal Navy Cordite Factory manufactured naval gun propellant and explosives such as cordite, picrite and tetryl and associated ammunition (Taylor et al., 2006). The most common sources of Pb pollution, are manufacturing plants and industries such as metal smelting, the production of paint, batteries, bearings, solders, seals, a wide range of electronic applications, weights, foils, wires, shields. Pb is used in a number of car components, including lead wheel weights, solder in electronics, and lead car batteries (Kendler and Pirone, 1994; Napp, 1995; Singh et al., 1997; Mukherjee, 2011). The sources of mercury pollution are manufacturing plants and industries that make wide use of mercury in the production of fluorescent lamps, some thermometers, batteries and switches, and other similar products (Shaub, 1993; Sang and Lourie, 1997; Floyd et al., 2002; Acosta-Ruiz and Powers, 2003; Cain et al., 2008). All waste water from the Holton Heath Industrial Estate is treated at the local sewage works that discharges into Poole Harbour. This industrial estate is home to a wide range of business including chemical companies, engineering workshops (e.g. garage services, car body repairs, pipework contractors, boats repairs, electric fencing products, stainless steel metal workers and suppliers of extruded aluminium products to the electrical, cable and tube industries), and road transport hauliers.   A series of pollution incidents in the area have been recorded relatively recently, two of which resulted in the temporary closure of a nearby sewage treatment works after a mystery chemical or other pollutant ‘knocked out’ the works. The Environment Agency in their comment on these incidents stated that "Chemicals and oils can cause serious pollution and damage sewage treatment works. We are asking companies to take a moment to ensure any chemicals on their site are kept safe. Chemicals must never be poured down any drain and companies should tell us when a spillage occurs" (Dunning, 2008).

Metals in the waters of Poole Harbour clustered into four groups; each contains a pair of metals (Figure 2.6). Hg and Pb formed a distinctive group; they were the only metals that followed a unique spatial pattern, as both showed the highest concentrations in Holton Heath (S05) then in northern embayments. As and Cr form the next distinctive group to lesser extent than the first group. Both these metals showed their highest concentrations in the eastern part of the harbour in samples from marinas and yacht clubs in the north and in Newtown Bay and Brands Bay in the south. The similarity of the last two groups was higher and metals belonging to these groups (Zn, Cu and Ni) showed their highest values mainly in the northern part of Holes Bay where very restricted water exchange.

According to the As and Cr concentrations in water samples, Poole Harbour could be divided to two main clusters: the eastern part including sites S11, S12, S14, S15, S16, and S17, and western part including the remaining sites (Figure 2.7). This clustering may be related to the sources of these metals that dominate each part of the harbour. While the northern embayments and the west part of the harbour are mainly affected by historical pollutants accumulated in the sediment and the discharge of sewage, the marinas and yacht yards in the east part and the bays in the south are affected by shipping traffic.

2.4.2. Metals in sediment

Historical data from the 1970s and 1980s showed that high levels of several metals were recorded in sediment of the northern part of Holes Bay and some metals e.g. Pb, Cu and Zn, were somewhat elevated in the southern part; while there was no evidence of contamination by metals elsewhere within the harbour (Boyden, 1975). Sediments from sites near urban areas were contaminated with one or more of the metals Cu, Pb, Zn, Ag, Cd and As (Thornton et al., 1975). Sn in sediments contamination increased from the mouth of the harbour to Holes Bay and exceed environmental quality targets designed to protect marine life in parts of the harbour (Langston et al., 1987). Turner (2000) reported that Poole Harbour has a greater sensitivity of sediment to point sources of trace metals in environments of relatively low tidal energy.

According to the CSQG, metal concentrations in sediments were generally, between the threshold effect levels (TEL) and the probable effect levels (PEL), except Cr which was below TEL in all sites, and Sn, which is not included in this guideline. This range is called “the possible effect range” within which adverse effects occasionally occur. Above normal concentrations of metals in sediment may reflect contamination sources due to past and present polluting activities (Thornton et al., 1975), however, the general health of sediment of Poole Harbour cannot be judged depending on the SQGs only as they cannot directly include fluctuating environmental factors such as grain size, pH-value, population stress or exposure time, which can affect, for example, bioavailability, toxicity and susceptibility (Hubner et al., 2009).

The highest concentrations of all metals (except for As) were recorded in samples from upper Holes Bay (sites S01 and S02). This is considered the most polluted area in the harbour, especially its eastern part (site 01) which is close to Poole sewage treatment works discharge point. These results agree with data from previous studies (White, 1991; Hubner, 2009). Sn, Hg and Ag were only recorded in upper Holes Bay, while they were below their detection limits in all other sites. Hg even exceeded the PEL guidelines, which reflects a serious local pollution of this metal in this area.

Wardlaw (2005) reported that sediments have accumulated toxic elements which had been discharged for decades, including Cd and Hg, and locally Ag, Cu, Zn. Holes Bay suffers from historical sources of pollution where a factory owned by the chemical company British Drug House Ltd. and a power station at Hamworthy (now demolished) used to discharge directly into it and polluted it with Sn from antifouling product used on the cooling water intake screens (Langston et al., 2003). Coal-based power stations contribute considerable Cu and Ni to sediments, possibly from cooling towers and coal storage areas (Birch et al., 2001). The Poole sewage treatment works (today operated by Wessex Water) discharges on the northern side of the same embayment through two streams (Humphreys and May, 2005; Hubner, 2009). The semi-enclosed nature of Holes Bay and its overall hydrological characteristics does not favour rapid self-restoration. The north part of Holes Bay is separated from the south part by Holes Bay Railway Bridge and they only connected through two channels which increase the isolation of the north part of the bay. Poor water exchange ensures that a considerable quantity of water discharged to the bay is retained inside its basin and the pollutants it carries precipitated and accumulated in the sediment.

Cluster analysis of the distribution of metals in sediment of Poole Harbour showed that the metals could be divided into four main groups which might be related to the source of these metals. As did not cluster with any other metal as it had a unique pattern of distribution in the harbour area (Figure 2.6). The highest concentration of As was recorded in the eastern part of Wareham Channel (site 06), not in Holes Bay, and it was approximately double the TEL. The narrow range of change in As concentrations between sites indicates that it may be primarily caused by non- point sources of pollution (e.g. ship traffic) rather than point sources (e.g. sewage discharge).The second cluster group included five metals (Cd, Ag, Cu, Hg and Sn). These metals share the same pattern of distribution as they were mainly elevated in upper Holes Bay and lower at all other sites; some of them even did not exceed the detection limit. The third group containing the last four metals, Cr, Pb, Zn and Ni. The highest concentrations of these metals were also in Holes Bay area, however, the other sites showed elevated values. The general relationship between concentrations of Pb and Zn in Poole Harbour was reported before by Turner (2000)

The cluster analysis of metal concentrations of sampling sites in Poole Harbour showed that sites in the north part of Holes Bay (sites 01 and 02) formed a very distinctive group (Figure 2.7) and metal contamination in this area is unique in its characteristics(semi-enclosed area and poor water exchange) and sources (historical sources and sewage discharge).

2.4.3. Metals in cockles

Sn was the only metal, of those analysed in the whole soft tissue of cockles, which was below its detection limit at all sites. Only three metals, Cr, Ni and Zn, were above their detection limits at all sites, while six metals were below the detection limit in at least one site. Samples from sites in Holes Bay (sites S01, S02 and S03) and Lytchett Bay (sites S04) showed that these areas had the most polluted organisms, with Hg and Pb detected in these areas and Ag detected in the sample from north-eastern part of Holes Bay only.

Generally, the metal concentrations in tissues of cockle samples were below that of sediment samples except for Ni and Ag. Ag concentration in cockles' tissue from site S01 in Holes Bay was approximately three fold that of sediment sample from same site while Ni concentrations were almost four times higher than that of sediment samples. A study of accumulation of Ag and Ni by cockles from Galician coast (northwest Spain) showed that cockles are good accumulators of these two metals and exceeded other bivalve species (e.g. blue mussel *Mytilus galloprovincialis*) (Saavedra et al., 2004).

Metal toxicity is often postulated to arise from reactions occurring in the cytosol (intracellular fluid found inside cells), through non-specific binding of the metal to non-thionein ligands, which are physiologically important molecules (metalloenzymes or small peptides such as glutathione) and are inactivated by metal binding (Mason and Jenkins 1995). Absence of EQS for metals in living organisms' tissues makes it difficult to specify whether the metal concentrations are dangerous or not. Although commercial cockle fishing grounds are restricted to outer harbour, illegal fishing is known to occur within the harbour (Drake and Bennett, 2011). Holes Bay is considered unclassified area (i.e. bivalve molluscs must not be subject to production or be collected) due to concerns about chemical contamination and Lytchett Bay is prohibited area due to bacteriological contamination (Food Authority: Borough of Poole, 2012).

The cluster analysis of the distribution of metals in cockles did not resulted in significant relationship between metals. The change in metal concentrations in sampling sites followed the same pattern of clustering as that of sediment samples. The sites in upper Holes Bay (sites S01 and S02) formed a distinctive group (Figures 2.7), and the biota in this area is directly affected by metal contamination of the sediment.

2.4.4. Metals in sponges

As opposed to cockles that live within sediment, Sponges' feeding habits make them particularly vulnerable to seawater quality. In this study, the metal concentrations in sponge samples were of a high magnitude when compared with that of the water of the same sites. Ag, for example, was below its detection limit in all water samples except for site S01 in upper Holes Bay; however, Ag could be detected in sponge samples from three different sites (sites S16, S17 and S18), which reflects the ability of these organisms to accumulate these metals.

This study showed that sponges accumulated Cu and Zn with a very high accumulation factor. Field studies have shown that sponges efficiently accumulate these two metals (Philp, 1999; Philp et al., 2003) and studies have shown that accumulation is a function of the quantity present in the environment and that bioconcentration factors may be very high (e.g. The enrichment factor of Cd, calculated as the ratio of metal concentration to that of sediments, for *S. inconstans* was 700) (Hansen et al., 1995; Cebrian et al., 2003; Perez et al., 2003; Rao et al., 2009; Pan et al., 2011).

Metals in sponges clustered into four groups. The first group contained three metals: As, Cr and Zn as these metals increased or decreased together from one site to another. The second group followed the same pattern of first one with the exception that metals of this group (Pb and Ni) were below their detection limits in site 18 (Lake yard). Cd and Ag formed the third group where their largest concentrations were recorded in site S17 (Holes Bay Cobbs Quay). The metal concentrations clustering in sponges samples showed that site S15 (Parkstone Bay) stand alone as separate cluster from all other sites with metals had their highest concentration in this site except Zn.

2.5. Environmental significance and conclusions

Comparing the results of this study with the pervious data on metals in water , sediment and cockles in Poole Harbour (Boyden, 1975; Thornton et al., 1975; Langston et al., 1987; Bryan and Langston, 1992) showed that the harbour environment is generally improving and concentrations of some metals are noticeably decreased (e.g. Sn and Pb) in all harbour areas and locally in Holes Bay, however, the harbour ecosystem still under pressure from metal contamination resulting from point sources of pollution as well as non-point sources. Metal contamination was shown in all environmental compartments. Water was polluted with As and Hg; these two metals usually exists as organic forms (e.g. arsenate, arsenite and methylated arsenic compounds, and chloride and hydroxide methyl mercury compounds that are more bioavailable than the inorganic forms and are known to bioaccumulate in organisms tissues. Sediment metals were mostly within “the possible effect range” within which adverse effect occasionally occurs.

The two species selected for biomonitoring were cockle *C. edule* which is an infaunal suspension feeder that live within sediment, and sponge *H. oculata* that its feeding habitat makes it particularly vulnerable to seawater quality to reflect the impact of contaminations in these two phases , water and sediment , on biota in Poole Harbour. Cockles had considerable concentrations of Ni, Ag and Hg in areas close to contamination sources. Although commercial fishing is prohibited in these areas, the contaminated cockles are still representing a risk to human consumption via illegal fishing, which is known to occur within the harbour, and also a risk to birds which feed on them. Finally, sponges accumulate metal (especially Cu and Zn) with very high magnitude (31-170 mg/kg of Cu and 69- 730 mg/kg of Zn).

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Many areas in the harbour were considered hot spots of metal contamination. Holton Heath for example was a hot spot for water pollution with Hg and Pb, whilst Holes Bay was a hot spot for sediment pollution with Hg. The enclosed nature of the harbour and its secondary embayments make it vulnerable to the effects of these metal sources which would disperse extremely slowly due to restricted tidal exchange and a substantial part of the pollution load remains in coastal areas close to land based contamination sources.

A systematic monitoring which covers all embayments is needed in this sort of marine environment. Although above EQSs concentrations of metals in water and sediment may reflect contamination sources due to past and present polluting activities, to monitor the effects of the full mixture of anthropogenically-derived chemicals found in the marine environments, it is considered important to measure additional information about the environment like toxicities and bioavailability and consider as well the biological effect that occurs in the species that may be exposed. Consequently, the monitoring should include biological monitoring techniques such as biomarkers (functional measures of exposure to stressors expressed at the suborganismal, physiological, or behavioural level) e.g. metallothionein, which is a metal-binding protein induced in direct response to metal contamination. An integrated management of the semi-enclosed coastal zones should be based on the overall hydrological characteristics of these sensitive areas and their abilities to self-restoration which is different than open coastal zones.

Chapter 3: Metallothioneins in common cockles (*Cerastoderma edule*) and sponges (*Haliclona oculata*) as biomarker of metal contamination in a semi-enclosed coastal area

3.1. Introduction

The spatial variations of metal contamination in biota, water and sediment within Poole Harbour were identified and quantified as an example of a heavily anthropogenically impacted semi-enclosed estuarine coastal area with a very low ability to disperse and flush contaminants that is affected by significant point and non-point sources of metal pollution (chapter 2). This study concluded that metal monitoring should include biological monitoring techniques such as biomarkers (e.g. MTs) to evaluate the impact of this type of pollution on biota within this sensitive environment.

The aim of this chapter is to examine the physiological significance of metal burdens within Poole Harbour and assess the potential use of MTs concentrations in two organisms: the common cockle (*Cerastoderma edule*) and Mermaid's Glove sponge (*Haliclona oculata*) for mapping the spatial extent of biological response to metal contamination.

3.2. Material and methods

3.2.1. Samples collection and storage

Sampling locations (Figure 2.1) were chosen to cover the whole Poole Harbour area including river estuaries and embayments, and to reflect the various sources of metal contamination as well as the selection of unpolluted, or less polluted sites (see Table 2.1). Samples were collected manually (sponges) or via a trailed dredge (cockles) during November and December 2009. Samples of biota were collected when present at each sampling site, but were not present at all sites (Table 2.1).

3.2.2. Metalothioneins analysis

The spectrophotometric method as described by Viarengo et al. (1997) and UNEP/RAMOGE (1999), with few modifications, was applied for detection of MTLPs in bivalves and for the first time to detect these molecules in sponges. This method has been reported to be a sensitive, time-saving, and low-cost technique able to detect metallothionein content in the tissues of marine organisms and has been inter-calibrated and standardized by a number of laboratories (UNEP/RAMOGE, 1999; Zorita et al., 2005). Three subsample (in order to minimize experimental errors) of about 1g were accurately weighed from soft tissues belonging to at least ten bivalves and whole tissue of sponges after they were physically homogenised using a pestle and mortar to produce one pool of homogenised tissue per site. Tissue samples were homogenised by homogenising buffer of 0.5 M sucrose, 20 mM Tris-HCl (pH 8.6), 0.006 mM leupeptin, 0.5 mM PMSF, and 0.01% ß-mercaptoethanol then centrifuged for 30 minutes at 28,600 × g (times gravity) at 4°C. The supernatant solution was purified with 1.05 mL cold ethanol (-20°C) and 80 µL chloroform (per 1 ml of supernatant) and centrifuged for 10 minutes at 6000 × g at 4°C. Then 40 µL concentrated HCl and three volumes cold ethanol (-20°C) and allowed the proteins to denature for one hour at -20°C. This was then centrifuged for 10 minutes at 6000 × g and the pellet saved. RNA was not added in the MT precipitation step as this has been shown to be unnecessary (Viarengo et al., 1997). The pellet then washed with a mixture of the previously described buffer solution (without the ß-mercaptoethanol), cold ethanol and 80 µL chloroform and centrifuged for 10 minutes at 6000 × g. The supernatant was discarded and the pellet was dried with N2 gas. The pellet was resuspended with 150 µL 0.025 M NaCl and 150 µL 1 N HCl with 4 mM EDTA. After resuspension, 4.2 mL of a solution containing 2 M NaCl and 0.43 mM DTNB and 0.2 M NaH2PO4 (pH 8.0) was added, and centrifuged for 5 minutes at 2500 rpm. The absorbance was read at 412 nm against standard solutions of reduced glutathione (GSH) using a UV-visible spectrophotometer.

