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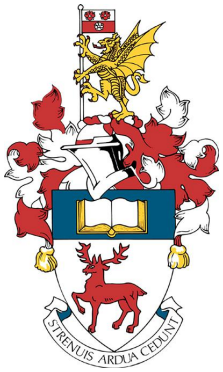

FACULTY OF ENGINEERING AND THE ENVIRONMENT

Civil, Maritime, and Environmental Engineering and Science

Centre for Environmental Sciences

Evaluating the potential of metallothionein as a reliable biomarker for metal pollution in selected marine organisms

by

James Francis Price Oaten

Thesis for the degree of Doctor or Philosophy (PhD)

May 2017

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF ENGINEERING AND THE ENVIRONMENT

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EVALUATING THE POTENTIAL OF METALLOTHIONEIN AS A RELIABLE BIOMARKER FOR METAL POLLUTION IN SELECTED MARINE ORGANISMS

James Francis Price Oaten

Metal pollution within coastal and estuarine environments is of concern due to its potential to decrease economic value to society, impact marine ecology, and reduce recreational use. Biomonitoring provides a valuable tool to monitor metal pollution as it allows the measurement of bioavailable metals, which have the highest potential to impact ecology and human health. Metallothionein (MT) is a biomarker of metal contamination; it is induced by metal exposure, binding and detoxifying metals within cells. MT has been included in numerous studies monitoring metal contamination using marine invertebrates. Causes of natural variation on MT concentrations such as reproduction, tissue mass, salinity, and temperature, are known issues in common bioindicator groups such as mussels and oysters. This can disrupt the relationship between MT and metal concentrations within marine invertebrates and cause MT to be a less reliable biomarker. Seasonal effects to MT concentrations are not definite in the Manila clam (*Ruditapes philippinarum*), an invasive clam species within the UK, which could be a promising bioindicator due to its increasingly global extent. Bioindicator species such as brown seaweeds (e.g. *Fucus spiralis*) have not been studied as a MT biomarker species despite their extensive use as a general metal bioindicator. Furthermore, standard protocols for the treatment of organisms before MT analysis have not been defined and studies are inconsistent. This may be causing further discrepancies between MT and metal concentrations due to changes in concentrations that may occur between sampling and MT analysis, perhaps due to stress endured during transportation or protein degradation during storage. Therefore, an aim of this PhD was to evaluate the potential of, and limits to, the use of MT in selected marine organisms. A further aim was to the refine the use of MT, in terms of both methodological protocols and seasonal sampling

strategies, to increase its reliability as a biomarker. It was found that the most appropriate treatment of organisms before MT analysis is to transport samples on ice from the field to the laboratory, and dissect as soon as possible thereafter. Depuration of organisms is not recommended before MT analysis, and storage at -20°C is acceptable for up to 10 weeks rather than the much lower temperatures used in some studies. It was found that gametogenesis in clam species in Poole Harbour during spring causes MT to be induced independently of metal exposure, compromising the reliability of MT. This suggests that the sampling of organisms should be restricted to a period of resting reproductive status. The potential of spiral wrack (*Fucus spiralis*) as a sensitive MT biomarker species was found to be limited, but may show promise in heavily contaminated environments, and warrants further research. Each of these findings can be implemented into international monitoring programmes as protocol in order to refine the use of MT. This thesis recommends that the shortcomings of current monitoring programmes are amended to include: evidence-based standard protocols for the pre-treatment of organisms; advisory restriction of sampling during gameteogenic periods; and the use of a range species as MT biomarker species.

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DECLARATION OF AUTHORSHIP

I, JAMES OATEN, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

EVALUATING THE POTENTIAL OF METALLOTHIONEIN AS A RELIABLE BIOMARKER FOR METAL POLLUTION IN SELECTED MARINE ORGANISMS

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. 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;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. 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;
5. I have acknowledged all main sources of help;
6. 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;
7. Parts of this work have been published (see below).

Signed: .....

Date: 23rd June 2017 .....

A version of Chapter 3 has been published in a scientific journal:

OATEN, J. F. P., HUDSON, M. D., JENSEN, A. C. & WILLIAMS, I. D. 2015. Effects of organism preparation in metallothionein and metal analysis in marine invertebrates for biomonitoring marine pollution. *Science of the Total Environment*, 518-519. 238-247.

A version of Chapter 4 has been published in a scientific journal:

OATEN, J. F. P., HUDSON, M. D., JENSEN, A. C. & WILIAMS, I. D. 2017 Seasonal effects to metallothionein response to metal exposure in a naturalised population of *Ruditapes philippinarum* in semi-enclosed estuarine environment. *Science of the Total Environment,* 575, 1279-1290.

Parts of chapter 4 have also been presented at the 8th International Conference on Marine Pollution and Ecotoxicology, 20th-24th June 2016, Hong Kong.

A version of Chapter 6 has been accepted for publication in a scientific journal:

OATEN, J. F. P., GIBSON, M. C., HUDSON, M. D., JENSEN, A. C. & WILLIAMS, I. D. 2017. Metal accumulation and metallothionein response in *Fucus spiralis. Journal of Environmental Pollution and Remediation.* In press.

Parts of chapter 6 have also been presented at the 6th International Conference on Environmental Pollution and Remediation, 18th-19th August 2016, Budapest, Hungary, and published in conference proceedings:

Proceedings of the 2nd World Congress on New Technologies (NewTech'16), Budapest, Hungary – August 18-19, 2016, Paper No. ICEPR 124.

A version of Chapter 7 was presented as a research poster at the Marine Protected Areas: Science, Policy and Management conference in Poole, UK, on the 15th-17th May 2017, winning 2nd place in the poster competition.

Acknowledgements

I would like to firstly express my gratitude to my supervisors: Ian Williams, Malcolm Hudson, and Antony Jensen. Their expertise and advice was highly valuable, and they motivated me throughout my project.

I'd like to thank John Humphreys for his assistance during field sampling, and Matthew Cooper for his support analysing trace metal concentrations. Thanks are also due to Peter Morgan and Anne Stringfellow for their advice and assistance in preparing sediment samples for trace metal analysis. Michelle Gibson deserves acknowledgements for her assistance with metallothioenin analysis, as does Lina Restrepo, Elena Lebedef, and Emily Thorne for their help dissecting clams.

Finally, I greatly appreciate the support from my parents, family and friends throughout this degree. Special thanks are due to Emma Scourfield.

The Engineering and Physical Sciences Research Council (EP/L505067/1) and Southampton Marine and Maritime Institute financially supported this PhD.

Definitions and Abbreviations

AChE – acetylcholinesterase

AVS – acid volatile sulphide

BEQUALM – Biological Effects and Quality Assurance in Monitoring Programmes

BRI – biomarker response index

CEFAS – Centre for Environment Fisheries Aquaculture Science

CSO – combined sewer overflow

CSQG – Canadian Sediment Quality Guidelines

DG – digestive gland

DNA – deoxyribonucleic acid

DOM – dissolved organic matter

EC – European Commission

EQS – environmental quality standards

EU – European Union

FAAS – flame atomic absorption spectrometry

FRAP – ferric reducing ability of plasma

G – gill

GSH - reduced glutathione

HELCOM – Helsinki Commission

ICES – International Council for the Exploration of the Sea

ICP-AES – inductively coupled plasma-atomic emission spectrometry

ICP-MS – inductively coupled plasma – mass spectrometry

IPPC – Integrated Pollution Prevention Control

JAMP – Joint Assessment Monitoring Programme

LMS – lysosomal membrane stability

LOD - limits of detection

MED POL – Mediterranean Pollution Monitoring Programme

MS – mass spectrometry

MSFD – Marine Strategy Framework Directive

MT – metallothionein

MTLP – metallothionein-like protein

NOCS – National Oceanography Centre Southampton

NRR – neutral red retention

PAH – polycyclic aromatic hydrocarbon

PEL – probable effect level

PMSF – phenylmethylsulfonyl fluoride

ROS – reactive oxygen species

RSPB – Royal Society for the Protection of Birds

RT-PCR – reverse transcript-polymerase chain reaction

SAC – Special Area of Conservation

SD – standard deviation

SH – sulphur-hydrogen

SPA – Special Protection Area

SSSI – Site of Special Scientific Interest

STW – sewage treatment works

TBT – tributyltin

TEL – threshold effect level

TOSC – total oxyradical scavenging capacity

UNEP/MAP – United Nations Environment Programmes/Mediterranean Action Plan

WFD – Water Framework Directive

WT – whole tissue

Chapter 1: Introduction

Metal pollution in the marine environment remains an important environmental issue. Of particular concern, mostly in developed countries, are legacy effects from historic sources of metal pollution that remain within an environmental system. This is prevalent in semi-enclosed estuarine environments where flushing and water exchange can be poor, resulting in contaminants residing in sediments (Dassenakis et al., 2003). There are also metal pollution issues associated with less developed countries and emerging economies due to rapid industrial expansion and limited regulation (Pan and Wang, 2012).

Biomonitoring of metal pollution is an important approach in assessing marine metal pollution. It allows many advantages over chemical monitoring whereby metals are measured directly from water or sediments. Biomonitoring provides a temporal and spatial synopsis of contaminants, and measures only bioavailable forms of metals (with the exception of metal adsorption to organism surfaces), which is what is of most concern as they have the greatest potential to impact biota, ecosystems and human health (Rainbow, 1995a). It also avoids issues of testing the water or sediments directly as factors such as tidal exchange, pH, and temperature may affect metal concentrations. Biomarkers, which are biological responses that indicate the effect of a contaminant to organisms, are often used to complement biomonitoring studies. Metallothionein (MT), a protein that is induced by and detoxifies metals by binding, is a particularly well-known biomarker for metal contamination (Amiard et al., 2006). It has been studied and utilised in marine invertebrates since the 1980s (Hennig, 1986, Engel and Roesijadi, 1987).

MT concentrations can be influenced by both biotic and abiotic factors, and natural variability in concentrations is accentuated seasonally (Geffard et al., 2005, Ivankovic et al., 2005, Bocchetti and Regoli, 2006). Consequently, MT may not accurately represent metal exposure to organisms. This reduces the reliability of using MT as a biomarker of metal contamination. Furthermore, the wealth of literature that exists on MT induction from metal exposure enlists various methodologies to analyse MT in organisms. In particular, the treatment of organisms and preparation of samples are inconsistent. This raises questions about the validity of employing MT for monitoring metal pollution, if methodological differences alter MT and metal concentrations.

An overarching aim of this thesis was to evaluate the potential of, and limits to, using MT as a biomarker for metal contamination in selected marine organisms. To account for seasonality, year-long sampling periods were adopted. Another aim was to determine if methodological procedures before analysis have the potential to create ambiguous results, and to make appropriate recommendations based on the results.

This thesis will address these issues in six following chapters, as follows:

- Chapter 2 forms the literature review. This describes background contextual literature, critically appraises related studies and highlights research gaps. From this, the detailed research aims and objectives are specified.
- Chapter 3 forms the first detailed study of this thesis. It focuses on addressing the issues of MT analysis associated with methodological inconsistency concerning the treatment and preparation of samples before analysing for MT.
- Chapter 4 consists of a study assessing the seasonal effects on MT concentrations in the Manila clam (*Ruditapes philippinarum*) in order to evaluate the potential of MT in a non-native species at the current limit of its distribution.
- Chapter 5 is a study investigating and comparing MT responses in *R. philippinarum* and sympatric native species *Ruditapes decussatus* and *Venerupis corrugata*. These species are lesser studied as bioindicators compared to other bivalves such as mussels and oysters.
- Chapter 6 is an exploratory study on the potential for MT to be used as a biomarker for metal pollution in spiral wrack (*Fucus spiralis*), which has not been studied previously.
- Chapter 7 discusses the outcomes of the work completed in this thesis, as well as previous works, and draws together the final conclusions of the thesis. It is written as a discussion style study that evaluates the reliability and the inclusion of MT as a biomarker in the context of international biomonitoring programmes.

Chapter 2: Literature review

2.1 Status of marine metal pollution

The issue of metal pollution in the marine environment has long been recognised by the scientific community. The amount of research on the fate of metals increased during the 1970s due to both critical issues such as Hg poisoning and the development of rapid analytical techniques (Bryan, 1980). Marine pollution research has now shifted and is focused on other pollutants we are becoming aware of, such as endocrine-disrupting toxicants (e.g. bisphenol A), or micro-plastics (Cole et al., 2011, Wei et al., 2011). However, metals are toxic, persistent, and non-degradable, and sediment pollution represents an on-going risk to coastal environments (Pekey, 2006). Anthropogenic metal pollution is prevalent throughout much of the world's coastal and estuarine environments, due to urbanisation and the industries that frequently surround them (Boldina-Cosqueric et al., 2010). Population growth and globalisation is likely to increase sources of metals to the coastal environments from further urbanisation, mining, industrial and domestic waste, *e*-waste recycling, and shipping. Increased sources of metals and pollution associated with rapid economic growth in emerging economies are evident throughout coastal China, for example, particularly close to industrial areas (Liu et al., 2007, Yu et al., 2008, Pan and Wang, 2012). Within Liaodong Bay, considered to be one of the most heavily metal polluted areas, few living organisms exist close to outfalls and there is a risk of metal poising to local people (Zheng et al., 2007, Pan and Wang, 2012). Furthermore, the composition of toxic metals may shift as the use of traditional metals are being replaced, such as the ban of tributyltin (TBT) being replaced by Cu and Zn in anti fouling paints (Evans et al., 2000, Turner, 2010).

The OSPAR Convention (named after the Oslo and Paris Conventions in 1992) is the mechanism by which 15 governments and the EU cooperate to protect the marine environment of the North East Atlantic (OSPAR Commission, 2016). OSPAR Commission produced a report on the status and trend of marine chemical pollution (OSPAR Commission, 2009). It reported that concentrations of Cd, Hg, and Pb in the marine environment have decreased from 1998 to 2007. However, in some coastal areas in the northeast Atlantic concentrations in sediments and biota still pose a risk of pollution effects: Pb, Cd and Hg biota concentrations exceeding EU dietary limits are generally linked to urban and industrial activity, for example around Denmark and several UK estuaries. Furthermore, metal accumulation in marine mammals, particularly Hg accumulation, is still of serious concern with Arctic populations of whales being exposed (AMAP, 2009). This evidences global spread of metal contamination.

Metal pollution is exacerbated in semi-enclosed environments, such as some estuaries, as metals tend not to disperse away from their source due to a lack of flushing within the system (Dassenakis et al., 2003). Isolated cases of metal pollution can result in severe impacts to the environment. For example, an influx of metals into the Fal Estuary, UK, caused by mining activity in the catchment has caused the most metal concentrated intertidal sediments in Britain (Bryan and Gibbs, 1983). Furthermore, the legacy of pollution is still evident in the Fal Estuary. Macro-invertebrate communities in the Fal are distinguished by high abundances of pollution-tolerant annelid species, which has clear implications for higher trophic levels (wading birds, demersal fish) (Warwick, 2001). Historical metal contamination can remain within sediments of poorly flushed, semi-enclosed systems and continue to be a risk of pollution, as is the case in Poole Harbour, UK (Aly et al., 2014), the Evoikos Gulf, Greece (Dassenakis et al., 2003), and Masan Bay, South Korea (Kwon and Lee, 2001).

Worldwide concern for marine metal pollution is reflected in international legislation. The European Union (EU) has established the Water Framework Directive (WFD 2000/60/EC) to protect and enhance the quality of all water bodies including coastal waters and estuaries (Marin-Guirao et al., 2005). Another initiative that has recently been developed is the European Marine Strategy Framework Directive (MSFD; Directive 2008/56/EC), which focuses on marine waters (Solaun et al., 2013). The ultimate objectives of these directives are to ensure 'good ecological and chemical status' for all European water bodies by 2015, but has been derogated to 2027 for water bodies failing to meet the original targets (European Commision, 2016). This is to be achieved by: eliminating 'priority hazardous substances', and contributing to achieving concentrations in the marine environment near to background values, for naturally occurring substances (Tueros et al., 2008). Of the list of 33 priority substances under the WFD (Directive 2008/105/EC), Cd, Hg, TBT, Ni, and Pb are included. The former three are considered 'priority hazardous substances' because of their persistence, bioaccumulation and/or toxicity or equivalent level of concern. Environmental Quality Standards (EQSs) for each of these contaminants are indicated for dissolved concentrations, both for 'annual average' and 'maximum allowable concentrations', yet concentrations within biota are only quoted for Hg. This is because water standards alone do not offer sufficient protection against Hg, due to its known toxicity, and therefore limits have been set for both water and biota, and member states which choose not to enforce biota standards should apply more stringent standards in water (Jurgens et al., 2013). However, the European Commission (EC) Regulation 1881/2006 sets out regulatory limits for contaminants in food in England. It states the maximum concentrations (wet weight) of Hg, Cd, and Pb permitted in bivalves for human consumption as 0.5 μg/g, 1 μg/g, and 1.5 μg/g, respectively. Other relevant legislation that regulates environmental metal concentrations include

the Bathing (76/160/EEC), Fish (78/659/EEC) and Shellfish (79/923/EEC) Waters Directives, and other legislation that covers metal substances or sources of metal pollution include Dangerous Substances (76/464/EC), Urban Wastewater (91/271/EEC) and Integrated Pollution Prevention Control (IPPC) (96/61/EC) Directives (Allan et al., 2006). The Shellfish Waters and Dangerous Substances Directives were repealed in 2013 and their requirements transferred to the WFD (Boyes and Elliott, 2014).

2.2 Metal pollution and toxicity

Metals fall into two categories: non-essential, such as cadmium (Cd), the metalloid arsenic (As), chromium (Cr), lead (Pb) and mercury (Hg); or essential such as copper (Cu), manganese (Mn), zinc (Zn), and iron (Fe). Figure 1 illustrates the biological effect of each type of metal. The former serve no biological function and are therefore considered more toxic at low concentrations. Once concentrations increase from zero (or rise above natural background concentrations) and reach a certain concentration (C_2 on Figure 1), adverse effects may occur, and at a certain concentration death will occur. Essential metals are required for the normal functioning of organisms, and therefore deficiencies in them can cause harm to organisms (concentrations less than C_1 on Figure 1). However, excess concentrations of essential metals can also cause a toxic effect (C_2 on Figure 1), until, if the dose is high enough, death occurs.