3.2.3. Statistical analyses

Statistical analyses were performed with PASW Statistics© 18. MT concentrations were presented by Box and whisker plots and were compared by the non-parametric Kruskal-Wallis test to determine statistically significant differences between sampling sites. Spearman’s rho correlation coefficients were used to determine the relation between metals and MT concentrations. Statistical significance was established at $P<0.05$ in all cases.

3.3. Results

3.3.1. Metallothionein concentrations

MTs concentrations measured in Poole Harbour gave a range of values between 28.0 μg/g (S12, Brands Bay) and 77.0 μg/g (S04, Lytchett Bay) in cockles, and between 23.4 μg/g (S16, Fisherman's Haven) and 55.7 μg/g (S18, Lake Yard) in sponges (Figure 3.1). Statistical analysis (Independent-samples Kruskal-Wallis test) showed that the distribution of MT across sampling sites was significantly different in cockles while it was not significant in sponges. MT correlation with metals measured in different environment components (water, sediment and tissue) showed no significant relation (Tables 3.1 and 3.2).

3.4. Discussion

Biomonitoring tests are frequently applied to assess the impact of pollution on a marine ecosystem. The physiological or population responses of biological organization are necessary for a clear indication of ecosystem health. However, detection of early warning responses at subcellular level, such as MTs production, seems to be very useful for the prevention of long-term ecological damage (Kalpaxis et al., 2004). In this study, MTs induction was used in an attempt to characterize biomarker of metal exposure that is commonly applied in pollution monitoring programs (Viarengo et al., 1999; 2007).

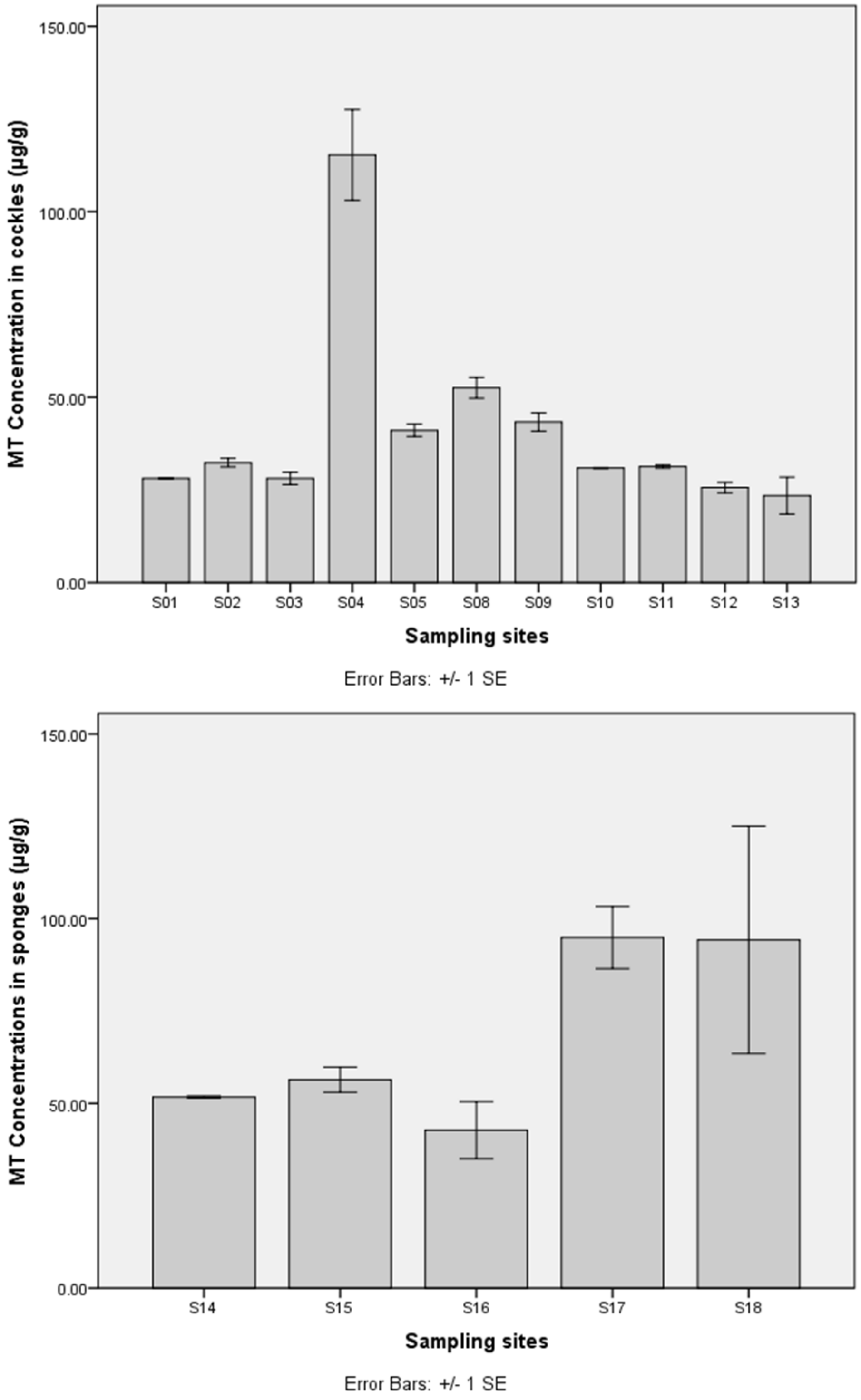

Figure 3-1: MTs concentrations (mean ± SE of three subsamples) in cockles and sponges collected from Poole Harbour during Nov-Dec 2009.

Previous studies of MT as biomarker of metal contamination in Poole Harbour were limited to unpublished preliminary trials conducted in the mid 1980's (Langston et al., 2003). These trials showed that induction of Cd-binding MT was significantly enhanced in mussels (*Mytilus edulis*) from Holes Bay when compared with that from an open coastal reference site at Godrevy in North Cornwall. This result was explained by the observation that most of the excess Cd in mussels from Holes Bay appeared to be sequestered by MT in an attempt to detoxify the metal (preventing interaction with enzymes and other vital molecules) in response to the elevated levels of bioavailable metal (Langston et al., 2003). The same study showed that another mollusc species (*L. littorea*) from Poole Harbour had a different response to metal pollution. Although MT induction in this species was again signified in response to increasing levels of contamination, in the more heavily contaminated areas (Holes Bay), Cd became associated with HMW (High Molecular Weight) ligands (e.g. enzymes) and LMW (Low Molecular Weight) fractions which includes amino acids and any free (potentially toxic) metal; this was explained as a result of saturation of MT (Langston et al., 2003).

The results of our study show that while some metal concentrations in cockles and sponges, and their surrounding environment (water and sediment) could be related to certain sources of metal contamination in Poole Harbour, MTs values in the soft tissue of cockles and whole tissue of sponges are not (Figure 3.1). Although MTs concentrations were significantly different in cockle samples at all sampling sites, no relation could be found between these concentrations and any of the tested metals (Table 3.1). Moreover, MTs concentrations in sponges were insignificantly different (i.e. relatively uniform) at all sampling sites (Table 3.2). Especially notable is the observation that some of the lowest MTs levels were detected in heavily polluted areas such as Holes Bay where some of the highest levels of all metals were measured in organisms' tissues.

These results may be explained on the basis that natural variability of MTs levels in cockles tends to hide any changes due to metal pollution. The low MTs response in biota in Holes Bay may be explained by that many, but not all, populations in polluted areas do have an increased tolerance (Amiard-Triquet et al., 2011). Cockles could have acquired tolerance as a result of detoxication mechanisms that include variety of physicochemical forms of storage. Previous study of MT produced by organisms (*M. edulis* and *Littorina littorea*) in Holes Bay showed that in the more heavily contaminated areas (Holes Bay), Cd became associated with HMW (High Molecular Weight) ligands (e.g. enzymes) and LMW (Low Molecular Weight) fractions which include amino acids and any free (potentially toxic) metal; this was explained as a result of saturation of MT (Langston et al., 2003).These mechanisms may produce results different from those “expected”, such as the binding to MTs by metals (Co, Ni, Fe and Mn) that, in an unpolluted environment, only occasionally bind to these proteins (Trombini et al., 2010). Although MTs are mainly involved in the adaptive cell mechanism of protection against metal toxicity, a wide range of other factors such as hormones, cytotoxic agents, physical and chemical stress could interfere with MT biosynthesis (Amiard et al., 2006). Moreover, it was reported that, in contrast with mammals or fish, in invertebrates, MTs frequently do not constitute the major intracellular sink for metals in target tissues (Mouneyrac et al., 2000).

From the results of this study, it is clear that the variations in MTs levels were not exclusively associated with metal concentrations and that other environmental factors could explain a percentage of MTs variability. MTs production in these organisms may not be an effective biomarker for metal stress in Poole Harbour and/or the metal concentrations measured may not be high enough to induce a stress response in these organisms. Therefore, care is needed in the interpretation of MTs levels from field studies.

Table 3-1: Spearman correlation coefficient of cockles' MT and metals measured in samples from Poole Harbour.

		As	Cd	Cr	Cu	Pb	Hg	Ni	Ag	Sn	Zn
Water	Correlation Coefficient	-0.518	-0.282	-0.245	0.436	0.064	0.264	0.009	N/A	N/A	-0.291
	Sig. (2-tailed)	0.102	0.401	0.467	0.180	0.853	0.433	0.979	N/A	N/A	0.385
	N	11	11	11	11	11	11	11	11	11	11
Sediment	Correlation Coefficient	0.000	-0.364	-0.301	-0.337	-0.032	-0.054	-0.155	0.100	-0.054	-0.028
	Sig. (2-tailed)	1.000	0.272	0.368	0.311	0.926	0.875	0.649	0.770	0.875	0.936
	N	11	11	11	11	11	11	11	11	11	11
Soft tissue	Correlation Coefficient	0.140	0.474	0.200	0.030	0.172	0.246	0.127	0.058	N/A	0.079
	Sig. (2-tailed)	0.700	0.166	0.580	0.934	0.636	0.493	0.726	0.873	N/A	0.829
	N	10	10	10	10	10	10	10	10	10	10

Table 3-2: Spearman correlation coefficient of sponges' MT and metals measured in samples from Poole Harbour.

		As	Cd	Cr	Cu	Pb	Hg	Ni	Ag	Sn	Zn
Water	Correlation Coefficient	-0.700	-0.300	-0.400	-0.300	-0.359	-0.700	0.200	N/A	N/A	-0.700
	Sig. (2-tailed)	0.188	0.624	0.505	0.624	0.553	0.188	0.747	N/A	N/A	0.188
	N	5	5	5	5	5	5	5	5	5	5
Tissue	Correlation Coefficient	-0.400	-0.300	-0.400	-0.300	-0.410	N/A	-0.300	0.154	N/A	-0.400
	Sig. (2-tailed)	0.505	0.624	0.505	0.624	0.493	N/A	0.624	0.805	N/A	0.505
	N	5	5	5	5	5	5	5	5	5	5

3.5. Conclusion and recommendations

Poole Harbour is a semi-enclosed ecosystem under pressure from metal pollution. MTs concentration was applied as a biomonitoring test to assess the impact of this type of pollution on biota (cockles and sponges). Pervious research has shown that metals accumulated in these organisms' tissues and detected in their surrounding environment (water and sediment) could be related to certain sources of metal contamination. The biomarker approach adopted in this study has shown that:

- MTs concentrations were significantly different in cockle samples at all sampling sites, while that of sponges were insignificantly different.
- No relation could be found between MTs concentrations in cockles and sponges, and any of the tested metals. Some of the lowest MTs levels were detected in heavily polluted areas such as Holes Bay.
- MTs levels in tested organisms from Poole Harbour are not exclusively associated with metal concentrations as other environmental factors could affect induction of this protein.
- While subsamples from each sample were analysed to ensure the validity of the method and to reduce error as the -SH group in MT can be easily oxidized leading to underestimation of MT, replicate of field samples should have been used to measure natural variation of MT rather than focusing on the average concentration for each site.
- Future studies of metal stress in Poole Harbour ecosystem may need to examine other indicator species and/or apply a different biomonitoring technique such as transplant experiments (caged organisms from a known low-metal location and pre-placed in clean equilibration medium). To facilitate interpretation of the results of this experiment, local populations could be sampled on the same dates as transplanted organisms.

Chapter 4: Biomonitoring metal pollution with caged and wild bivalves

4.1. Introduction

As no clear relation could be found between MT production in wild organisms and metal pollution in Poole Harbour, another approach based on transplant experiments along with sampling local populations on the same dates as transplanted organisms (to facilitate interpretation of the results) was applied. Transplanted organisms were used as active biomonitoring tools with the advantage of assuring comparable samples, reducing the variability normally encountered in field studies (Nasci et al., 2000). Active sampling also has other advantages, as a number of parameters can be controlled prior to sampling. Age, sex, physiology and organism health can be controlled (Nicholson and Lam, 2005).

In this chapter, the relationship between selected metal concentrations in sediment and MT induction in caged (*Mytilus edulis* and *Tapes philippinarum*) and wild (*Cerastoderma edule*) bivalveswas investigated. At the sites in Poole Harbour, UK, the impacts of metal pollution on aquatic organisms and conflicting evidence for the use of biomarkers as a method for environmental monitoring were investigated and evaluated.

4.2. Materials and methods

4.2.1. Sampling locations

Five sites were selected for study in Poole Harbour: four sites close to CSO outfalls and a sewage treatment works (P1–P4), and one site in an area that had no outfalls in close proximity (Newton Bay, P5) (Figure 4.1 and Table 4.1).

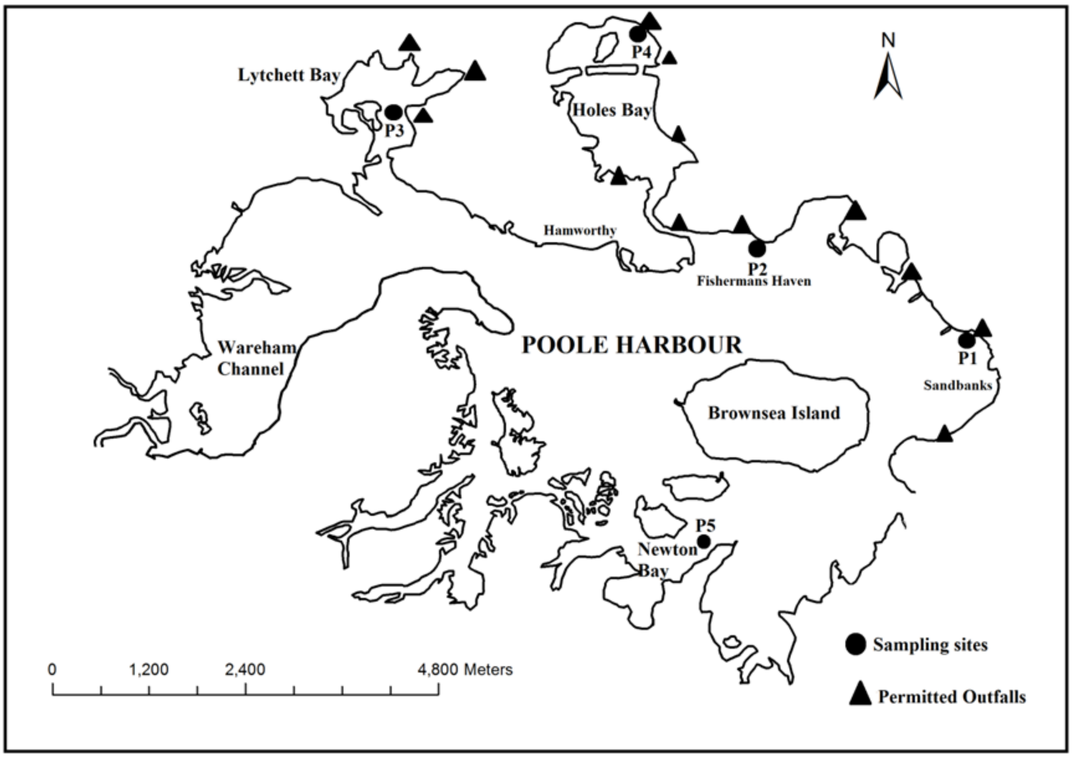

Figure 4-1: Locations of sample sites and permitted outfalls in Poole Harbour.