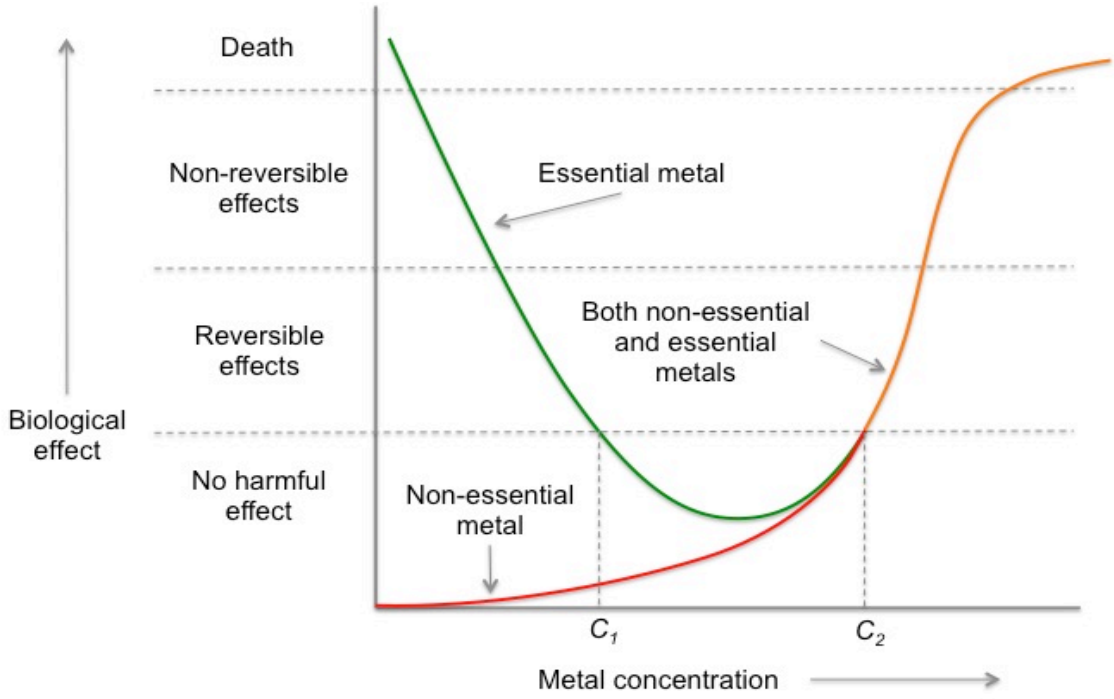


Figure 1 Biological responses to essential and non-essential metals. Normal metabolic activity occurs between concentrations C_1 and C_2 (adapted from Connell et al. 1999).

Most metals in marine environments usually occur at low concentrations yet can still impact marine organisms at cellular levels, due to the duration of exposure, disrupting ecology on larger, ecosystem scales (Bryan, 1979) (Table 1). Pb has been shown to impact the growth of the blue shrimp crustacean (*Artemia* spp.) and increase the mortality rate of the common mussel (*Mytilus edulis*) at approximately 10 mg/l (Bryan, 1976, Ansari et al., 2004). Pb poisoning in humans can lead to neurological damage where symptoms include nausea, coordination loss, and impairment of memory, as well as coma and death (Goyer, 1993). Effects of Cd toxicity have been observed at dissolved concentrations of approximately 5 μg/l in the Bristol Channel where *Patella vulgata* suffered a reduced ability to utilise glucose (Shore et al., 1975). Antarctic limpets were shown to lose all digestive gland structure and experience cell autolysis at 0.5 mg/l after 72 hours (Najle et al., 2000). Although Cu is an essential metal, it is also potentially one of the most toxic metals to a wide spectrum of marine life (Ansari et al., 2004). Experimental evidence suggests that a number of aquatic species are sensitive to Cu in the concentration range 1 to 10 μg/l (Bryan, 1976). These effects are rarely observed in the environment due to generally low concentrations, and possibly due to organism detoxification responses and factors affecting biological uptake. However, there is still a need to monitor metals in the environment as shifts in their anthropogenic use occur. For example, deleterious effects of tributyltin (TBT) from antifoulant paints on oysters (Alzieu, 1991) and neogastropods (Bryan et al., 1987) have been observed and their presence has led to environmental degradation in many coastal areas globally (Bryan and Langston, 1992). France first banned TBT in 1985 over environmental concerns, followed by the UK in 1987 on vessels less than 25 m (Bray et al., 2012). A global ban was not ratified until 2009 (Sonak et al., 2009). However, Cu and Zn are being used more to replace the role of TBT as an antifoulant on watercraft, and we may now see a further increase in these metals in estuarine environments (Turner, 2010). Estuarine environments are also used for recreational activities and seafood harvesting resulting in a potential threat to human health, primarily through ingesting metal-contaminated biota, and economic resources (Ansari et al., 2004).

Certain metals can cause epigenetic effects. This refers to the heritable changes in gene expression without mutating DNA sequences (Henikoff and Matzke, 1997, Cheng et al., 2012). This can have enormous significance for the long-term survival of exposed populations (Anderson et al., 1994). For example, oxidative stress, DNA methylation, and histone modifications caused by metals such as nickel (Ni), Cr, and As can lead to chromosomal damage and silencing or reactivation of gene expression (Salnikow and Zhitkovich, 2008, Chervona and Costa, 2012). Carcinogenic effects are thought likely to stem from this, promoting the survival and expansion of genetically/epigenetically-altered cells (Salnikow and Zhitkovich, 2008). These effects can also be used as molecular markers to inform on metal exposure (Varotto et al., 2013).

Table 1 Range of effects of a toxicant to a population (adapted from Connell et al. 1999).

		Dose or concentration	Exposure period	Predicted response
		Very low	Very long (many years)	No detectable effects
		Low	Long (months/years)	Death of sensitive individuals Sub-lethal effects in survivors
		Intermediate	Intermediate (days)	Equal number of deaths and survivors Severe effects in some survivors
		High	Short (hours/days)	Few resistant individuals survive
Increasing dose	**Increasing exposure period**	Very high	Very short (hours)	Death to all members of the population

The bioavailability of metals, and thus toxicity, can be affected by marine biology and marine chemistry (Rainbow, 1997). The exposure pathway to the organism is important. Organisms that inhabit the water column, such as seaweeds, are likely to be subjected to dissolved concentrations of metals (Rainbow and Phillips, 1993). Sediment metals are more likely to be accumulated by benthic organisms, through ingestion of particles (Bryan and Langston, 1992). Suspension feeding organisms, such as mussels and oysters, take up metals both directly from seawater and suspended particles during feeding (Rainbow, 1995a). The primary way in which organisms accumulate metals is through the diet, but can also be taken in through permeable body surfaces (Rainbow, 2007). Therefore, feeding and habitat are important considerations that affect bioavailability. Physio-chemical effects to metal bioavailability are summarised in Table 2. Sediment composition is an important control over bioavailability. Organic matter and Fe/Mn oxide or hydroxide formation within sediments may adsorb metals and isolate them from biota, unless ingested by detrital feeding organisms, and are therefore important controlling parameters (Bryan and Gibbs, 1983, Rainbow, 2006). However, complexation of trace metals with dissolved

organic matter (DOM) occurs in estuarine and open ocean environments (Donat et al., 1986). This causes metals to remain within the water column, increasing bioavailability to filter feeding organisms that ingest DOM (Guo et al., 2001). The equilibrium between dissolved metal concentrations in overlying and interstitial waters and metals adsorbed to particles and sediments largely controls bioavailability (Bryan and Langston, 1992). This can be altered by abiotic factors such as pH, salinity, dissolved organic matter, dissolved oxygen, and redox conditions. Furthermore, chemical speciation of metals can influence bioavailability. For example, free metal ions, such as the free cupric ion (Cu^{2+}), are known to be the most bioavailable inorganic forms but only make up a small percentage of total dissolved concentrations (Zamuda and Sunda, 1982, Bryan and Langston, 1992, Campbell, 1995). This is because they are able to traverse biological membranes and bind to biological ligands (Peijnenburg and Jager, 2003). Metal speciation can be influenced by a number of factors such as pH, temperature, salinity, and DOM (Campbell, 1995, Guo et al., 2001, Griscom and Fisher, 2004, Mubiana and Blust, 2007).

Table 2 Effects and outcome of factors affecting metal bioavailability.

Factor	Effects	Metals affected	Outcome	Reference
Sediment particle size	Small particles have a larger surface area for metal binding, increasing metal concentration	All	Small particles increase bioavailability	Rainbow (2006)
Fe/Mn concentration and oxide and hydroxide formation	Precipitates and adsorbs metals onto surface and sinks to sediments	Ag, As, Cu, Pb, Zn, Cd	High Fn/Mn concentrations reduce metal bioavailability	Bryan and Gibbs (1983), Bryan and Langston (1992)
Particulate organic matter (e.g. humic substances)	Metals have high affinity to organic matter and bind to them in sediments	Cu, Ag, Pb	Particulate organic matter decreases bioavailability, but may be available to deposit feeding organisms	Bryan and Langston (1992), Rainbow (2006)
Dissolved organic matter (DOM)	Metals are complexed with DOM and remain available to be ingested by filter feeding organisms	Cu, Zn, Cd, Fe, Ni, Mg	High DOM concentrations increases bioavailability to filter feeding organisms	Donat et al. (1986), Guo et al. (2001)
Acid-volatile sulphide (AVS)	High AVS concentrations in sediment results in co-precipitation of metals with insoluble sulphides (e.g. pyrite)	Cd, Cu, Hg, Pb, Zn	High AVS concentrations decrease bioavailability	Bryan and Langston (1992), Morse (1994), Cooper and Morse (1998)
Metal speciation	Free metal ions are most bioavailable form as they can traverse biological membranes and bind to biological ligands	Most	Higher free ion concentrations increase bioavailability (affected by many factor e.g. temperature)	Bryan and Langston (1992), Campbell (1995), Peijnenburg and Jager (2003)
Anoxic/reducing conditions	Sulphide mineral formation during anoxic conditions acts as sink for metals	As, Cu, Hg	Anoxic conditions decrease bioavailability	Morse (1994), Cooper and Morse (1998)
pH	Increase in pH encourages formation of oxides and hydroxides precipitating metals from solution to sediment, and decreases free metal ion concentration	Cu	High pH decreases bioavailability	Bryan and Langston (1992), Rainbow (2006)
Dissolved oxygen	High dissolved oxygen concentration causes oxidation and release of metals from suspended particles and sediment	Cu	High dissolved oxygen concentrations increase bioavailability	Cooper and Morse (1998), Mubiana et al. (2005)
Salinity	Low salinity causes less inorganic complexation of metals with chloride ions	Zn, Cd, Cu	Low salinity Increases bioavailability	Bryan and Langston (1992), Rainbow (1995b)
Extracellular polymers (bacteria)	Sediment bacteria containing extracellular polymers (polysaccharides) can accumulate metals which are then ingested by organisms	Cu, Pb, Cd	Presence increases bioavailability	Schlekat et al. (1998), Bhaskar and Bhosle (2006)
Interaction between metals	Presence of certain metals may reduce uptake of metals through competition for uptake sites	Metal ions of similar size and charge (e.g. Cd and Ca)	Presence of certain metals may decrease bioavailability of other metals	Ng and Wang (2004)