Table 4‑1: Description of sampling sites and associated sources of pollution.

Code	Site	Coordinates	Description	Pollution Sources
P1	Sandbanks (Whitley Lake)	50° 42.149` N 01° 56.659` W	A wide sandy bay enclosed by the Sandbanks peninsula. Almost the entire shoreline is protected by concrete or stone embankments.	• Road surface run-off • Two yacht clubs • 1 CSO pipe
P2	Fisherman’s Haven	50° 42.678` N 01° 58.598` W	Situated at the east end of Poole Quay.	• 3 CSO pipes • Boat traffic from nearby marina and the main harbour channel • Moored boats
P3	Lytchett Bay	50° 43.647` N 02° 02.284` W	The Sherford River estuary which has become increasingly isolated as a result of land reclamation.	• Sewage treatment works • 1 CSO pipe
P4	Holes Bay	50° 44.012` N 01° 59.688` W	The largest secondary embayment entering Poole Harbour which has narrow entrance compounding restrictions to the flow. Consequently, a sheltered nature and reduced currents, particularly in the upper bay, encourage siltation.	• 2 CSO pipes • Sewage treatment works • High concentration of Industry around the bay. • Historical pollution sources • Marina
P5	Newton Bay	50° 40.425` N 01° 59.602` W	Located in the central part of the south shore.	• Boating channel

N: North, W: West, CSO: Combined Sewer Overflow

4.2.2. Selection of organisms and caging

Clean organisms of a uniform size (29-24 mm width; *M. edulis* and *T. philippinarum*) , from Rock, Cornwall, were taken for transplantation to the relevant locations. Samples were kept in isothermal containers during transportation. A specifically designed cage (40×30×11cm) made of plastic and nylon (to prevent possible metal contamination) was used to contain samples. The cages were perforated to allow water to flow freely through the cage, with bricks attached to prevent movement and to encourage *M. edulis* byssus thread growth (Nicholson and Lam, 2005). Garden canes with pink tape wrapped around the top were used to highlight the cages' locations.

At each site, 24 *M. edulis*, and 34 *T. philippinarum* were placed in each cage. Cages were placed from a small boat. Sampling took place July-August 2010. A sample of 7 *M. edulis* and 10 *T. philippinarum* were removed after two weeks (only three of the sites were visited at that time due to logistical problems, as a boat was unavailable to visit Newton and Lytchett Bays), and the remainder after five weeks, in addition to native samples of cockles, *C. edule* were collected (depending on availability) during that time. Samples were packed in ice and kept between 0-4°C during transportation to prevent degradation of the MT protein (Amiard et al., 2006). Sediment samples for analysis of metals were taken when samples were placed.

4.2.3. Sediment metal analysis

Sediments were stored at -20°C. Before analysis, sediment was placed in a freeze-dryer (VirTis benchtop K©) for 72 hours to remove water content. Accurately weighted 1±0.09 g of each dried sample was subjected to digestion in *boiling Aqua Regia* (a 3:1 v/v mixture of concentrated, 35%, hydrochloric acid and concentrated, 68%, nitric acid) for 48 hours in heating blocks in order to bring the metals into solution. The digested sample was filtered and diluted to a known volume before being analysed. Determination of Zn, Pb, Cd and Cu, which are considered as priority pollutants in wastewater and CSO (Gasperi et al., 2008), was made by atomic absorption spectrometry (AAS) (Varian© SpectraAA-200) according to an accepted standard method (Standing Committee of Analysts, 2006).

Photos of the cages (a) with bricks attached to prevent movement (b) and byssus thread growth after two (c) and five weeks (d).

4.2.4. Determination of MT

The MTs concentrations were not measured in samples before transplantation because MTs have a normal role in the metabolise of essential metals; it is expected that natural factors could interact with metals in governing the concentrations of MTs in organisms, consequently, the relevance of MTs as biomarkers must be examined taking into account the signal-to-noise ratio (Cairns, 1992) in which the signal, the MT concentration in response to metal exposure (i.e. MTs in samples transplanted in polluted sites), must be clearly distinguished from the noise (i.e. MTs in samples transplanted in unpolluted sites). Therefore, it is common practice to compare the MTs concentrations along pollution gradient after exposure time only and consider the MTs concentrations in the unpolluted sites as the baseline rather than the initial concentrations before deploying (Stien et al., 1998; Bodin et al., 2004; Dondero et al., 2006; Gagnon et al., 2006; Damiens et al., 2007; Pytharopoulou et al., 2008; Guidi et al., 2010; Tsangaris et al., 2010b; de los Ríos et al., 2012)

Analysis was performed on seven *M. edulis* and ten *T. philippinarum* from each study site after two and five weeks, in addition to at least ten wild *C. edule*. All samples were stored at -20°C to prevent protein loss. Samples were blended using a pestle and mortar prior to analysis. Three accurately weighted 1± 0.09 g subsamples were made for each emulsified sample. The spectrophotometric method as described by Viarengo et al. (1997) and UNEP/RAMOGE (1999), with few modifications (not adding RNA in the MT pelletization step as this has been shown to be unnecessary) was applied for detection of MTs in bivalves (see 3.2.2 for detailed procedure).

4.3. Results

4.3.1. Metals in sediment

Concentrations of Cd at all sites were below the detection limit (2.5 mg /kg) in sediment, whilst the concentrations of three other metals (Cu, Pb and Zn) showed variation at different sites in Poole Harbour (Figure 4.2). These data indicate that Holes Bay (P4) had the highest concentrations of metals, followed by Lytchett Bay (P3), Fisherman’s Haven (P2), Newton’s Bay (P5) and Sandbanks (P1). The total metal concentration range was 22.7 to 601.6 mg/kg, presenting a wide variation in concentrations between sites. Previous studies confirmed this pattern of distribution of sediment containing metals (Figure 4.2) ; (see Chapter 2) and (Hubner, 2009). All metal concentrations at Holes Bay and Lytchett Bay exceed the Threshold Effect Level (TEL) proposed by the Canadian Sediment Quality Guidelines (CSQG) which is the standard used by the Environment Agency in the UK (Hubner et al., 2009). Zn exceeded the Probable Effect Level (PEL) proposed by the CSQC at Holes Bay only (Figure 4.2).

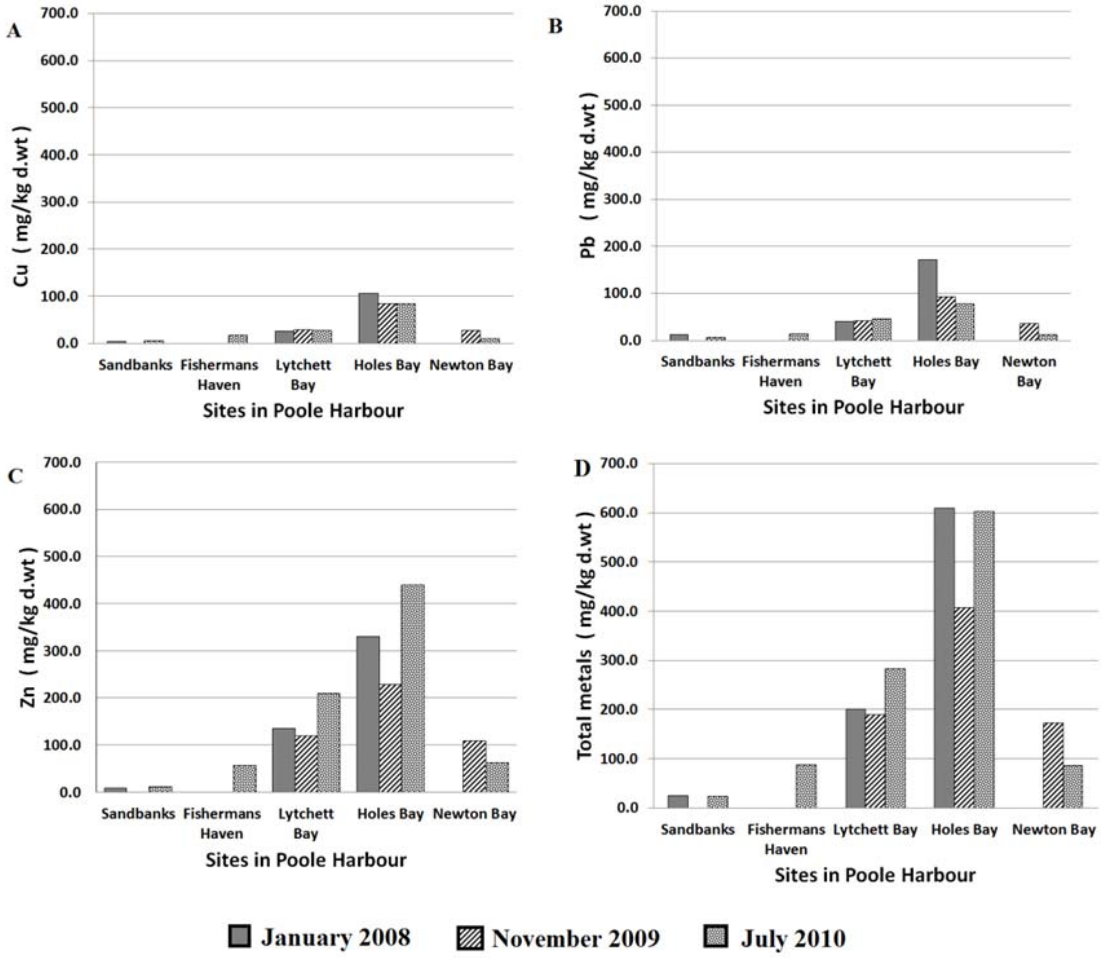

Figure 4-2: Concentrations of copper (A), lead (B), zinc (C) and total metals (D) in sediment samples from sites in Poole Harbour during January 2008 (Hubner, 2009), November 2009 (chapter 2) and July 2010 (this study).

4.3.2. Metallothioneins Concentrations

Metallothionein concentrations in transplanted mussels and clams showed a wide range of values which could be related to species, time and site. The difference in mussel MT concentrations (Figure 4.3) for the two week samples indicates that Holes Bay had the highest concentration of MT (102.0 µg/g), followed by Sandbanks (58.7 µg/g) and Fisherman's Haven (29.8 µg/g). Two week clam samples showed the same order where Holes Bay had the highest concentration (66.0 µg/g) followed by Sandbanks (42.7 µg/g) then Fisherman's Haven (36.1 µg/g).

The five week samples showed that the highest MT concentrations in both species were recorded at Lytchett Bay (226.7 and 90.1 µg/g in mussels and clams, respectively) whilst the lowest concentrations were recorded in Newton Bay (32.1 and 25.6 µg/g in mussels and clams, respectively) (Figure 4.3).

MT concentrations measured in wild cockles in Poole Harbour gave a range of values from 25.5 µg/g (P5, Newton Bay) to 763.0 µg/g (P3, Lytchett Bay).

4.3.3. Relationship between MT and metal concentrations in sediments

Scatter plots of MT values in mussels and clams at sampling sites were produced to check for any relationship (Figure 4.4). These plots revealed a linear relationship between MT and metal concentrations, indicating that a linear regression model might be appropriate. However, samples with very high metal concentrations (Holes Bay) did not fit with this model (Figure 4.4). As the scatter plots indicated that the relationship deviates from linearity because of samples taken from Holes Bay, Pearson correlation coefficients for MT in five week mussels and clams samples and wild cockles, and metals were calculated using different approaches: i) for all sampling sites and ii) for sampling sites excluding Holes Bay (Table 4.2). The results showed that when Holes Bay was included in the analysis (i), the only significant relationship was reported between MT in cockles and metal concentrations, but when this site was excluded (ii), more significant relationships were evident.

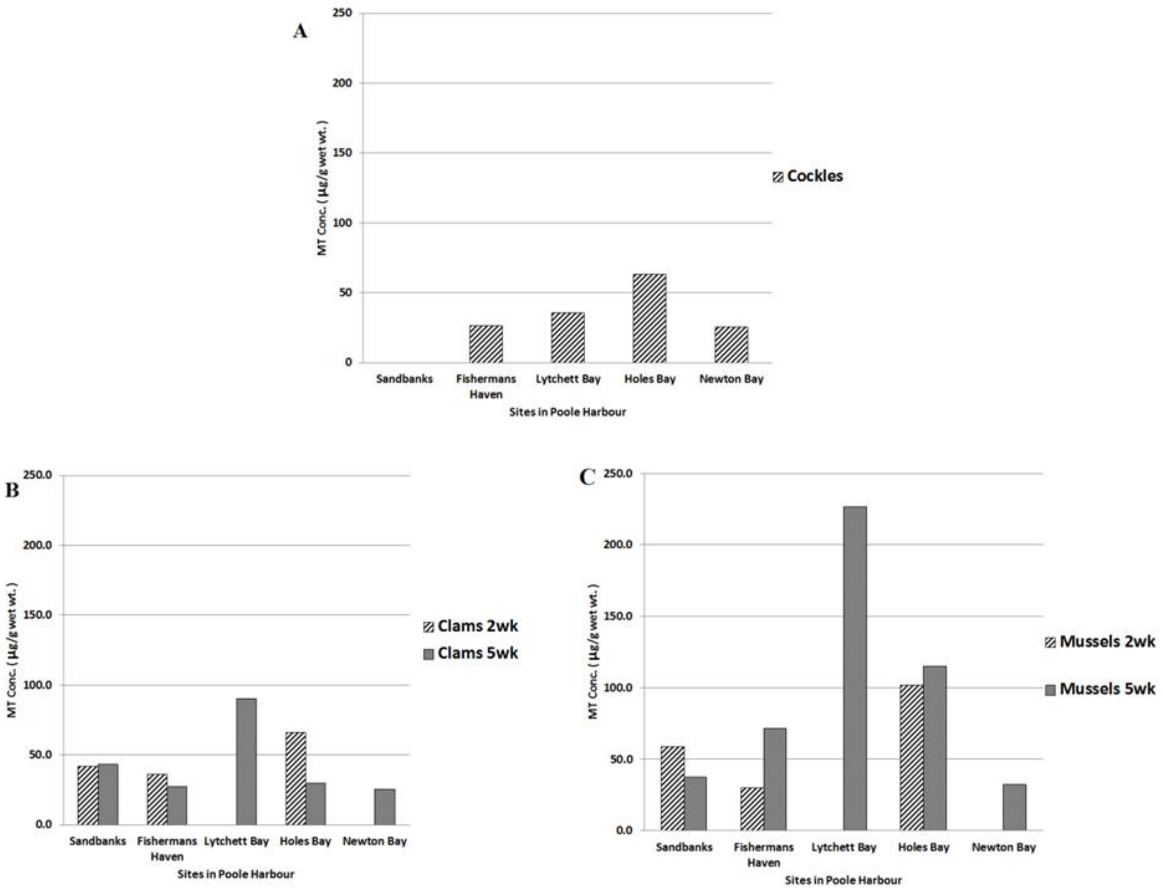

Figure 4-3: Metallothionein (MT) concentrations in (A) wild cockles, (B) two week (2wk) and five week (5wk) clams and (C) mussels in Poole Harbour during July 2010. NB two-week samples were not taken at Lytchett Bay and Newton Bay, and no wild cockles were present for sampling at Sandbanks.