2.3 Biomonitoring and biomarkers of pollution

Physical and chemical variables within coastal environments are used to provide an indication of marine metal contamination, however limitations have been identified in determining biological effects, which is of ultimate concern (Kirby et al., 1998). Furthermore, measuring concentrations within sediments or seawater is vulnerable to fluctuations due to physical influences such as tidal exchange, and chemical influences such as salinity, pH and dissolved oxygen, and may result in non-representative results. The use of marine organisms to monitor metal contamination is a way to gain representative information, and pollution monitoring has seen a shift towards biological monitoring (biomonitoring), from measuring contaminants directly in seawater or sediments (Lam and Gray, 2001). Biomonitoring provides a synoptic measure of exposure to elements of direct ecotoxicological relevance and allows spatial and temporal derivations to be examined more readily than traditional techniques (Rainbow, 1995a). Many species are well known bio-accumulators (see section 2.3) and are characterised by efficient detoxification methods, such as intracellular compartmentalisation, or metal inactivation by binding to protein molecules (Demuynck et al., 2004). High bioaccumulation of metals generally highlights the most bioavailable elemental species. This is important as it reflects the metal that is most available to cause harm to organisms, ecosystems and the human population.

Studies are often conducted focusing on the concentration of metals within the tissue of organisms. This can be complemented by the use of biomarkers. An ecotoxicological biomarker is: 'a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)' (Depledge, 1994). In an environmental context, biomarkers are advantageous as they demonstrate toxicants have entered organisms, have been distributed between tissues, and are eliciting a toxic effect (Shugart et al., 1992). Therefore, they indicate potential effects and integrated toxicities of specific pollutants (Zhou et al., 2008). Furthermore, biomarkers are regarded as early warning signals of pollution as changes at molecular level occur at a threshold lower than concentrations that can be detected by monitoring change in a species, population or community (Won et al., 2008). As such, they enable the recognition of the potential consequences of pollution before more serious consequences occur, such as species mortality and ecosystem disruption.

Biomarkers can be either "effect biomarkers", which demonstrate adverse effects to an organism, or "biomarkers of exposure" that reflect the exposure to a contaminant (Galloway et al., 2008). Examples of effect biomarkers include:

- Lysosomal stability, which is a measure of cellular damage caused by a variety of xenobiotic chemicals including metals, and;
- Oxidative stress, which also responds to a variety of xenobiotics, and is a measure of free radicals that damage cells.

Examples of biomarkers of exposure include:

- Imposex (also an effect biomarker), which indicates reproductive interference in littoral waters from organotins, such as the effect of TBT on dog whelks where female reproductive organs transform to non-functional male gonads; and
- Metallothioneins (MTs), which are proteins induced by metals to sequester and prevent damage to an organism.

Further examples are presented in Tables 3 and 4.

Biomarkers indicate the biological impact caused by pollutants, which offers extra information on the severity of pollution not offered by measuring concentrations of a pollutant (i.e. direct measurement of concentrations in biological tissue or the environment). Effective and reliable biomarkers must therefore be sensitive to both contaminant availability and early biological effects (Van der Oost et al., 2003). Van der Oost et al. (2003), Hagger et al. (2006), and Le et al. (2016) describe a set of criteria for biomarkers. They include: 1) availability of reliable (quality assured) and low-cost measurements; 2) well-characterised dependence of the response on exposure dose and time; 3) sensitivity to pollutant exposure and effects; 4) known influence of confounding factors; 5) well-defined basal line in non-contaminated situations; and 6) established relationship between the response and effects on the organism. Essentially, this means that a predictable response to a contaminant is required of a reliable biomarker, which is not seen in a non-contaminated environment, and other influencers do not affect its concentration.

Table 3 Biomarkers of effect included in Natural England's suite of assays (adapted from Galloway et al. 2008).

Method	Issues addressed	Biological significance	References
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 (integrated response)	Styrishave and Depledge (1996), Galloway et al. (2002), Brown et al. (2004)
Scope for growth (includes clearance rate, oxygen consumption etc.)	Responds to a variety of contaminants	Integrative response; a sub-lethal measure of energy available for growth	Widdows et al. (2002), Halldorsson et al. (2005)
Micronuclei	Not contaminant-specific, although agent would be genotoxic	A measure of deoxyribonucleic acid (DNA) damage which may have higher consequences due to loss of DNA material	Heddle et al. (1983), Scarpato et al. (1990), Bolognesi et al. (1999), Hagger et al. (2005)
Lysosomal stability (including neutral red retention (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	Coles et al. (1995), Cajaraville et al. (2000), Brown et al. (2004), Ringwood et al. (2004)
Immunocompetence	Not contaminant-specific; will respond to a wide range of environmental contaminants	Measures factors that influence susceptibility to disease	Dyrynda et al. (1998), Auffret et al. (2004), Parry and Pipe (2004)
Oxidative stress (including total oxyradical scavenging capacity (TOSC) and ferric reducing ability of plasma (FRAP))	Not contaminant-specific; will respond to a wide range of environmental contaminants	Measures the presence of free radicals	Livingstone et al. (1992), Camus et al. (2004), Regoli et al. (2004)

Table 4 Biomarkers of exposure included in Natural England's suite of assays (adapted from Galloway et al. 2008).

Method	Issue addressed	Biological significance	References
Acetylcholinesterase (AChE) inhibition	Organophosphates and carbamates or similar molecules	Measures exposure to organophosphates and carbamate pesticides	Radenac et al. (1998), Cajaraville et al. (2000), Rickwood and Galloway (2004)
MT induction and metal partitioning	Measures induction of MT protein by certain metals (e.g. Zn, Cu, Cd, Hg)	Measures exposure and disturbance of Cu and Zn metabolism	Leung and Furness (1999), Geffard et al. (2002), Galloway et al. (2004b)
Imposex (also a biomarker of effect)	Specific to reproductive effects of organotins	Reproductive interference in coastal (littoral) waters	Bauer et al. (1997), De Wolf et al. (2001), Galloway et al. (2004b)
Polycyclic aromatic hydrocarbon (PAH) urine metabolites	Polycyclic aromatic hydrocarbons (PAHs)	Measures exposure to and metabolism of PAHs	Watson et al. (2004a), Watson et al. (2004b)

2.4 Metallothionein in bioindicators

Fowler et al. (1987) separated MTs into three classes. Class I comprises all proteinaceous MTs similar to mammalian MT based on cysteine locations. Class II incorporates all proteinaceous MTs that are not closely related to mammalian MTs. Class III includes non-proteinaceous MTs also known as phytochelatins. Mammalian MTs take the form of a single polypeptide chain formed of 61 amino acid residues (Binz and Kagi, 1999) (Figure 2). They contain thiol groups (sulphur-hydrogen) associated with high cysteine content (33%), which allow them to bind to metals (Amiard et al., 2006). Once bound by sulphur atoms in thiolate clusters, usually to divalent metals, no free thiol groups exist and MT forms a tetrahedral geometry (Romero-Isart and Vasak, 2002). Two subunits exist: a stable α-domain that can incorporate four divalent metal cations, and the β-domain that can only accommodate three (Romero-Isart and Vasak, 2002). The tertiary structure is highly dynamic, and ions can exchange easily within the more reactive β-domain, which may also involve exchange with ions bound to intracellular ligands (Coyle et al., 2002). MTs other unique features such as, low molecular weight, heat stability and a non-enzymatic nature enables them to be differentiated from most other proteins (Langston et al., 1998).

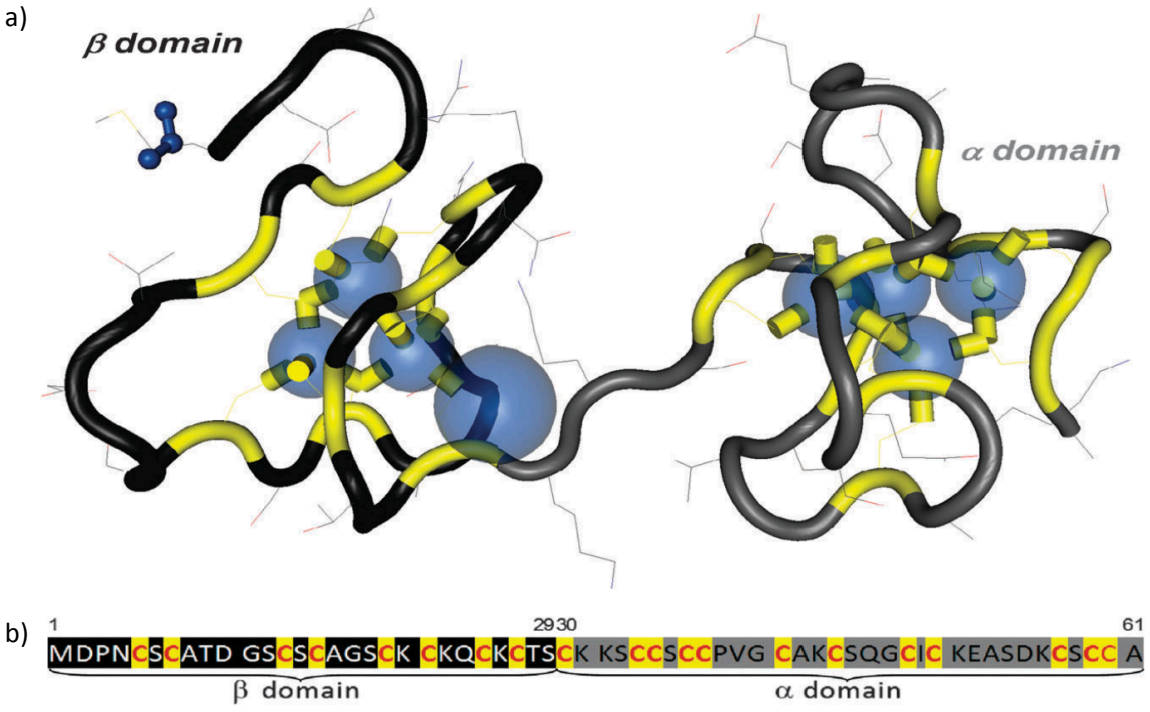


Figure 2 Structural characterisation of α and β domain of rat MT-2 based on data from Expasy database, showing a) 3D representation of tertiary structure of rat MT-2 (pdb ID 4MT2), and b) the amino acid sequence of rat MT-2 (P04355) (adopted from Babula et al. 2012).

Functions of MT include the regulation of intracellular availability of essential metals to metal-dependent processes (metalloenzymes, respiratory pigments, nucleic acids and membranes) (Roesijadi, 1996), gene regulation (Roesijadi, 1994), protection against ionizing radiation (Cai et al., 1999), and a more general antioxidant role (Viarengo et al., 2000). However, the primary function of MT is generally agreed to be to bind to excess essential metals or non-essential metals to prevent toxic effects, such as oxidative stress, to an organism (Amiard et al., 2006). Palmiter (1994), and Roesijadi (1996) propose that regulation of MT genes is mediated by the replacement of Zn from target ligands suffering inappropriate metal binding due to increased metal exposure, as toxic metals such as Cd have a higher affinity than Zn to the protein. The free Zn ion then binds to a Zn-sensitive inhibitor, which allows a constitutively active metal transcription factor to transcribe the MT gene. Incoming toxic metals then replace Zn bound to MT, and Zn rebinds to ligands ready to respond to on-going metal exposure. It is this detoxification and metal-binding role that allows MT to be used as a biomarker of metal exposure, as its concentration increases in response to metal accumulation (Langston et al., 1998). Since the 1980s, the potential of MT to be used as a biomarker of metal contamination has been recognised, particularly in marine molluscs (Hennig, 1986, Engel and Roesijadi, 1987). MT induction as a response to metal exposure is well documented in many species due to its role in the detoxification of toxic metals (Amiard et al., 2006).

Mollusca invertebrates such as mussels (*Mytilus* spp.) and oysters (*Crassostrea* spp.) are commonly used as bioindicators (Bebianno and Langston, 1992, Regoli and Orlando, 1994a, Langston et al., 1998, Astley et al., 1999, Amiard et al., 2007). They are resistant to pollution, efficient bio-accumulators, sessile, and long-lived (Rainbow, 1995a). Bivalve molluscs predominantly reflect bioavailable metal concentrations in water and particulate matter, as they are filter feeders and their gills are in contact with the water. Furthermore, MTs inducted from these species are defined as Class I MTs, allowing biomarker response to be evaluated in the context of human health (Langston et al., 1998). The structure of oyster MT is almost identical to mammalian MT; the first 27 cysteine residues reflect mammalian forms. MT in mussels is also very similar, sharing the same amino acid composition with oysters (29% cysteine, 16% glycine and 13% lysine) (Carpene, 1993). Many studies have reported correlations between MT in mussels and oysters and metal exposure (Frazier and George, 1983, Viarengo et al., 1985, Fowler et al., 1986, Bebianno and Langston, 1991, Geffard et al., 2001, Geffard et al., 2002, Mourgaud et al., 2002, Tanguy et al., 2002, Amiard et al., 2004, Zorita et al., 2007b, Pytharopoulou et al., 2008). Some metals, such as Cd and Hg, induce specific isoforms of MT in these species (Geret and Cosson, 2002, Tanguy and Moraga, 2001). George and Olsson (1994) considered *Mytilus* sp. to be the only promising invertebrate candidate for MT based biomonitoring. Due to the suitability of

these organisms as bioindicators, their use in biomonitoring has been thoroughly explored and they have been used in extensive biomonitoring programmes such as the Mussel-Watch programme (Goldberg et al., 1978). Other mollusc species have been studied and show similar MT responses to metals. These include winkles (*Littorina littorea*) (Bebianno et al., 1992, Leung and Furness, 1999), cockles (*Cerastoderma edule*) (Baudrimont et al., 2006, Aly et al., 2014), and clams (*Ruditapes decussatus, Ruditapes philippinarum*) (Bebianno et al., 1993, Bebianno et al., 2000, Hamza-Chaffai et al., 2000, Bocchetti et al., 2008).

Other organisms that have been used as bioindicators with MT include crustaceans (Legras et al., 2000, Galloway et al., 2004b), annelids (polychaetes) (Berthet et al., 2003, Ng et al., 2008), coelenterates and echinoderms (Amiard et al., 2006), and sponges (Aly et al., 2014). These taxa offer different characteristics that make them suitable for specific biomonitoring applications. For example, annelids inhabit and feed from marine sediment and therefore reflect sediment metal contamination, through ingestion of sediments or through body surfaces (Bryan and Langston, 1992). Sponges are sedentary filter filters and therefore reflect dissolved metal concentrations. With respect to crustaceans, convincing evidence of relationships between MT and metal concentrations has been reported (Galloway et al., 2004b). However, they are also mobile, which can raise questions over representativeness of on-site collection, yet may also provide a spatial synopsis of contamination.

2.5 Metallothionein in clams

Less attention has been given to clam species in biomonitoring studies compared with mussels and oysters, though they have been shown to be useful bioindicators, and have been gaining more attention recently. Many studies have reported a positive response of MT to metal exposure in the grooved carpet shell clam, or European clam (*Ruditapes decussatus*) in the digestive gland and gills to Cd, Zn, and Cu (Hamza-Chaffai et al., 2000, Geret et al., 2003, Bebianno et al., 2004, Serafim and Bebianno, 2007a). However, Smaoui-Damak et al. (2004) showed no correlations between Cd and MT in the gills stating body mass to be a more important moderator of MT, but other metals that may influence MT induction were not studied (Serafim and Bebianno, 2010). Although with fewer examples, the Manila clam (*Ruditapes philippinarum*), which is indigenous to the Indian-Pacific region but has spread to northern European and Mediterranean waters through introductions for cultivation (Delgado and Perez-Camacho, 2007), has also been shown to exhibit induction of MT following metal exposure (Zhao et al., 2010, Won et al., 2012). MT concentrations in response to metal exposure in the pullet carpet shell clam (*Venerupis corrugata*) have only been examined once before, but showed positive responses (Velez et al., 2016). There are differences in the response of MT and metals in different tissues in

clams. MT in the gills seems to respond quicker to metal exposure, and digestive gland MT responds to overall accumulation of metals over longer time periods (Serafim and Bebianno, 2007b). Some studies suggest correlations between MT and metal concentrations are stronger in the gills as it is the first point of contact with contaminants (Geret et al., 2003, Bebianno et al., 2004, Cravo et al., 2013), yet other studies recommend the use of the digestive gland (Smaoui-Damak et al., 2009, Zhao et al., 2010). It is also shown that physiochemical forms of storage (soluble and insoluble fractions) of metals are an important aspect of analysis. The majority of these studies were completed in the laboratory. Few studies were completed in the field, and in these cases MT response to varying amounts of metal contamination are less certain due to biotic and abiotic moderators (Moschino et al., 2012, Cravo et al., 2013).