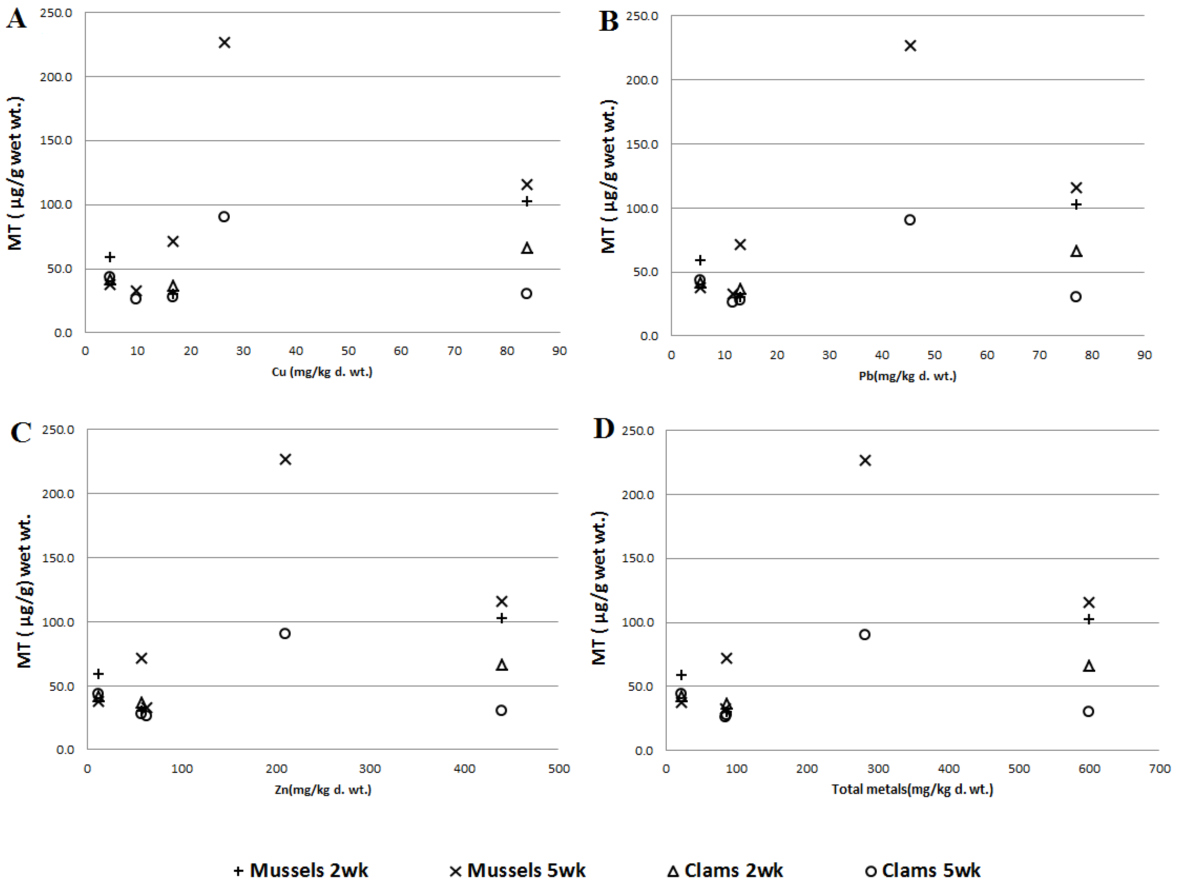

Figure 4-4: Scatter plots of MT values in two (2wk) and five (5wk) week mussels and clams versus the corresponding values of copper (A), lead (B), zinc (C) and total metals (D) in sediment of Poole Harbour during July2010.

Table 4-2: Pearson correlations between MT in five week mussel and clam samples, and wild cockles, and metals in sediments (significance shown in brackets).

		Cu	Pb	Zn	Total Metals
Mussels MT	i	0.359 (0.553)	0.613 (0.272)	0.524 (0.365)	0.515 (0.374)
	ii	0.924 [a] (0.076)	0.984* [a] (0.016)	0.961* [a] (0.039)	0.968* [a] (0.032)
Clams MT	i	-0.090 (0.886)	0.209 (0.736)	0.103 (0.869)	0.091 (0.884)
	ii	0.721 [a] (0.279)	0.901 [a] (0.099)	0.859 [a] (0.141)	0.859 [a] (0.141)
Cockles MT	i	0.995** (0.005)	0.965* (0.035)	0.989* (0.011)	0.991** (0.009)
	ii	0.935 [a] (0.23)	1.000* [a] (0.018)	0.995 [a] (0.062)	0.999* [a] (0.033)

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

[a] Person correlations for samples excluding Holes Bay

4.3.4. Cluster analysis

Cluster analysis was performed to test how sampling sites in Poole Harbour could be grouped according to their metal concentrations and MT response of transplanted organisms. To verify the integrity of cluster results, an ANOVA was run using the cluster groupings as categorical values. According to the metal profile only, Holes Bay was the only member of its cluster followed by Lytchett Bay in a separate cluster; then the other three sites showed some similarity (around 80%) (Figure 4.5A). ANOVA results showed that all metals directed this clustering ($p<0.05$). The MT concentrations in five week mussel and clam samples showed that Lytchett Bay formed a standalone group whilst Holes Bay and Fisherman's Haven showed some similarity (around 70%); and Sandbanks and Newton Bay were more similar (around 90%) (Figure 4.5B). ANOVA results of this clustering showed that only mussels' MT directed this clustering ($p=0.038$) rather than clams' MT ($p= 0.054$). When both metals and MT data were used, the results showed that Lytchett Bay and Holes Bay formed a distinctive group, whilst Fisherman's Haven and Newton Bay formed another separate group and finally Sandbanks stood alone (Figure 4.5C). However, ANOVA results showed that not the metals nor MT concentrations where significantly different between sites in these clusters ($p>0.05$).

4.4. Discussion

The results have shown that the two semi-enclosed embayments (Holes Bay and Lytchett Bay) to the north of Poole Harbour are under pressure from metal pollution (Figures 4.3). These two areas were considered "hot spots" of metal pollution where Cu, Pb and Zn exceeded the Threshold Effect Level (TEL) according to the Canadian Sediment Quality Guidelines (CSQG); this means that these metals are in the possible effect range within which adverse effects occasionally occur (CCME, 2001). The enclosed nature of these two areas makes them vulnerable to the effects of point sources of pollution (e.g. CSO pipes) as well as non-point sources (e.g. historically polluted sediment) that would disperse extremely slowly due to restricted tidal exchange and affect environmental quality (see chapter 2). The tidal prism in Hoes Bay is about 40% greater than in the outer Harbour. Consequently, a sheltered nature and reduced currents, particularly in the upper bay, encourage siltation (Langston et al., 2003; Wardlaw, 2005).

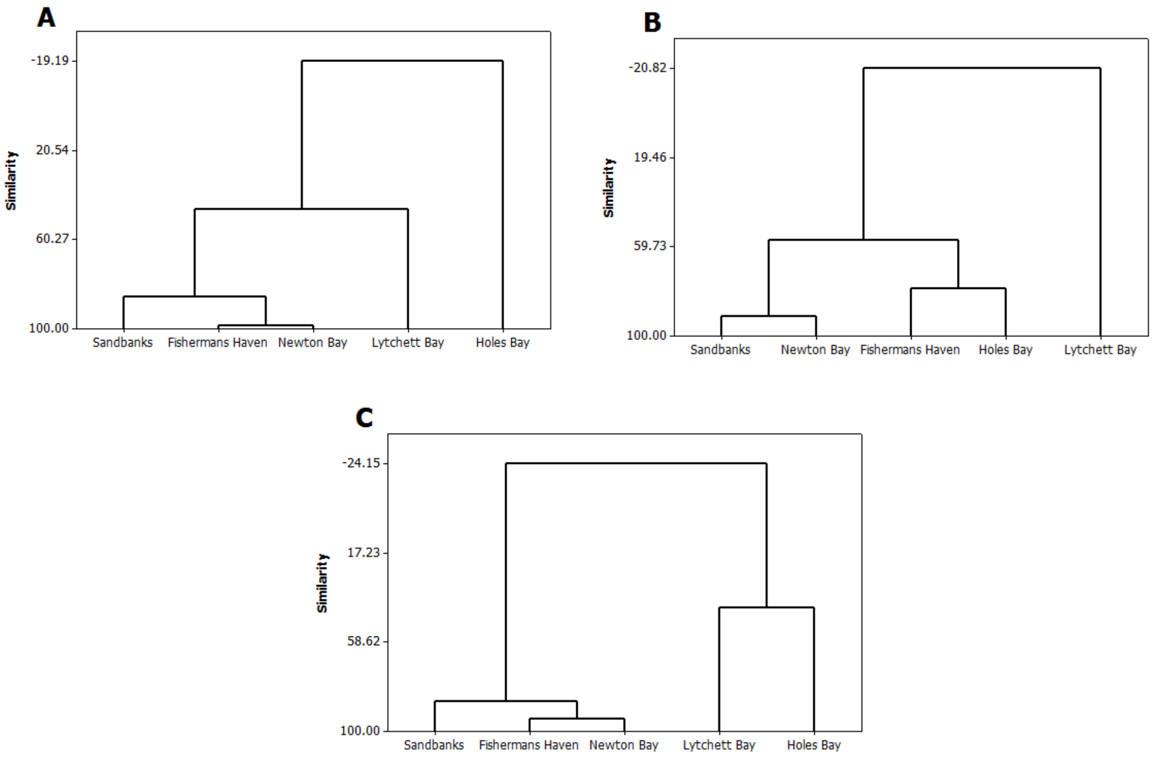

Figure 4-5: Dendrograms, using Ward linkage, eucliden distance, showing how sampling sites were related according to their metal profile (A), MT concentrations (B) and both (C).

Sewage Treatment Works (STWs) and CSO discharges may be considered one of the main sources of metal pollution in Poole Harbour. The harbour receives treated waste water from three main STWs that serve the communities of Poole, Lytchett Minster and Wareham. There were plans for the sewage treatment works to be updated during 2005- 2010 to meet European Union Standards (Drake and Bennett, 2011); however, effluent and overflow still discharges into the Harbour (Wardlaw, 2005). The Harbour has a small tidal range (1.8 m at spring tides, 0.6 m at neaps) and a double high water which means that water levels are often above mean tide level for 16 out of 24 hours. The northern embayments of Poole Harbour have minimal tidal ranges and are not flushing containments out to sea; instead they are mostly re-suspended and deposited in the same location (Wardlaw, 2005). Gasperi et al. (2008) reported that in their study, Cu, Pb and Zn concentrations in CSOs were raised, which they explained as a result of the high metal concentrations found in runoff, or in-sewer deposit erosion. They also reported that a major proportion of Cu originates from deposit erosion, whilst runoff proves to be the major source of Cd, Pb and Zn and for street runoff, vehicles and vehicle leaching constituted the leading contributors to metal loads (Gasperi et al., 2008). These two factors, the limited geographical dispersion of CSO discharges and the high concentrations of the tested metals in the discharges, may explain the high metal concentrations in Holes Bay and Lytchett Bay (P4 and P3). The low concentration of metals at the remaining sites may be because they are missing one of these factors. Newton Bay (P5) is a semi-enclosed area (albeit less enclosed than the northern bays) but it does not receive CSO discharges. Sandbanks (P1) receives CSO discharges from more than one pipe (Figure 5.1); however, it is very close to the harbour's opening so it is supposed to be well flushed. Fisherman's Haven (PS2) also receives CSO discharges via more than one pipe which discharges into an open coastal area, allowing exchange of water and reducing the impact of CSO. This area of Poole Harbour has a strong tidal current (Tidal currents peak at around 1m sec^{-1} near the Harbour mouth (Langston et al., 2003)) that can remove contaminants rapidly before settlement can occur, explaining the lower metal concentrations at these two sites (Wardlaw, 2005).

The advantage of biomarkers is that unlike chemical analysis, they can show the effects of a range of contaminants (Livingstone, 2001) and can demonstrate interactive and combined effects due to the numerous environmental factors that can characterise a location (Amiard et al., 2006). To understand the sub-lethal effects of metal contaminants on the marine environment, MT was used as biomarker of metal exposure. MT production is an early warning response at sub-cellular levels, which seems to be very useful for the prevention of long-term ecological damage (Kalpaxis et al., 2004).

In chapter 3, it was reported that as no relation could be found between MT production in wild cockles and metal pollution in Poole Harbour, a different biomonitoring technique may be needed. It was suggested transplant experiments along with sampling local populations on the same dates as transplanted organisms to facilitate interpretation of the results of this experiment.

In this study, transplanted mussels and clams were used as active biomonitoring tools with the advantage of assuring comparable samples, reducing the variability normally encountered in field studies (Nasci et al., 2000). Active sampling (collecting organisms from indigenous populations in a clean area or after passing cleaning process and transplanting them into a polluted area for a certain period of time) also has advantages, as a number of parameters can be controlled prior to sampling. Age, sex, physiology and organism health can be controlled (Nicholson and Lam, 2005). The cages worked effectively, remaining in position. After two weeks, a diverse range of ecology was found to have developed, including prawns, crabs, and at one location, the growth of sponges. There was also algal growth and other marine fauna species; *M. edulis* adapted to the boxes and had good attachment to the bricks and to the plastic box itself by the byssus thread. Very low mortality was observed during the five weeks of caging at all sites (0- 6 individuals per cage), and dead animals were excluded from analysis. Wild cockles were sampled at the same time to study the long-term effects of pollution on the biomarker expression and compare it to that of the non-native organisms.

The relationship between MT induction in the two transplanted species (mussel and clam) and metal pollution (Cu, Pb and Zn concentrations) was examined. The results show that MT concentrations in transplanted organisms were lower than expected in heavily polluted areas such as Holes Bay where the highest concentrations of all metals were detected in sediment (Figure 4.4). Omitting Holes Bay data leads to a stronger linear relationship with metals in the sediment (Table 4.2). Two week samples from Poole Harbour showed no significant correlation with observed metal concentrations in sediment samples, whilst five week mussels MT was significantly related ($P \leq 0.05$) to Pb, Zn and total metal concentrations (Table 4.2). No relation was found between clams MT and metals suggesting that MTs production in Manila clam may not be an effective biomarker for metal stress in Poole Harbour and/or need longer time to induce a stress response in these organisms.

In their recent field study of Manila clams within the lagoon of Venice, Moschino et al. (2012) highlighted the weak correlation between the environmental exposure and the internal dose of metals and organic contaminates and their scarce tendency to affect biological responses (e.g. MTs concentrations) at hierarchical levels in this species which was explained as result of the development of adaptive mechanisms. In experimental studies, MT induction in Manila clams has been shown to occur after two days at concentrations of 100 μg l_{-1} Cd and Cu (Bordin et al., 1997). In *M. edulis*, MT induction occurred after one day at concentrations of 15-80μg l^{-1} of Cd and Cu (Roesijadi et al., 1989). MT induction in *M. edulis* can also occur at lower metal concentrations and over a short time period. However, within bivalve species, MT concentrations can vary significantly within the biological matrix, shown conclusively in *M. edulis* (Amiard et al., 2006). Variations in temperature, salinity and turbidity can alter MT induction; these must be monitored during analysis of seasonal MT concentrations (Bodin et al., 2004). Biological parameters that can affect MT induction include; age, sex, physiology and health of an organism (Nicholson and Lam, 2005).