Three studies, forming part of a larger study, have compared metal accumulation and MT in *R. decussatus* and *R. philippinarum* sampled from the Ria de Aveiro, northwest Portugal (Figueira et al., 2012, Freitas et al., 2012, Figueira and Freitas, 2013). Figueira and Freitas (2013) examined the element accumulation and health risk of consumption for each species. Findings include that *R. philippinarum* accumulates lower amounts of metals (Cd, As, Hg, Pb) relative to *R. decussatus*; depuration causes the element burden to decrease more in *R. philippinarum* than in *R. decussatus*; and subcellular distribution of elements in *R. philippinarum* is lower in the soluble fraction (i.e. cytosol and proteins), where they are toxic and readily available. Freitas et al. (2012) specifically studied the effects of depuration to element and MT concentration on each species. Concentrations of elements in organisms were low for both species. Slightly lower MT concentrations were observed following depuration compared to initial concentrations in the field, though not significantly different. This may be due to the distinct metal allocation between soluble and insoluble fractions (i.e. cell walls, exoskeleton and metal concretions). Figueira et al. (2012) studied the toxicological effects and bioaccumulation patterns of both species exposed to Cd in the laboratory. They conclude that *R. decussatus* had a higher capacity to increase the expression of MTs when exposed to Cd, compared to *R. philippinarum*. As a result, *R. philippinarum* presented higher oxidative stress, despite a much higher accumulation of Cd in *R. decussatus* in the soluble fraction (metal-sensitive fractions). However, there is evidence to suggest that *R. philippinarum* is more tolerant to physical stress than the native species (Tanguy et al., 2008). Whilst these comparisons are very insightful, these species have not been compared in detail in the field in an environment where natural stressors may cause varying levels of oxidative stress, and may alter metal accumulation and MT response.

2.6 Metallothionein in seaweeds

Certain lesser-studied species may possess characteristics that may overcome issues associated

with seasonal MT variability in other species and therefore may prove to be useful bioindicators with MT. Seaweeds are advocated as bioindicators of metal pollution in temperate coastal waters (Bryan and Hummerstone, 1973, Forsberg et al., 1988, Rainbow and Phillips, 1993, Vasquez and Guerra, 1996, Burger et al., 2007). This is due mainly to their high abundances and immobility (Rainbow, 1995a). They often dominate metal contaminated habitats as they are resistant to metal pollution, and they have an ability to accumulate metals so their intracellular concentrations reflect time-integrated contamination loads in the marine environment (Vasquez and Guerra, 1996). As a consequence, seaweeds, particularly brown seaweeds (fucoids), are established sentinels for metal contamination and are exploited for biomonitoring (Owen et al., 2012). However, literature on marine macroalgae MT response to metal exposure is limited, compared to other organisms. The MT gene has only been identified in a brown seaweed called bladder wrack (*Fucus vesiculosus*) by Morris et al. (1999), who suggested that a protective mechanism against metal exposure exists for this species. The study revealed that recombinant MT (in *Escherichia coli*) binds with Cd, and Cd can be displaced by Cu under anaerobic conditions. Induction of the MT gene followed laboratory exposure to elevated Cu. A recent follow-up study revealed complexities of the MT gene and MT *in vivo*, using *F. vesiculosus* from the Severn and Fal Estuaries (Owen et al., 2012). It showed the gene for MT to be exhibited following exposure to 30 µg/l of Cu. Other studies found MT to bind readily to As, Cd, and Zn, *in vivo* (Merrifield et al., 2006, Ngu et al., 2009). Sea lettuce (*Ulva linza*) has been found to respond to metal exposure by producing peptide thiols, such as glutathione and phytochelatins (class III MT) that act as a detoxifying mechanism (Malea et al., 2006). However specific MT induction in response to metal exposure has not been documented in this species. No seaweed species has been analysed for MT concentration in a biomonitoring context despite their obvious potential to be international universal bioindicators.

2.7 Metallothionein use and reliability

Although MT induction in a number of species has been used as a biomarker of metal contamination, there are variations in MT induction and response to metal exposure among different metals, organs, species, and exposure scenarios (Amiard et al., 2006). Furthermore, the absence of MT induction following metal exposure, and insignificant relationships between MT and metal concentrations, raises questions concerning the validity of using MT as a biomarker (Le et al., 2016). There are studies that suggest confounding factors may influence MT response independently of metal exposure (Geffard et al., 2001). Seasonal variability in MT concentration may be due to biological and ecological factors such as weight (and/or size and/or age), sex, sexual maturity or reproductive stage, or physical environmental factors such as salinity, pH, or

temperature (Geffard et al., 2005). It is therefore pertinent to examine the signal-to-noise ratio, in which the signal, the change in MT in response to metal exposure, is distinguishable from the noise, the natural variability in MT due to the aforementioned factors, both biotic and abiotic (Geffard et al., 2005).

The disruption caused by the reproductive cycle on MT response to metals has often been reported, however, different mechanisms have been described in bivalves. Some studies suggest that the tissue mass of the mantle and digestive gland increases as the gonads develop during sexual maturation, as gonadic tissue is indiscrete from the mantle and digestive gland. In turn, this causes a decrease in MT concentrations due to biological dilution (Raspor et al., 2004, Raspor et al., 2005). Another line of evidence suggests that the development of gametes in the gonads (gametogenesis) causes increases in MT due to hormonal induction (Baudrimont et al., 1997, Baudrimont et al., 2006, Mao et al., 2012), or that there are high MT concentrations in gametes in gonadal tissue to provide a protective mechanism during larval stages to increase survival (Meistertzheim et al., 2009). The body condition, or condition index, of bivalves, measured by the ratio of whole weight including shell to the weight of soft tissue, may also effect MT concentrations. As MT production is governed more generally by protein metabolism, it stands to reason that bivalves in poor body condition will have lowered physiological capability to produce MT (Mourgaud et al., 2002). The condition index of bivalves tends to vary seasonally due to altering food availability, temperature stress, and energy expenditure following spawns (Geffard et al., 2005, Ivankovic et al., 2005, Meistertzheim et al., 2009).

Aside from biological factors, MT concentrations can also be sensitive to specific environmental factors. Differing temperatures have been shown to effect MT concentrations. Serafim et al. (2002) report MT concentrations to increase in the gills of *Mytilus galloprovincialis* at higher temperatures citing increased rates of metabolism and metal accumulation, though Bocchetti et al. (2008) report a reduced capacity in *R. philippinarum* and *M. gallprovincialis* to produce anti-oxidant enzymes during warmer periods. Salinity variation and hypoosmotic stress is also shown to influence MT concentrations in *M. galloprovincialis*, with an inverse relationship between MT concentrations and salinity, which is tentatively linked to subcellular distribution of metals and general antioxidant defence capacity (Hamer et al., 2008). Fluctuations in crab MT concentrations seem to be more closely related to variations in protein metabolism in response to salinity changes than to variations in accumulated metal concentrations (Legras et al., 2000).

Due to confounding factors that alter MT response to metal exposure, MT as a biomarker has been used incorrectly in the past. There are instances where its reported successful use is false, as the effects of non-metallic influences on MT concentrations were not recognised or considered.

For example, Bebianno and Machado (1997) report significant correlations between MT and Cu and Cd concentrations in *M. galloprovincialis*, concluding MT in mussels could be a useful early warning signal of Cd and Cu contamination. However, samples were obtained from Portugal during April and May 1994, and it is stated that animals were in pre-spawning stage and in Maturation State III, meaning gametes are mature and ready to spawn (Lubet, 1959). There was no mention or consideration of the fact that the advanced stage in the reproductive cycle of mussels could affect MT concentrations. Fewer instances of this have occurred using clams and other bivalve species, probably because these are used in modern studies, and rarely neglect seasonal effects on MT and metal concentrations. However, there are instances where MT use has been discouraged, but confounding factors explaining its poor relationship with metals have not been properly established. For example, Moschino et al. (2012) suggest a cautious use of MT in *R. philippinarum* due to low sensitivity and responsiveness to anthropogenic metal contamination, ascribed to biological and seasonal factors. Although acknowledgement that this is possibly due to reproductive state and condition index is provided as clams were sampled during June and October, it is not suggested that MT in this species may be more responsive to metals during other seasons.

MT is part of a recognized group of biomarkers used in monitoring programmes at European level and is included in Natural England's suite of assays (Galloway et al., 2008), the United Nations Environmental Programme Mediterranean Action Plan (UNEP/MAP) MED POL (UNEP/MAP, 2015), and OSPAR Joint Assessment Monitoring Programme (OSPAR Commission, 2013). However, the issues surrounding MT reliability have yet to be fully resolved or incorporated into monitoring programmes. Confounding factors are recognised to be of importance in OSPAR Commission's JAMP (OSPAR Commission, 2013), and advise sampling to be undertaken outside of the spawning period of *M. galloprovincialis* and *M. edulis*. However, despite the growing amount of research on the issues with MT as a biomarker, there is still a lack of consensus among studies as to the effects of confounding factors, and consequently MT seems to dissatisfy the criteria for a reliable biomarker (section 2.3), at least in part. Such issues must be further examined if MT as a biomarker is to become an established and dependable tool in biomonitoring. It is essential to accommodate, or be able to discount, environmental noise into the experimental design of studies if MT is to satisfy the criteria for a reliable biomarker.

2.8 Methodologies for measuring metallothionein and metals

Methods to measure MT include electrochemical methods, spectrophotometric methods, chromatography, saturation-based methods, immunological methods, electrophoresis, and reverse transcript-polymerase chain reaction (RT-PCR) (Shariati and Shariati, 2011). A UV

spectrophotometric method has been selected for MT analysis during this study. It was developed by Viarengo et al. (1997) and validated by numerous laboratories (UNEP/RAMOGE, 1999, Zorita et al., 2005). It is deemed as a repeatable, sensitive, time saving, and low cost technique. Consequently, it satisfies part of the criteria for MT to be a reliable biomarker (criterion 1 in section 2.3). MT is evaluated utilizing a partially purified metalloprotein fraction obtained by ethanol/chloroform fractionation of the tissue homogenate. Complete MT precipitation is obtained and precautions are taken to avoid oxidation of the sulphur-hydrogen (SH) groups, contamination by soluble low molecular weight thiols, and enzymatic protein degradation that is a risk during sample homogenization. Ellman's SH reagent denatures MT by low pH and high ionic strength, and is used to quantify MT concentration spectrophotometrically.