The results of this study show that MTs values in the soft tissue of wild cockles could be related to all metals (Table 4.2) including that of heavily polluted Holes Bay. However, MT concentrations were generally lower than that of transplanted organisms (Figures 4.3 and 4.4). This low MTs response in wild biota was explained earlier (chapter 3) on the basis of that, a population could have acquired tolerance as a result of detoxification mechanisms that include a variety of physicochemical forms of storage, while non-native samples were not adapted to contaminants present within the environment, providing an improved indication of levels of contamination (Amiard et al., 2006).

The results of this study also indicate that under extreme conditions of metal pollution, the relationship between metal concentrations and MT deviates from linearity: a threshold appears to be reached beyond which the physiological capacity of organisms introduced to polluted areas is exceeded .In both transplanted bivalves, MT induction increased to peak levels with increased metal concentration, and then declined with continued increases in the concentration (Figure 4.6). Evidence shows that MT analysis produces inconsistencies and contradictions at sites where metal concentrations are known to be high, but a low MT concentration is produced (Pellerin and Amiard, 2009). It has been shown that if metal concentrations are extremely high, bivalve MT induction is actually reduced, resulting in inaccuracies at high metal concentrations, probably as a result of a toxic effect preventing detoxification processes from being fully functional (Amiard et al., 2006).

Machreki-Ajmi et al. (2008) showed that at most contaminated site the insoluble fraction of the digestive gland of *C. glaucum* bound much more cadmium than the soluble fraction (containing MT). Moreover, they reported that when concentration of total Cd in the digestive gland rose above 1 µg/g wet weight, Cd in the insoluble fraction become predominant compared to soluble fraction. This means that MTs can bind only a limited fraction of metal and that the relationship between MTs and metal levels is relevant only up to a certain degree of metal contamination. Furthermore, compared to other sites, the significant increase in Cd concentration in the soluble fraction of the digestive gland of cockles from the most contaminated site was not accompanied by a significant increase in MT concentrations. This could be a result of MT saturation with the toxic metals which start to bind other proteins in the cell.

Previous study of MT produced by organisms (*M. edulis* and *L. littorea*) in Holes Bay showed that in the heavily contaminated areas (Holes Bay), Cd became associated with HMW (High Molecular Weight) ligands (e.g. enzymes) and LMW (Low Molecular Weight) fractions which include amino acids and any free (potentially toxic) metal; this was explained as a result of saturation of MT (Langston et al., 2003). The results of studies of the MT mRNA expression as a biomarker of metal pollution imply that dose-related responses of MT expression occur only with doses of metals that do not cause damaging effects to the physiological functioning of organisms (Wu and Hwang 2003) (Chang et al. 2007).

A field study of the freshwater bivalve *Corbicula fluminea* showed that after three weeks of transplantation in high polluted area, MT concentrations did not have any significant difference in comparison with reference organisms which was explained as the defence capacities of the bivalves were overwhelmed as result of the too rapid metal bioaccumulation causing very high concentrations in the soft body, leading to rapid and severe structural and functional damage (Baudrimont et al., 1999). When the binding capacity of MT has been exceeded, the metal leaks to other parts of the cell combining with other cell fractions. If organisms were exposed to high amount of metals, the basal levels of MT would not be sufficient for protection and de novo MT synthesis is required, however, this de novo synthesis of metallothionein may be slower than the rate of metals influx (Ladhar-Chaabouni et al., 2012).

Consequently, the suitability and applicability of MT content in tested bivalves' tissues as a biomarker of exposure to metal pollution is questionable, indicating important limitations in reflecting the metals exposure levels at highly polluted environments. Therefore, the use of MT contents as a monitoring tool for assessing the environmental metal contamination is inadequate."

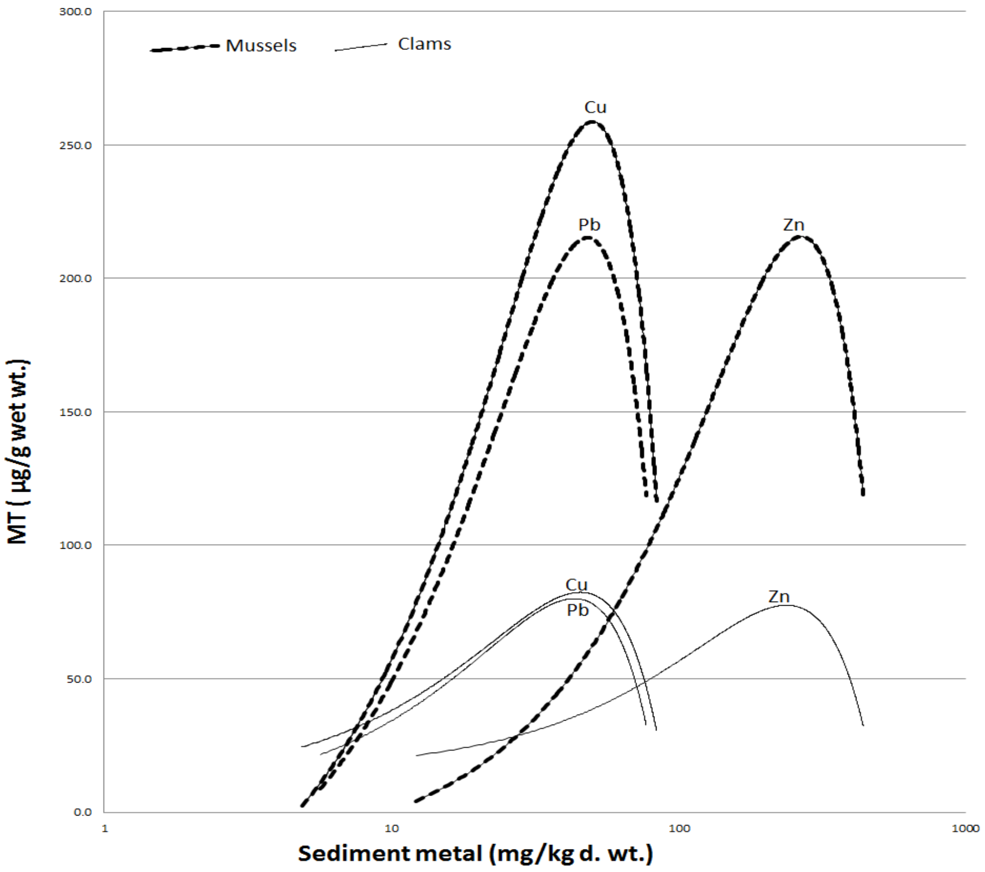

Figure 4-6: Metal -response curves for MT induction in transplanted mussels and clams. The values used to construct the curves were derived from measurements of MT of five week samples and metal concentrations of sediment from all sampling sites. The curves were fitted using polynomial curve fits implemented in Excel 14.0.6112.

As all the four sites (P1 to P4) are anthropologically impacted, a cluster analysis was performed to test how they could be grouped according to the impacts from metal sources and how they could be compared with the clean site (P5). The results showed that clustering relating to pollution impacts differs according to the data used to construct the clusters. According to the metal profile of sediments only, Holes Bay stood alone as it is a heavily polluted, semi-enclosed area affected by sewage discharges and historical sources of pollution. The second cluster, focussed on Lytchett Bay, is also a semi-enclosed area but it is mainly affected by sewage discharges only. Although P1 and P2 receive CSO discharges, they show similar sediment quality to a site that does not (P5), which indicates that they are not seriously impacted by these discharges (Figure 4.5A).

Clustering according to MT concentrations in five week transplanted mussel samples showed some differences depending on how these organisms are affected by metal pollution. Lytchett Bay forms a stand-alone group, with organisms that are highly impacted as they produce higher MTs concentrations. Holes Bay and Fisherman’s Haven showed some similarity with MT induction of moderate values. Sandbanks and Newton Bay showed similarly low MT induction (Figure 4.5B).

Both metals and MT data were used to characterise how the environment is impacted by metal pollution. But the clusters resulted were not verified by ANOVA and they were not statistically valid.

4.5. Conclusions

Active sampling has proven to be an effective method to assess the impacts of metals on molluscs. However, natural variability needs careful consideration when designing a sampling programme as this can lead to different MT induction. Induction of MT in transplanted organisms can provide an early indication of contamination at low concentrations of metal pollution; however, under heavily polluted conditions, this relationship deviates from linearity as MT induction increases to peak levels with increased metal concentration, and then declined with continued increases in the concentration. MT production in mussels was an effective biomarker when analysing the impacts of pollutants over a short period of time (five weeks), whilst in Manila clam it was not; this is probably because the metal concentrations may need a longer time to induce a stress response in these organisms. MT concentrations in wild cockles could be related to metal pollution; however, they were generally lower than that of transplanted organisms as native organisms show some tolerance to pollution- this may be due to habituation, or natural selection producing a pollution tolerant local population.

Chapter 5: Bioindicators of metal pollution in industrialised estuarine environments

5.1. Introduction

Most research on the potential of MT synthesis in biomonitoring has focussed upon mussels and other bivalves (Amiard et al., 2006), with less attention on the development of suitable benthic bioindicators to represent bioavailable metals in sediments (Durou et al., 2007). It is well-known that the more widespread the distribution of a single biomonitoring species, the greater its value as a cosmopolitan biomonitor providing cross-reference across large geographical areas (Rainbow, 1995). However, different species show a wide range of responses to certain type of pollution, and indeed, they are tolerant of a variable amount of pollutants, consequently, it becomes more difficult to elect a species to be a bioindicator of certain type of pollution. Therefore, this chapter aimed to assess the suitability of three species to be bioindicators of metal pollution in heavily and moderate polluted industrialised estuarine environments (the Fal estuary and the Solent, UK). The study investigated the potential of MTs as biomarkers of metal pollution in *Hediste diversicolor*, a benthic worm in an area with a long history of metal pollution, and *Patella vulgata* as novel bioindicator in the Solent estuarine environments, and compare them to a well-studied bioindicator, *Mytilus edulis* , which was available in the both sites.

5.2. Materials and methods

5.2.1. Sampling Sites

The Fal Estuary is situated in Cornwall, south-west England, at the western entrance of the English Channel; see Figure 5.1. The estuary is a ria (drowned valley) system fed by numerous rivers and the five main creeks (Mylor, Restronguet, Pill, St Just and Percuil), all of which flow into Carrick Roads (Pirrie et al., 2003). Metal mining has taken place in Cornwall since the Bronze Age, with tin and copper mined extensively since the 1200s (Pirrie et al., 2003). Mine wastes were frequently discharged into rivers such as the Carnon (which drains a formerly very productive tin mining area into Restronguet Creek), which then flow into the estuary (Clark, 2001). These wastes contained large quantities of metals which were then deposited in the sediments; this deposition and sedimentation has resulted in the severe contamination of the

Carnon River and Restronguet Creek (e.g. Cu and Zn concentrations reached to 1,300 and 23,000 µg/l respectively) (Neal et al., 2005). Past mining has caused the intertidal sediments of the Fal to contain the UK's highest concentrations of metals (Warwick, 2001).The Wheal Jane incident was a very visual mine water pollution event which affected the Fal Estuary in January 1992 and resulted in the establishment of an ongoing water treatment system (Banks et al., 1997; Younger, 2002; Neal et al., 2005). Contaminated sediments continue to be transported from Restronguet Creek into Carrick Roads and into the other creeks, by the dominant tidal flows (Pirrie et al., 2003). This deposition and transportation has created a marked pollution gradient between the five main creeks of the Fal Estuary, causing otherwise similar creeks to experience concentrations which differ by orders of magnitude; as demonstrated in Table 5.1 (Warwick, 2001).

The Solent estuarine environments (Figure 5.1), which encompass four coastal plain estuaries and four bar-built estuaries, are situated in the southern region of England. The estuary is fed by three major rivers: Itchen, Test and Hamble. The environmental quality in the Solent estuaries has deteriorated over time through human activities including power plant combustion, traffic, manufacturing, oil refinery and sewage discharge (Romeril, 1979; Armannsson et al., 1985; Croudace and Cundy, 1995; Cundy and Croudace, 1995a; Cundy et al., 2003). In order to improve the Integrated Coastal Zone Management in the Solent sub-region of the South East, the Solent Forum, which includes the Solent estuarine environments, was established in 1992 (Fletcher et al., 2007). There are several harbours in this area which allow the transportation of raw materials such as coal, oil, gas and other chemicals and act as source of pollution. The Esso refinery at Fawley has its own mile-long marine terminal handles 2000 ship movements and 22 million tons of crude oil annually. There is also a BP Oil Terminal at Hamble, linked to Fawley by pipeline. During the past decades, there has been an increase of industrial activity associated with metal pollution in the coastal areas in the Solent estuaries. The refinery at Fawley started in 1921 and it was expanded by Esso in 1949-1951. In the first decade of the twenty-first century, it handled over 20 Mt of crude oil per year and was the biggest in UK (Croudace and Cundy, 1995). The waste from the oil refinery contained a large amount of metals which deposited and accumulated in the estuaries. Consequently, elevated amounts of copper and lead were found in Hamble estuary (Cundy and Croudace, 1995b). Furthermore, the intensive development for recreational purposes and capital dredging works worsened the problems (Cundy et al., 2003). As a result of more than a century of intense harbour and industrial activities, the presence of contaminated tributaries containing sewage effluent and other diffuse sources or line sources (i.e. roads) of pollution, this estuarine area is contaminated with metals. Worse still, research on faunal biodiversity has indicated that this area is characterized by a low species richness, low diversity and domination by a few species (number of species in some areas was 4-12 /0.2 m2) (Houston et al., 1983).

5.2.2. Collection of samples

Sampling locations in Fal Estuary were decided after consultation with Truro Harbour Commissioners and Falmouth Marine School; site locations are shown in Figure 5.1, and sites descriptions in Table 5.1. Land-based sampling was conducted around the estuary on 16th July 2010. To obtain a comprehensive coverage of the estuary, 11 sites were identified and seven were sampled. *H. diversicolor* were sampled from seven sites while mussels were only available in two sites (Table 5.1). Spot sediment samples (no replicates) were collected from all the seven sites. Organisms and sediment stored in a cool bag for transport before being frozen prior to analysis.

Based on the likely influence of anthropogenic activities, 8 sampling locations were identified in the Solent Estuary (Figure 5.1). These sites were selected to give wide coverage of the estuary in order to ensure that some sites were situated near industrial areas, whilst others were in areas far from anthropogenic sources of metal pollution (Table 5.1). Sampling was conducted on 10th, 22nd and 24th June, 2011. *M. edulis*, *P. vulgata* and sediments samples were collected (Table 5.1). Once collected, they were stored on ice in cool bags and shipped to the laboratory within 8 h. After that, they were frozen at −20°C in order to prevent degradation before analysis.

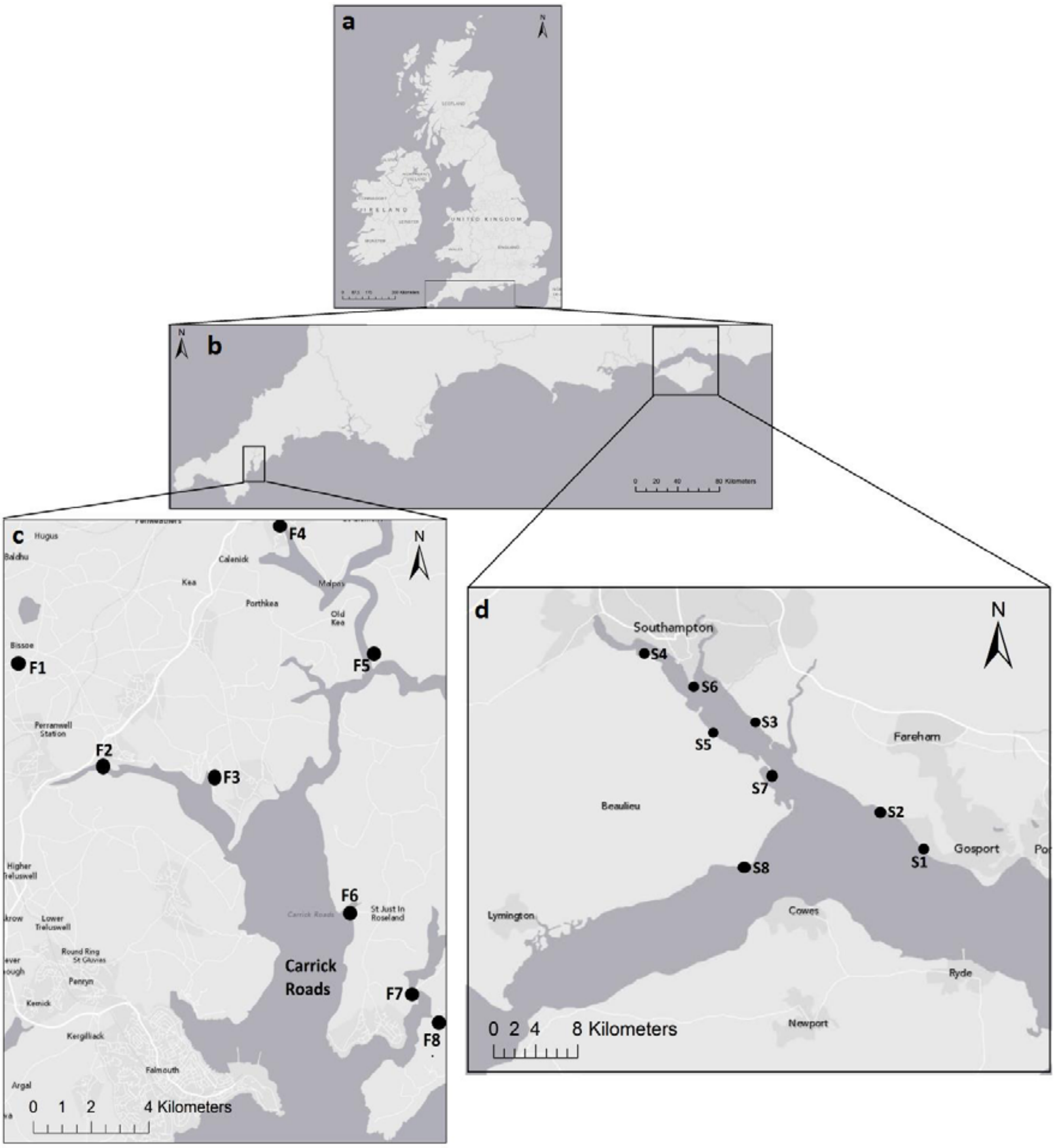

Figure 5-1: Maps showing the location of the (c) Fal and (d) Solent Estuaries in (b) south-west coast of England, (a) UK; F1, Bissoe; F2, Devoran; F3, Penpol Creek; F4, Malpas; F5, Channel; F6, St Just Creek; F7, Percuil; F8, Froe; S1, Lee-On-the-Solent; S2, Hill Head; S3, Hamble Oil Terminal; S4, Cracknore Hard; S5, Hythe Marina Village; S6, National Oceanographic Centre (NOC); S7, Fawley Power Station; S8, Lepe. (ArcGIS 10 maps)

5.2.3. Determination of Sediment Metal Content

Frozen sediment samples in plastic containers were placed in a freeze-dryer (VirTis benchtop K) until completely dry (around 72 hours). Upon removal, each sample was ground to a fine powder with a pestle and mortar. Approximately 1 g (±0.001 g) of each sample was transferred into individual boiling tubes. The samples were then prepared for analysis using the United Kingdom Environment Agency's acid digestion method (Standing Committee of Analysts, 2006). Standards of known concentrations were prepared using the metals Cu, Pb and Zn; they are known to cause metal contamination and values can be compared with previous studies. Metal absorbance in standards and samples was measured using a flame atomic absorption spectrophotometer (Varian SpectraAA-200).

5.2.4. Determination of Metallothioneins Concentration

Organism samples were thawed and prepared as follows: *H. diversicolor* - separated from their sediment and homogenised appropriate amounts of soft tissue; mussels and limpet -the soft tissues from at least 10 individuals were homogenized separately after being separated from sediments and rapid dissection. To minimise errors in quantification of MT determination, three subsamples of approximately 1 g were taken for each sample.

Samples were then prepared and analysed according to the spectrophotometric method developed and described by (Viarengo et al., 1997; 1999) and applied for the United Nations Environmental Programme's MED POL biomonitoring programme (UNEP/RAMOGE, 1999). Standards of known concentrations were prepared using reduced glutathione (GSH). Absorbance was measured at 412nm by a UV-Visible spectrophotometer (Cecil 3000 scanning spectrophotometer) (Viarengo et al., 1997; 1999) (see 3.2.2 for detailed procedure).

Table 5-1: Description and samples collected from sites within the Fal and Solent Estuaries, UK.

Area	**Site name**	**site code**	**Latitude & longitude**	**Description**	**Samples collected**
The Fal Estuary	Bissoe	F1	N 50°13'47.1" W 05°07'31.2"	Small, muddy, freshwater stream; next to a current concrete manufacturer, a disused arsenic works and upstream of the old Wheal Jane mine discharge channel.	Sediment
	Devoran	F2	N 50°12'33.5" W 05°05'15.6"	Mudflats; in the upper northern section of Restronguet Creek.	Sediment *& H. diversicolor*
	Penpol Creek	F3	N 50°12'19.9" W 05°04'08.2"	Mudflats; in the central northern section of Restronguet Creek.	Sediment *&H. diversicolor*
	Malpas	F4	N 50°15'23.4" W 05°02'34.1"	Mudflats; in the north of the estuary near Truro, out of the influence of the main contaminated sediment movement.	Sediment *& H. diversicolor*
	Channel	F5	N 50°13'58.3" W 05°01'01.8"	Pontoon; in Truro River, close to the commercial mussel beds.	*M. edulis*
	St Just Creek	F6	N 50°10'52.9" W 05°01'05.2"	Shoreline with sand, shingle and coarse sediments; in St Just Creek.	Sediment
	Percuil	F7	N 50°10'00.9" W 04°59'58.9"	Rocky shoreline with some fine sediment; in Percuil Creek.	Sediment, *M.edulis & H. diversicolor*
	Froe	F8	N 50°09'45.9" W 04°59'10.6"	Mudflats in Percuil Creek.	Sediment *&H. diversicolor*
Solent Estuary	Lee-On-the-Solent	S1	N 50°48.109′ W 01°12.261′	Shoreline with gravels and coarse sediment, quite far from sources of pollution.	Sediment, *M.edulis & P. vulgata*
	Hill Head	S2	N 50°48.803′ W 01°13.525′	Mudflats containing fine sand and coarse sediments, near a sewage pipe.	Sediment, *M.edulis & P. vulgata*
	Hamble Oil Terminal	S3	N 50°51.137′ W 01°19.367′	Mudflats containing coarse sediments, in the vicinity of an important oil jetty.	Sediment, *M.edulis & P. vulgata*
	Cracknore Hard	S4	N 50°53.780′ W 01°25.542′	Mudflats partially covered with rock, brick and concrete, near an important industrial complex, including a gas power station with a coolant outfall, a large waste incinerator and various other heavy industrial activities.	Sediment *& M.edulis*
	Hythe Marina Village	S5	N 50°52.497′ W 01°23.860′	Shingle beaches with coarse sand and sediment, in the vicinity of a marina and local sources of urban pollution.	Sediment, *M.edulis & P. vulgata*
	National Oceanographic Centre (NOC)	S6	N 50°52.496′ W 01°23.859′	Sea walls near the dock head.	*M.edulis & P. vulgata*
	Fawley Power Station	S7	N 50°48.973′ W 01°19.466′	Mudflats near the intake point cooling water.	Sediment, *M.edulis & P. vulgata*
	Lepe	S8	N 50°47.056′ W 01°21.640′	Shingle beaches with fine sand and sediment	Sediment, *M.edulis & P. vulgata*

5.3. Results

5.3.1. Sediment metal concentrations

The concentrations (mg/kg dry weight) of the three metals investigated (Cu, Pb and Zn) were determined in sediment samples from the Fal and Solent Estuaries and results are presented in Figure 5.2.

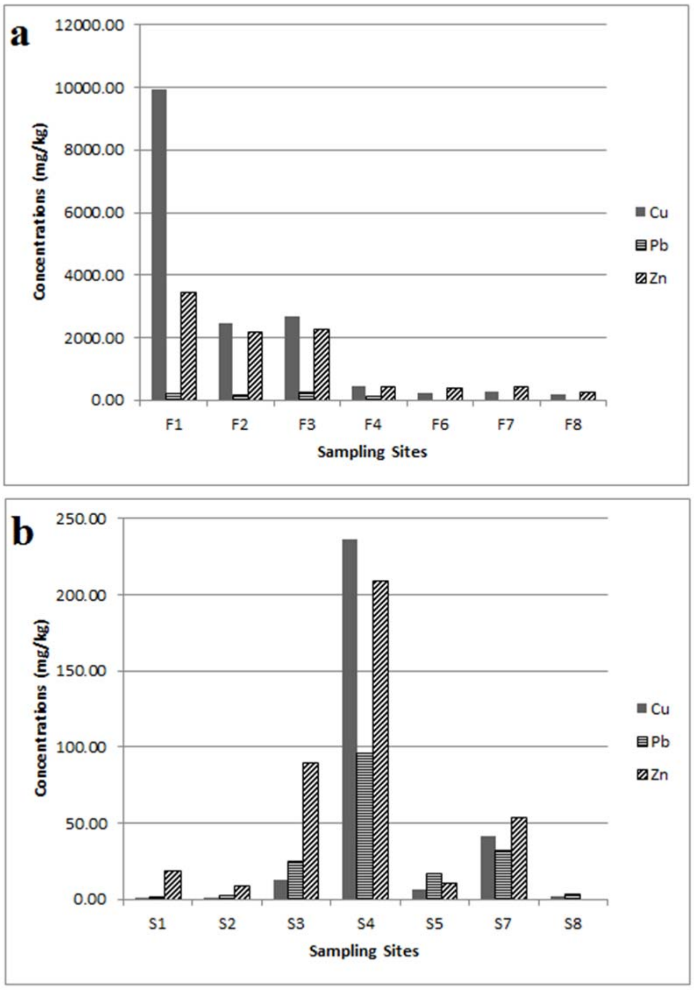

Figure 5-2: Concentrations of copper, lead and zinc in sediment samples from sites in the Fal (a) and Solent (b) Estuaries during July 2010 and June 2011. F1, Bissoe; F2, Devoran; F3, Penpol Creek; F4, Malpas; F6, St Just Creek; F7, Percuil; F8, Froe; S1, Lee-On-the-Solent; S2, Hill Head; S3, Hamble Oil Terminal; S4, Cracknore Hard; S5, Hythe Marina Village; S7, Fawley Power Station; S8, Lepe. (N.B. different Y axis scale is used in each graph.)

The concentrations showed variation at different sites in both locations (Figure 5.2). These data indicate that at the Fal Estuary, Bissoe (F1) had the highest concentrations of Cu and Zn whilst Penpol Creek (F3) had the highest concentration of Pb. (P1). The lowest concentrations of Cu and Zn were recorded in Froe (F8) while the concentration of Pb was found in St Just Creek (F6). The total metal concentration range was 507 in Froe (F8) to 13654 mg/kg in Bissoe (F1), presenting a very wide variation in concentrations between sites. All metal concentrations at all sites in the Fal Estuary exceeded the Threshold Effect Level (TEL) (18.7 mg/kg for Cu, 30.2 mg/kg for Pb and 124 mg/kg for Zn) proposed by the Canadian Sediment Quality Guidelines (CSQG) which is the standard used by the Environment Agency in the UK (Hubner et al., 2009). All metals exceeded the Probable Effect Level (PEL) (108 mg/kg for Cu, 112 mg/kg for Pb and 271 mg/kg for Zn) proposed by the CSQC at all sites except Pb at sites F6, F7 and F8; and Zn at site F8. This indicates that the sediment of this area is heavily polluted with these metals.

In the Solent, sediments from sites S1, S2 and S8, which are situated far (more than 10 km) from sources of pollution especially Cu and Pb from the chemical industrial complex and oil refinery at Fawley (Romeril, 1979; Armannsson et al., 1985; Croudace and Cundy, 1995; Cundy and Croudace, 1995a; Statham, 2000; Cundy et al., 2003), had the lowest concentrations of metals. For all metals, the concentrations varied from 5.68 at Lepe (S8) to 542.56 mg/kg dry weight at Cracknore Hard (S4), which is located near an important industrial complex, including a gas power station with a coolant outfall, a large waste incinerator. Cu concentrations exceeded the TEL at one site only (Fawley PowerStation (S7)) and exceeded the PEL at one site (Cracknore Hard (S4)). Pb exceeded the TEL at the same sites but did not exceed the PEL at any. Zn concentrations exceeded the TEL at two sites; Hamble Oil Terminal (S3) and Fawley PowerStation (S7) and exceeded the PEL at Cracknore Hard (S4). This indicates that the sediment of Cracknore Hard (S4) is heavily polluted with these metals whilst Hamble Oil Terminal (S3) and Fawley PowerStation (S7) show a lesser degree of pollution. Power stations can pollute the aquatic environment with metals through discharge of cooling system effluent includes primarily heated water, biocides used to prevent slime build-up and fouling in the condensers, and metals leached from the condensers(Majewski and Miller, 1979).

5.3.2. Metallothioneins

The MT concentrations (µg/g wet weight) determined for all organisms and the overall mean and standard deviation for each site are presented in Figure 5.3.

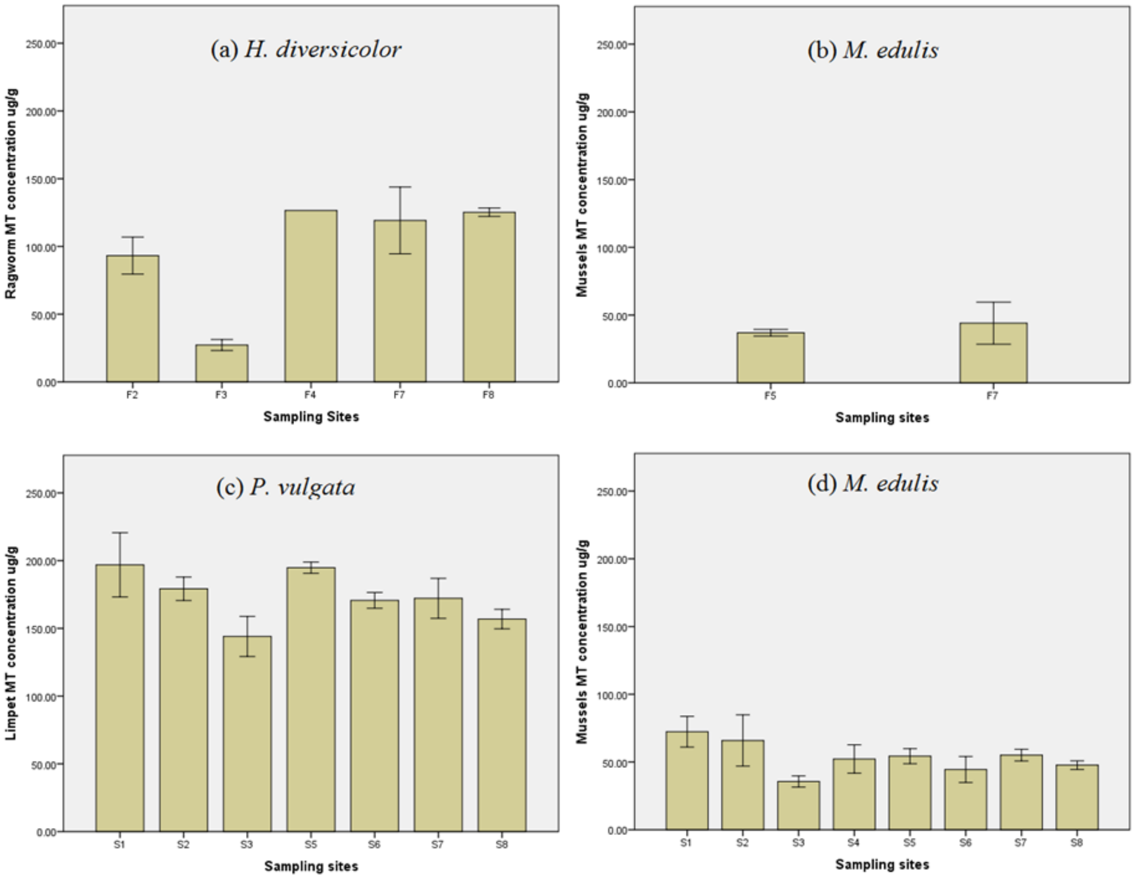

Figure 5-3: MTs concentrations (mean ± SE of three subsamples) in (a) *H. diversicolor* and (b) *M. edulis* collected from the Fal Estuary during July 2010, and (c) *P. vulgata* and (d) *M. edulis* collected from Solent Estuary during June 2011 (N.B. sample of *H. diversicolor* from site F4 was sufficient for one measure only).