Evidence-based standardisation on the methodologies for sampling and treatment of organisms before MT analysis does not exist. Consequently, studies adopt different procedures for the transportation of organisms from field to laboratory, depuration of organisms, dissection of tissues, and storage of samples before analysis. This is despite advisory procedures for transportation of organisms and storage temperatures suggested by (Davies and Vethaak, 2012). It is currently unknown if these methodological inconsistencies are affecting MT and metal concentrations between the time of sampling and MT analysis, perhaps due to stress endured during transportation or protein degradation during storage. If this is the case, results may be ambiguous and unreliable: an issue that raises concerns given their use in international monitoring programmes. This may compromise the reliability of MT as a biomarker and fail criterion 1 of a reliable biomarker (section 2.3). Therefore, it is important to investigate the magnitude of these potential effects, and to recommend the most appropriate procedure for the treatment of organisms before MT analysis.

In most analytical laboratories, trace element analysis is performed using atomic spectroscopy techniques because it offers advantages such as low limits of detection, and they provide the total amount of element present in the sample (Baffi et al., 2002, Brown and Milton, 2005). Since this technique requires sample introduction as a liquid solution, biological samples have to undergo digestion from dried samples in acid (Enamorado-Báez et al., 2013). There are many different acids that can be used but usually include nitric acid (HNO_3), hydrochloric acid (HCl), and sulphuric acid (H_2SO_4). Hydrogen peroxide (H_2O_2) can also be employed to mineralize organic matter, which also results in less spectral interferences (Sucharova and Suchara, 2006). The main atomic spectroscopy techniques are flame atomic absorption spectrometry (FAAS), inductively coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma-atomic emission spectrometry (ICP-AES), which are described by Brown and Milton (2005). FAAS sprays a sample into a flame where it dissociates into constituent atoms. Electromagnetic radiation in the

UV/visible part of the spectrum is then directed through the flame where it is absorbed in a manner characteristic of the atoms present. Advantages of FAAS include its lack of interference, low cost, and simplicity of use. However, it can be less sensitive with certain refractory elements (e.g. Al, B, Ti) because the flame is not hot enough for complete atomization. Measurements can also be time-consuming as each element has to be measured separately, which also means a large quantity of sample needs to be prepared. ICP-MS ionizes a sample using intense argon plasma and detects various elemental species using conventional mass spectrometry (MS) techniques. It offers high sensitivity, low limits of detection, and multi-elemental analysis. This allows a smaller sample to be digested and prepared. Therefore, ICP-MS was selected for measurement of metals in organism tissue and sediments in this study. However, this technique is still very expensive, and suffers from atomic and isobaric interferences. Interferences were overcome by addition of In/Re and Be which correct for matrix effects, which are a suppression or enhancement of analyte signal caused by concentrations of matrix elements, and instrument drift (Zheng et al., 2003, Millour et al., 2011, Karandashev et al., 2014).

2.9 Study areas

2.9.1 Poole Harbour

Poole Harbour is one of the largest marine inlets in the UK. It is located on the south coast of England in Dorset (Figure 3). Its water area is approximately 36 km^2 at high water spring tide, and has a shoreline of 100 km with five islands (Humphreys and May, 2005). The harbour itself is very shallow, with a depth varying between 0.5 m below and 2.5 m above chart datum, with a low tidal range of 1.7 m (mean springs) (Aly et al., 2014). However, the entrance to open water is only 370 m wide, which creates a poorly flushed system allowing siltation and sediments to reside in the harbour (Aly et al., 2013).

Historically, polluting point sources within Poole Harbour have mostly been eliminated or improved, and water quality is considered to be good, achieving statutory quality standards outlined by the European Union (Wardlaw, 2005). However, due to the restricted tidal exchange, chemical contaminants are still present in the sediment and continue to be a source of pollution (Langston et al., 2003b). Holes Bay, a secondary embayment to the northeast of the Harbour, is of particular concern. It has a narrow entrance restricting flow and exchange of water and consequently suffers from siltation creating a build up of contaminants, including metals. This problem is exacerbated in the northern part of Holes Bay as a railway bridge connecting eastern and western shores further restricts water exchange. Organotins such as TBT and other metals such as Cu, Cd, Hg, and Zn are present within the area (Wardlaw, 2005). Sources include boats

and shipping (particularly close to marinas), historic trade discharges, major sewage treatment works (STWs), and previously contaminated sediments (within Holes Bay, for example) (Wardlaw, 2005). Holes Bay in Poole Harbour is a closed fishery due to environmental pollution (Aly et al., 2013). Although regulatory enforcement largely prevents illegal fishing, there is still a risk of human consumption of shellfish from this area.

Poole Harbour has a range of important habitats including intertidal mudflats, salt marsh, fen habitats, coastal grazing marsh, and lowland heath-land (The Poole Harbour Steering Group, 2007). It is designated a Site of Special Scientific Interest, (SSSI), a European Special Protection Area (SPA), and a Wetland of International Importance (Ramsar site). The area is therefore protected under the Wildlife and Countryside Act 1981 and under the Natural Habitat Regulations 1994 (The Poole Harbour Steering Group, 2007). The area also has a bass (*Dicentrarchus labrax*) nursery area.

This area has a range of habitats, with a clear contamination gradient. This allows for a useful study site to test the response of species to varying degrees of metal contamination. Additionally, the UK is one of the northernmost locations where the invasive Manila clam, *R. philippinarum*, has naturalised (Humphreys et al., 2015). It was introduced to Poole Harbour in 1988 for aquaculture (Jensen et al., 2004). Its natural distribution is within the Indian-Pacific region but it is now common throughout much of the world including European waters (Delgado and Perez-Camacho, 2007). Therefore, seasonal effects on MT concentrations are unknown in this species at this latitude, which need to be established to refine its use as a MT biomarker species. This would be advantageous from a biomonitoring perspective, as this species inhabits coastal regions in the Indian-Pacific region and Atlantic and Mediterranean coasts, and therefore offers a widely available bioindicator.

2.9.2 Southampton Water

Southampton Water is an estuary on the south coast of the UK that is fed by the River Itchen, the River Test, and the River Hamble (Figure 4). It is subject to military, commercial, and industrial activity and serves a large number of recreational activities. A major source of metal contamination includes effluent discharge from the Esso oil refinery at Fawley and has been a contributor of hydrocarbon, Cu, and possibly Pb contamination to the area (Cundy and Croudace, 1995). However, effluent quality improvements since the 1970 have led to marked reductions in discharge (Cundy et al., 2003). The persistence of the antifoulant TBT and the Cu-based products used at sites surrounding shipping and recreational boating facilities is also an issue in the area (Galloway et al., 2004b). Industry on the River Test near Marchwood and Cracknore Hard include

a military port, sewage works and a waste transfer station (Solent Forum, 2013). Southampton Water is also a major UK port and is subjected to large volumes of shipping which is a potential source of contamination (Solent Forum, 2013).

Habitats within Southampton Water, outlined on a Joint Nature Conservation Committee (JNCC) database, include extensive mud flats and salt marshes together with adjacent coastal habitats such as saline lagoons, shingle beaches, reedbeds, and grazing marsh. Mud flats have rich invertebrate fauna and form an important food resource for estuarine birds. In summer, it is an important breeding ground for gulls and terns, and in winter it holds a large assemblage of geese, ducks and waders. The area is therefore designated as a Special Protection Area (SPA), and a Special Area of Conservation (SAC) (JNCC, 2014). Similarly to Poole Harbour, this area is also a bass (*Dicentrarchus labrax*) nursery area.

The dock at the National Oceanography Centre Southampton (NOCS), University of Southampton, was selected as a site to test the effects of the pre-treatment of organisms before MT analysis. It was a key requirement for the site to be located close to the laboratory (at the University of Southampton) in order to set up an experiment that allowed quick transportation from the field to the laboratory, and to compare that to a long transportation time, which was simulated.