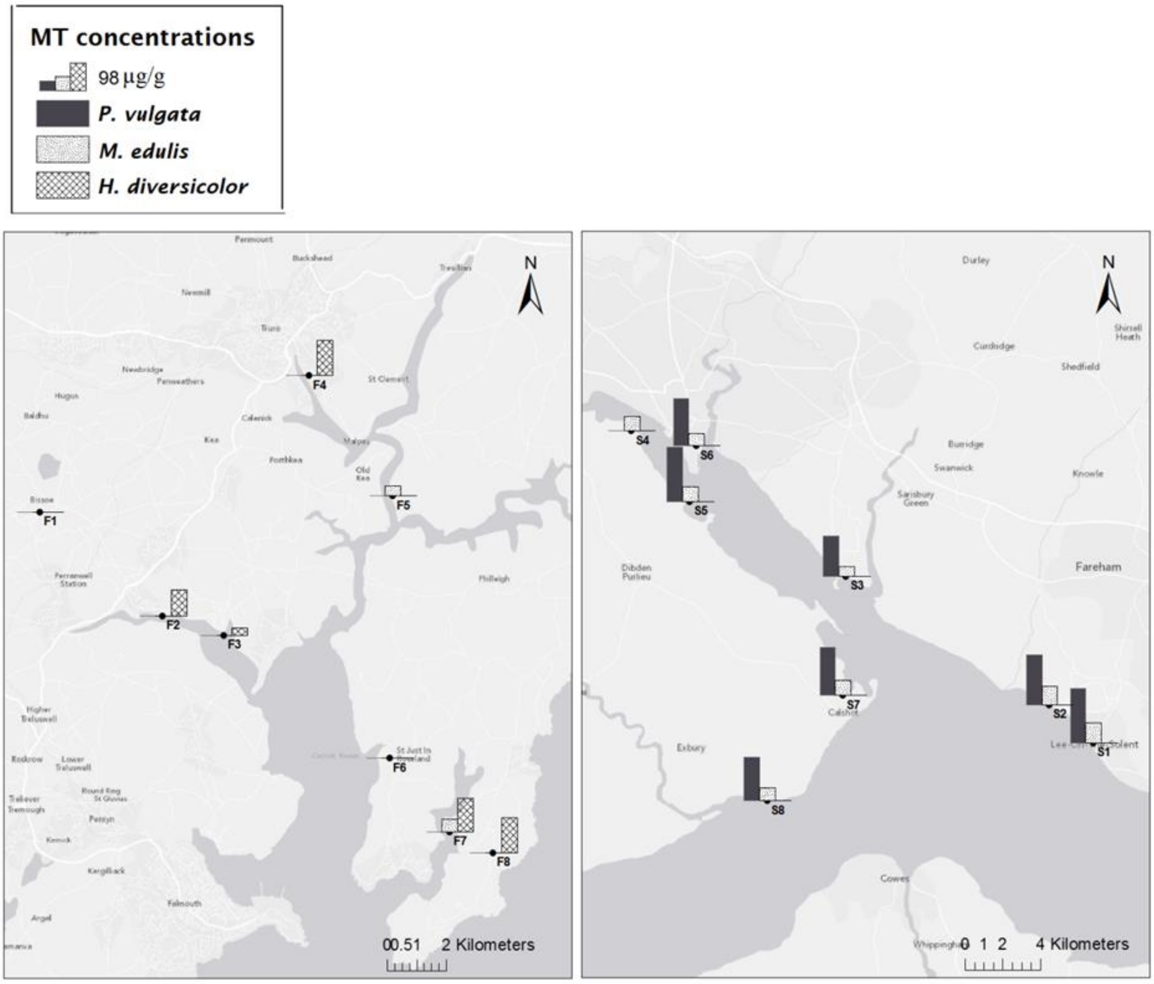

Figure 5-4: Maps showing MTs concentrations in *H. diversicolor* and *M.edulis* collected from the Fal Estuary (left) during July 2010, and *P. vulgata* and *M. edulis* collected from Solent Estuary (right) during June 2011.

In the Fal Estuary, differences in MT concentration in *H. diversicolor* were observed with concentrations ranging between 27.21µg/g at site F3 to 126.53 µg/g at site F4. *M. edulis* samples were only collected at two sites F5 (36.98 µg/g) and F7 (44.07µg/g). In the Solent, MT concentrations in mussels (*M. edulis*) varied from 35.62 to 72.37 µg/g wet weight (Figure 5.4). As to limpets (*P. vulgata*), the figure ranged from 144.04 to 196.91 µg/g wet weight. The highest concentrations of MT were found in Site 1 for both mussels and limpets. Similarly, the lowest values were in Site 3 for the two organisms.

5.3.3. Relationship between MT and heavy metal concentrations in sediments

Correlation coefficients to show the relation between MT in *H. diversicolor* in the Fal Estuary and *M. edulis*, and *P. vulgata* in the Solent Estuary, and metals in sediment were calculated (Table 5.2) after applying the Kolmogorov-Simrnov test of normality.

5.4. Discussion

5.4.1. Metal pollution of Sediments

The results of this study have confirmed the presence of marked metal pollution gradient in the Fal Estuary between the ‘clean’ sites (sites F4, and F6 - F8) in the north and east of the estuary and the ‘polluted’ sites (sites F1 - F3) in the west of the estuary. The data collected in this study is comparable to that of other studies as comparisons were made with data provided by the EA (Environment Agency, unpublished, data from 2005-2006); see Table 5.3. The comparisons showed that for Cu and Zn (and to a lesser extent, Pb) the concentrations measured were consistent with the six-year-old EA data which is still representative of the pollution gradient because the gradient has not altered for decades, with trends observed in the 1970s still seen today. . For this study, it was important to measure sediment metal concentrations as *H. diversicolor* is a benthic invertebrate which bioaccumulates metals from sediment.

Table 5-2: Pearson correlations between MT in *H. diversicolor* collected from (a) the Fal Estuary, and *P. vulgata* and *M. edulis* collected from (b) Solent Estuary and metals in sediments (significance shown in brackets).

a) The Fal Estuary	Cu	Pb	Zn	Total metals
Pb	0.666 (0.102)			
Zn	0.903** (0.005)	0.879** (0.009)		
Total metals	0.992** (0.000)	0.746 (0.054)	0.951** (0.001)	
H. diversicolor MT	-0.853 (0.066)	-0.909* (0.032)	-0.849 (0.069)	-0.857 (0.064)

b) Solent Estuary	Cu	Pb	Zn	Total metals	*P. vulgata* MT
Pb	0.973** (0.000)				
Zn	0.933** (0.002)	0.965** (0.000)			
Total metals	0.985** (0.000)	0.990** (0.000)	0.980** (0.000)		
P. vulgata MT	-0.208 (0.693)	-0.350 (0.496)	-0.579 (0.2290)	-0.487 (0.328)	
M. edulis MT	-0.131 (0.78)	-0.285 (0.536)	-0.336 (0.462)	-0.24 (0.605)	0.817* (0.025)

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 5-3: Metal concentrations (mg/kg d.w) in sediment samples collected in this study (sites F3, F4 and F6 - F8) and by the Environment Agency (EA) (sites EA3, EA4 and EA6 - EA8) from similar locations across the Fal Estuary, UK (Environment Agency, unpublished, data from 2005-2006).

		Sediment metal concentrations (mg/kg)					
		Cu		Pb		Zn	
Site name	site number	Sample	EA	sample	EA	sample	EA
Bissoe	F1	9955.14		236.47		3462.40	
Devoran	F2	2478.58		193.00		2173.99	
Penpol Creek	F3/EA3	2668.97	2210	282.92	208	2285.86	2230
Malpas	F4/EA4	456.50	343	127.32	148	438.18	548
Channel	EA5		263		89.60		429
St Just Creek	F6/EA6	223.68	354	33.44	130	394.96	602
Percuil	F7/EA7	291.05	167	56.35	69.70	441.24	275
Froe	F8/EA7	195.45	67.30	50.67	39.10	261.65	166

In the Solent, metal concentrations in sediments from different sites were also measured (Figure 5.2). The highest concentrations of Pb, Zn and Cu were recorded at Site 4. As expected, the lowest values were recorded at Sites 1, 2 and 8, which were designated as clean sites according to the results of previous studies and data from the EA (Environment Agency, unpublished data from 2008-2011). These results were similar to previous studies and data from the EA (Environment Agency, unpublished, data from 2008-2011) except that the metal concentrations at Sites 1, 2 and 5 decreased over time (Table 5.4). By comparing the concentrations of metals in our studies with historical data, it is possible to suggest that the concentrations of metals in most all of the contaminated sites (3, 4, and 7) have not changed significantly over the past few decades.
The correlation coefficients between Pb, Zn and Cu were statistically significant (Table 5.2). In general, the distribution of metals in sediments is related to the sources of metals and sediment composition. If the ratios of metals from different sources are similar, metals with similar distribution patterns may be highly related to each other. For Pb, Zn and Cu, the major chemical forms in sediments are associated with residual fractions and Fe-Mn oxide (2001; Li et al., 2007), which may be a reason for the strong indication of association (the high correlation coefficients).

Metals accumulated in sediments exist in five geochemical forms: exchangeable; carbonate-bound; Fe-Mn oxides bound; organic matter bound and residual metal phase, which can influence their mobility, biological availability and chemical behaviours (Li et al., 2007). Metals in the residual fraction are the least dangerous with their low bioavailability. Before triggering MT induction and other biological response, metals should be in the bio-available form and absorbed by organisms. Metals’ assimilation efficiency (related to bioavailability) but not the absolute concentration is found to be positively correlated to the metallothionein-like proteins (MTLPs) fraction (Cheung and Wang, 2005).

Table 5-4: Comparison of metal concentrations in sediments collected in this study, by Environment Agency, and related literature.

No	Site names	Metal concentrations/ ug/g dry weight						Sources
		Cu	**Cr**	**Ni**	**Zn**	**Cd**	**Pb**	
S1	LEE-ON-SOLENT	0.4	5.07	14.37	18.56	0	1.11	Present study
		10	-	17	101	0.09	-	(Armannsson et al., 1985)
S2	HILL HEAD	1.32	9.88	14.38	8.92	0	2.15	Present study
		3.8	-	0.6	-	8.2	57.1	(Armannsson et al., 1985)
S3	HAMBLE OIL TERMINAL	12.64	10.36	70.58	89.69	0.02	29.35	Present study
		24.5	-	-	71.4	-	-	(Romeril, 1979)
		9.1	-	61.9	-	6.5	37.4	(Al-Rasheid and Sleigh, 1995)
		41	-	16	71	0.16	-	(Armannsson et al., 1985)
S4	CRACKNORE HARD	236.64	21.84	37.61	209.54	0.07	136.96	Present study
		51	42	48	143	8	81	(Sharifi et al., 1991)
		48.3	-	-	152	-	-	(Romeril, 1979)
S5	HYTHE MARINA VILLAGE	6.53	5.96	22.26	10.76	0.58	11.92	Present study
		122	48	58	396	9	128	(Sharifi et al., 1991)
		16.2	-	49.4	-	29	29.1	(Al-Rasheid and Sleigh, 1995)
S6	NOC	113.4	101.48	39.88	125.2	0.23	42.3	EA Data 2008
S7	FAWLEY POWERSTATION	41.15	19.4	33.2	54.27	0	33.56	Present study
		16.9	29.07	17.45	50	0.24	19.13	EA Data 2008
		-	-	-	-	0.47	-	EA Data 2009
		-	-	-	-	0.89	-	EA Data 2010
S8	LEPE	2.08	6.43	19.13	0	0	4.51	Present study

5.4.2. The studied species as bioindicators of metal pollution in industrialised estuarine environment

The application of MT induction in bioindicators is complicated as the effects of environmental and physiological factors need to be understood and accounted for in each bioindicator to reflect the true concentration of induced MT and avoid bias in biomonitoring data (Viarengo et al., 1999). Consequently, in contrast to the general hypothesis that metals induce MT synthesis, many recent researches reported that MTs induction is not always correlated with metal pollution (Ladhar-Chaabouni et al., 2012; Moschino et al., 2012; Paul-Pont et al., 2012; Ramšak et al., 2012; Strogyloudi et al., 2012), or even that a negative relation between MT and metals concentrations is found (Bebianno et al., 2003). Therefore, in this study, MTs induction in relation to metal pollution was investigated in three different species: *M. edulis*, a filter feeder mussel; *H. diversicolor*, a benthic *polychete* and *P. vulgate*, a herbivorous gastropod that lives on rock surfaces. This study questioned the suitability of these species as bioindicators of metal pollution in industrialised estuarine environments in the light of contrary results of recent studies.

M. edulis

Among estuarine organisms, mussels are common bioindicator species in ecotoxicological studies. Due to its worldwide distribution it has been used in many monitoring programs, such as the Mussel Watch Program (Goldberg, 1975). As sedentary filter feeders, mussels have the potential to bioaccumulate and bioconcentrate pollutants from the aquatic environment (Acker et al., 2005). Mussels have also been shown in several studies to express these various biomarkers in response to stressful conditions including exposure to metals (Viarengo et al., 1999).

In the Fal Estuary, *M. edulis* was only present at two sites (F5 and F7) and mainly absent at sites close to historical mining activities (sites F1, F2 and F 3), so it was difficult to thoroughly assess their potential in this study. Although results suggest that there is more MT induction at site F7 than at site F5; see Figure 5.3b., without further sampling it is impossible to verify the correlation with the sediment pollution gradient. The absence of this species at sites with high concentration of metals indicates that metal pollution at these sites exceeds its tolerance threshold and therefore it is not a suitable bioindicator at this level of pollution.

On the other hand, in the Solent Estuary, our results showed that MT concentration in mussels was highest at the clean Site S1 whilst the lowest values were reported at Site S3, a moderately polluted site (Figure 5.3). Besides metal concentrations, other factors (e.g. size, age, food abundance, seasonal changes, reproductive status, temperature, salinity bioavailability of metals) have been shown to have additional influence on MT concentrations in mussels (Schmidt et al., 2012). Based on the systematic investigation in mussels, MT concentrations are suggested to be varying between different organs. Because MT is induced by metals, tissues which have the highest ability to accumulate metals generally have the highest MT concentrations (Arockia Vasanthi et al., 2012). For mussels, MT concentrations in the digestive gland are higher than those in the gills (Amiard et al., 2006). As a result, MT in specific organ may change significantly while those in other organs remain stable. In this study, analysing MT in whole tissues may neglect tissue variation. Furthermore, different metals may have different effects on MT induction e.g. Cu and Zn appeared to be the most potent MT inducers whereas Ni and Ag had a much less potent effect (Amiard et al., 2006).

H. diversicolor

H. diversicolor is a benthic species well adapted to stressful and polluted environments and has potential to be a good bioindicator (Sole et al., 2009). *H. diversicolor* has already been established as a good bioindicator of sediment bioavailable metals through measurement of bioaccumulated concentrations, but the potential of using MT synthesis in them has only recently been recognised (Mouneyrac et al., 2003; Durou et al., 2007). *H. diversicolor* is a keystone estuarine species due to its role in bioturbation and through being a major constituent of mudflat biomass and an important food source for crustaceans, fish and birds (Berthet et al., 2003). Some populations of *H. diversicolor* have adapted to metal pollution by becoming metal-tolerant (Berthet et al., 2003).

Statistical analysis showed that in *H. diversicolor*, the relationship between metal pollution and MTs is very strongly negative, so as the metal concentration increases, MT concentration decreases. These negative relationships are unexpected as it was assumed that as metal concentrations increased so would MT. It is clear from Figure 5.3 that MT concentration varies between sites. However, the 'cleaner' (e.g. site F8 in Percuil Creek) samples contained more MT in H. diversicolor than the more 'polluted' (e.g. site F3 in Restronguet Creek) ones. Restronguet Creek's *H. diversicolor* population is known to be metal-tolerant, but this may have been achieved through mechanisms other than MT synthesis; for example, through reduced metal uptake and increased excretion, or incorporation in insoluble deposits (Berthet et al., 2003). However, it has been observed that MTs and/or metallothionein-like proteins (MTLPs) represent a relatively important store of metals and usually contribute to metal detoxification in some way (Berthet et al., 2003). Many studies have observed the presence of MTs or MTLPs in *H. diversicolor*, but none have yet confirmed that they are induced by metals (Durou et al., 2007; Sole et al., 2009). This means that the presence alone of MTs and MTLPs does not necessarily imply that their synthesis was metal-induced. It has also been observed that the concentration of MT is not necessarily entirely representative of its involvement in metal detoxification. This is due to the high turnover rate of MT in invertebrates, through which many MT-metal complexes are broken down and the metals stored in insoluble deposits (Mouneyrac et al., 2003). A study by Mouneyrac et al (2003) agrees with the findings of this study by measuring significantly higher CHSTC (cytosolic, heat-stable, thiol-rich, compounds), including MT, concentrations in *H. diversicolor* samples in a clean site (Blackwater Estuary, Essex) than a polluted site (Restronguet Creek). Another reason for the lower MT concentrations observed in Restronguet samples is the increased metal-tolerance of *H. diversicolor* there relative to other creeks. It was observed that, under laboratory conditions, upon exposure to high Cu concentrations, *H. diversicolor* secrete mucus. This physiological defence response is much more pronounced in metal-tolerant individuals, and it is thought that this secretion may bind to dissolved metals, reducing their bioavailability (Mouneyrac et al., 2003).

P. vulgata

P. vulgata is a herbivorous gastropod that lives on rock surfaces and is widespread in many estuarine regions. They can accumulate metals which makes them susceptible to environmental pollution (Bini et al., 2008). Although an old study of *P. vulgata* from a polluted environment (Bristol Channel, UK) revealed induction of MT as response to elevated Cd concentrations (Noellambot et al., 1980), MT in this species has not been further investigated as biomarker of metal pollution.

MT concentrations in limpet from the Solent estuary were the highest at the clean Site S1 whilst the lowest values were at Site S3. Furthermore, a significant positive relationship between metal concentrations and MT was not detected (Table 5.2). Although the amino acid sequence of MT in *P. vulgata* has shown some similarity with class I MT of mussels and oysters (Noellambot et al., 1980), there is little information about MT concentrations relation with metal pollution in this species. The results of study of another limpet (*P. aspera*) reported that Cu and Cd, but not Zn, showed a relation with MT concentrations (Bebianno et al., 2003) .However, the same study stated that MTs concentrations were affected by body size and weight.

In order to minimize the potentially toxic effects of metals, gastropods have developed many other pathways besides MT induction. For example, when challenged with high concentrations of metals, gastropods can react by sequestering the harmful element in pyrophosphate-based granules and thus reduce the bioavailability and detoxify the metals (Leung et al., 2002). In addition, chronic exposure to Zn and Pb have been shown not to relate to MT induction and cellular ligands such as phosphates are responsible for sequestration of metal ions and detoxification in these organisms (Zaldibar et al., 2008). Adaptive phenomenon at the cellular level in metal-stressed aimed at increasing their cell turnover has also been shown to be a detoxification mechanism (Zaldibar et al., 2007). All these indicate that metal concentration is not the only factor that influences MT induction and MT production is not the only pathway of metal detoxification; therefore, *P. vulgata* is not a good candidate to be a bioindicator of metal pollution in studied estuarine environment.

5.5. Conclusion

The main outcomes of the present study highlight the unsuitability of *M. edulis, H. diversicolor*, and *P. vulgata* as bioindicators of metal pollution in both heavily and moderately polluted industrialised estuarine environments. The study showed that the environmental metal exposure in these areas has limited tendency to be related to MTs induction in these species. In these species, the influence of biotic and abiotic factors (e.g. size, age, food abundance, seasonal changes, reproductive status, temperature, salinity) governs the induction of MTs rather than metal pollution (Legras et al., 2000; Bodin et al., 2004; Amiard et al., 2006; Schmidt et al., 2012). Moreover, the detoxification processes and the existence of metal-tolerant populations at polluted sites limit MT application as a biomarker in these species under such circumstances (Mason and Jenkins, 1995; Mouneyrac et al., 2003; Demuynck et al., 2004; Ng et al., 2008). The effects of local environmental and physiological factors which vary from area to another makes it hard to suggest a cosmopolitan bioindicator species that can effectively represent different types of environments and hence can be used as cross-reference across large geographical areas.

Chapter 6: General Discussion

The primary aim of this study was to investigate and compare the ability of the selected invertebrates' biomarker (metallothionein) responses to assess the general environmental health of an estuary. This study introduced some new species (sponges, limpets and ragworms) as bioindicators and investigated whether their metallothioneins could act as a biomarker for pollution in the area of study (estuaries in the south coast of England). It also compared these species and bivalves in order to investigate whether there was any relationship between metal pollution and metallothionein concentrations. This study tried to answer a specific question: are MTs good biomarkers of metal exposure in monitoring programmes in the study area?

Estuaries are regions of high productivity, important to the life cycles of many birds, fish and invertebrates, including commercially important coastal species (Geffard et al., 2005a), and are subject to a growing list of environmental regulations. Estuaries worldwide receive much of the waste discharged by mankind into aquatic environments as result of the industrialised, agricultural, and urbanized nature of these estuaries and their catchments, (Cundy et al., 2003). Within the sea, almost all pollution is concentrated into estuaries and near shore coastal zones, so when environmentalists or politicians talk about pollution in the sea, they are often really discussing pollution in estuaries (McLusky and Elliott, 2004). Despite having profound effects on the estuarine ecosystem, long term "chronic" pollution is often sanctioned by governments, and is less likely to feature in newspaper headlines. "Hot-spots" of acute pollution are usually readily detectable, and often incur public concern, but, for example, an oil spill at the mouth of an estuary will command immediate attention because of the pollution risk, whereas an industrial discharge containing only a small percentage of oil will rarely feature in the public domain, despite the fact that over long timescales, more oil could enter the estuary from the industrial discharge than is spilt from a stricken tanker (McLusky and Elliott, 2004).

Metals are often important components of pollutant releases to estuaries. Although some metals are essential for living organisms, and occur naturally in the environment, metals have complex environmental chemistry. Metals do not degrade and their chemical speciation, bioavailability, bioaccumulation, and toxicity are key issues in assessing the hazards they may cause (Peijnenburg and Vijver, 2007). All environmental media have naturally occurring mixtures of metals, and metals are often introduced into the environment as mixtures. As a result of industrialization, current concentrations of metals may be elevated in environmental compartments (water, sediment and biota) relative to levels occurring naturally (Walker et al., 2006).

Whilst research related to metal pollution continues intensively and is evolving rapidly, some areas still lack sufficient information for a representative assessment of the quality of a specific environment to be carried out. Thus, specific approaches may become outdated or may otherwise require modification to reflect the best available science. Technologies now exist to measure extremely low concentrations of metals in water, biota and sediments. Nevertheless, in many aquatic habitats, metal concentrations are still close to the limits of detectability and dissolved concentrations usually vary over time, particularly for example, in estuaries with differential inputs of river and sea water at different states of the tide, and differential river flow according to recent rainfall in the catchment which typically varies seasonally. Each measurement represents a single point in time that may be very different from the dissolved concentration present at that exact location the day before or the day after. It is thus necessary to design effective longitudinal sampling programmes that can account for such variation and give a more realistic picture of the amount of dissolved metal present in a habitat. Such sampling programmes are achievable but are expensive (Rainbow, 2006).

On the other hand, the physicochemical characteristics of sediments greatly affect their ability to accumulate metals, so that different sediments will reach different concentrations from identical dissolved sources of metal (Bryan and Langston, 1992; Turner, 2000). For example, particle size and organic carbon content have major effects on metal accumulation by the sediment. Sediments high in organic carbon content will bind more metals than those with low organic carbon, and sediments with particles of high surface area will also accumulate more metals than particles of low surface area. Thus muds (small particle size, high organic content) accumulate more metals than sands (large particle size, low organic content). In the mixing zones of estuaries, river-borne iron and manganese will precipitate from solution as hydroxides as a result of increasing pH. As oxides and hydroxides of iron sink to be incorporated into the sediment, many metals such as Ag, As, Cu and Pb (Turner, 2000) adsorb onto them. Correspondingly, differences in the iron oxide contents of aquatic sediments will cause differences in the capacity of the sediments to accumulate metals from solution (Bryan and Langston, 1992).

Therefore, the use of biological markers or biomarkers measured at the molecular or cellular level have been proposed as sensitive 'early warning' tools for biological effect measurement in environmental quality assessment (Rainbow, 2006). The selected biomarkers should indicate that the organism has been exposed to pollutants (exposure biomarkers) and/or the magnitude of the organism's response to the pollutant (effect biomarkers or biomarkers of stress). One of the most important features of molecular/ cellular biomarkers is that they have the potential to anticipate changes at higher levels of biological organisation, i.e. population, community or ecosystem. Thus these 'early warning' biomarkers can be used in a predictive way, allowing the initiation of bioremediation strategies before irreversible environmental damage of ecological consequences occurs. Biomarkers are then defined as short-term indicators of long term biological effects (Depledge, 2009).

The literature on MTs as biomarkers of metal pollution in the area of study was limited to one published research article (Galloway et al., 2004) and unpublished data (Langston et al., 2003) which leads to the challenging prediction of the efficiency of MTs as biomarker in the studied estuaries. This study examined several potential candidates as metal biomonitors in estuaries of south England coast. It was found that invertebrates show a wide range of metal accumulation patterns; correspondingly different invertebrates collected from the same habitat show very different accumulated metal concentrations and hence metallothionein induction. It is therefore meaningless to compare MT concentration between organisms of different taxa. As can be seen from mussels *(M. edulis)* and limpets *(P. vulgata)* collected from the same waters (the Solent - Chapter 5), what can be considered a high concentration in the two species can differ by one or two orders of magnitude. In the case of these two organisms it may reflect their biology– one *(M. edulis)* is a filter feeder; the other (*P. vulgata*) is a grazer – so although they share the same environment they are exposed to different amounts of pollutants through the food chain.

The responses of estuarine organisms to pollution range from acute adverse effects to minimal effects. At the highest level of pollution, the responses of the animals and plants are easily recognised, since the results are acute and may be lethal to all forms of life. At a lower level of pollution, the sensitive fauna are eliminated, but tolerant species may thrive and become more abundant. The estuarine ecosystem thus shows distortions of conditions and organisms (McLusky and Elliott, 2004). Measurement of these distortions is the basis of the biological monitoring of pollutant effects. At the lowest level of pollution, only subtle changes in the physiology and biochemistry of the organisms may occur. Such subtle changes may be crucial in the long term, but can be difficult to detect (Monserrat et al., 2007).

All species are tolerant of a certain amount of environmental variation and, as shown in chapters 3 and 5, estuaries are variable and estuarine organisms are typically more tolerant to wider environmental variation than organisms from other systems. The tolerance to pollution will certainly vary from species to species, and will often vary between individuals of the same species. Given that estuarine species are already particularly tolerant to environmental variation compared to marine or freshwater species (Monserrat et al., 2007), then the capacity of the estuarine ecosystem to accept pollutants, which enhance natural variation, such as organic matter, is relatively great. Because estuaries are such highly variable and thus perhaps resilient systems, it becomes more difficult to detect anthropogenic stress in the biota separately from natural stress and from natural variability (Borja et al., 2012). Ideal estuarine bioindicators (usually in the form of measured responses of a species, its population, or its community) should be gathered from species that are broadly distributed between estuaries and regions (Bortone, 2005).

The evidence from this study (Chapters 3 and 5) suggests that the population could have acquired tolerance as a result of detoxification mechanisms that include variety of physicochemical forms of storage. These mechanisms may produce results different from those “expected”, such as the binding to MTs by metals that, in an unpolluted environment, only occasionally bind to these proteins (Trombini et al., 2010). These organisms may also have other mechanisms than binding to MTs to deal with higher levels of metals, such as glutathione, or metals become associated with HMW ligands (e.g. enzymes) and LMW fractions which includes amino acids and any free (potentially toxic) metal (Langston et al., 2003) making MTs non-indicative of metal stress. Although MTs are mainly involved in the adaptive cell mechanism of protection against metal toxicity, a wide range of other factors such as hormones, cytotoxic agents, physical and chemical stress could interfere with MT biosynthesis (Amiard et al., 2006). Furthermore, it was reported that, in contrast with mammals or fish, in invertebrates, MTs frequently do not constitute the major intracellular sink for metals in target tissues (Mouneyrac et al., 2000).

Moreover, it was found in this study (Chapter 4) that under extreme conditions of metal pollution, the relationship between metal concentrations and MT deviates from linearity: a threshold appears to be reached beyond which the physiological capacity of organisms introduced to polluted areas is exceeded. In transplanted animals from a clean environment, MT induction increased to peak levels with increased metal concentration, and then declined with continued increases in the concentration (see Figure 4.6). Evidence shows that MT analysis produces inconsistencies and contradictions at sites where metal concentrations are known to be high, but a low MT concentration is produced (Pellerin and Amiard, 2009; Mao et al., 2012). It has been shown that if metal concentrations are extremely high, bivalve MT induction is actually reduced, probably as a result of a toxic effect preventing detoxification processes from being fully functional (Amiard et al., 2006).

This study could explain the confounding results of many recent publications (Moschino et al., 2010; Ladhar-Chaabouni et al., 2012; Paul-Pont et al., 2012; Ramšak et al., 2012; Strogyloudi et al., 2012). These studies have shown that MT is not induced in invertebrates challenged with metals, or even that a negative relation between MT concentrations and metal levels is found. This is in contrast to the general hypothesis that metals induce MT synthesis (Bebianno et al., 2003). The results show that metallothioneins were not exclusively associated with metal concentrations and other environmental factors may govern MTs variability and that MTs production in wild and transplanted estuarine invertebrates that were investigated in this study was not effective biomarkers for metal pollution.

Conclusions and recommendations

This study has shown that MT concentrations in different estuarine invertebrates vary between sites; however, it does conclude that the many uncertainties surrounding MT indicate that it may have no potential as biomarker in estuarine environments. It is also apparent that the importance of both the alternative detoxification processes and the existence of metal-tolerant populations at polluted sites may complicate or invalidate its application as a biomarker.

Most of the tested species are established bioindicators of metal bioavailability through the measurement of bioaccumulated metal concentrations, so this could be their role in biomonitoring rather than using MT induction (Mouneyrac et al., 2003). It is recommended to have a battery of biomarkers, rather than using one biomarker, which allows the evaluation of the physiological integrity of all species present in a given ecosystem. Batteries of biomarkers should be capable of assessing the toxic effects of the highest possible number of environmental contaminants (Lagadic, 1997).

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