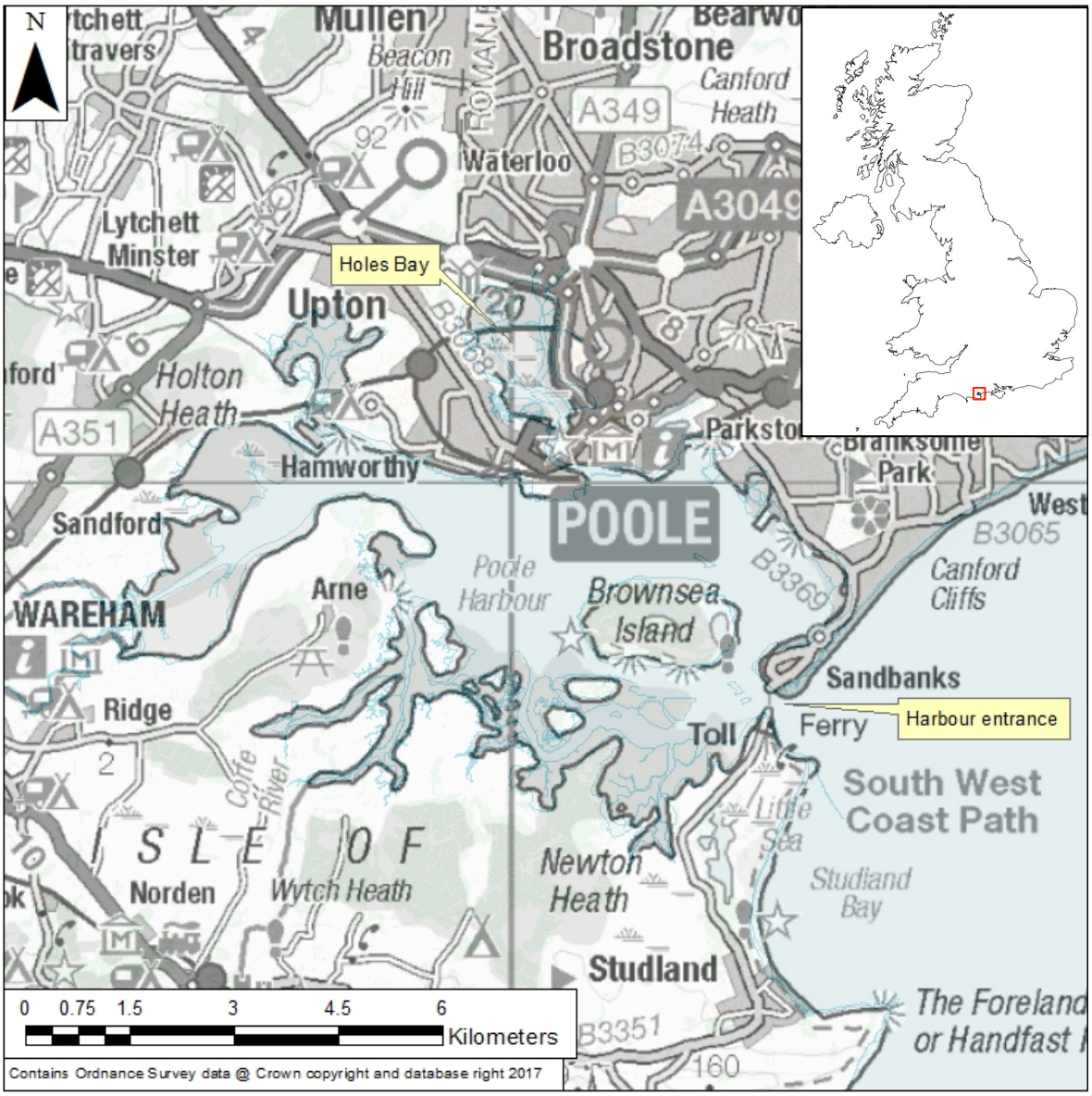


Figure 3 Map of Poole Harbour, indicating its location on the south coast of England, UK.

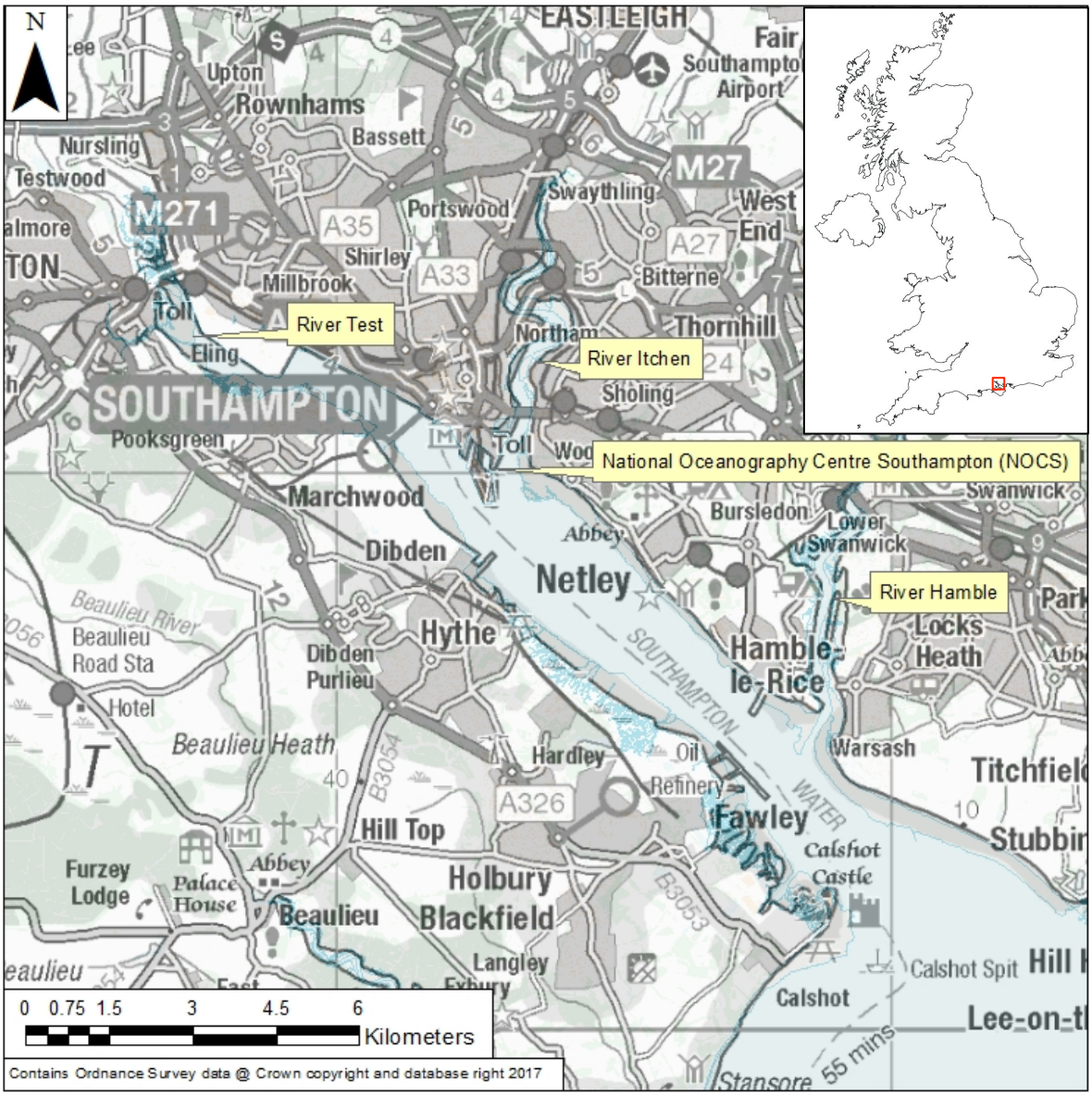


Figure 4 Map of Southampton Water, indicating its location on the south coast of England, UK.

2.10 Research aims and objectives

There is a wealth of literature that highlights the promise and use of MT as a biomarker to complement biomonitoring studies. However, there is a growing amount of evidence regarding confounding issues that affect confidence in its use as a reliable biomarker in terms of seasonal effects to natural variation that alter MT induction separately from metal exposure, and methodological inconsistencies. Therefore, a key overarching aim of this thesis was to address these issues, and evaluate the potential of, and limits to, using MT as a biomarker for metal pollution in selected marine organisms. The outcome of this will help to constrain and refine the use of MT in biomonitoring studies and international monitoring programmes.

2.10.1 Chapter 3: Effects of organism preparation in metallothionein and metal analysis in marine invertebrates for biomonitoring metal pollution

Due to a lack of a standard method for the treatment of organisms before MT analysis, there is potential for methodological inconsistencies to alter MT concentrations. This may mask true MT responses. In order to increase the robustness of the use of MT as a biomarker, these issues need to be addressed and resolved. A consistent approach needs to be identified in order for the use of MT to be relied upon in biomonitoring studies and international monitoring programmes.

An initial study aimed to determine if different pre-treatments, such as targeting the digestive gland or whole soft tissue, transportation method, depuration, and storage temperature are significant enough to affect MT and metal concentrations, and to recommend the most appropriate methods.

The objectives to achieve this were to:

- Actively sample *M. edulis* from Southampton Water, whereby organisms are transplanted from a reference site to the study site and sampled thereafter.
- Apply different pre-treatments to organisms including transportation on ice, transportation in seawater, depuration, and rapid dissection in the field and determine if they affect MT and metal concentrations significantly.
- Store dissected organisms at -20°C and -80°C and determine if samples suffer MT degradation and lower storage temperatures.
- Measure MT and metals in both the digestive gland and whole tissue and analyse the differences between each biological matrix.

From these objectives, the most appropriate pre-treatments for MT analysis were recommended which can be implemented into MT biomarker studies and international monitoring protocols. These findings have been published (Oaten et al., 2015), and employed in the continuation of this project.

2.10.2 Chapter 4: Seasonal effects on metallothionein responses to metal exposure in a naturalised population of *Ruditapes philippinarum* in a semi-enclosed estuarine environment

R. philippinarum is a lesser-studied bivalve for metal biomonitoring in the marine environment. Its use offers a widely available bioindicator, as this species inhabits coastal regions in the Indian-Pacific region, to which it is native, and Atlantic and Mediterranean coasts. However, as it is a non-native species in the UK, seasonal effects to MT responses are not fully understood at this latitude, hindering its use as a bioindicator.

This chapter aimed to evaluate the potential of *R. philippinarum* as a MT biomarker species at the northernmost extent of its distribution, and determine seasonal effects to MT concentrations. It also aimed to evaluate the risk of its consumption relating to tissue metal concentrations in Poole Harbour.

The objectives to achieve this were to:

- Sample *R. philippinarum* from sites with varying degrees of metal contamination in Poole Harbour across seasonal boundaries, for one year.
- Record environmental parameters and physiological changes to *R. philippinarum* that are known to vary seasonally.
- Measure MT and metal concentrations within tissue, and sediment metal concentrations from each site.
- Determine if concentrations of MT and metals are related, or alter due to seasonal changes (environmental and physiological).
- Determine if the concentrations of metals within *R. philippinarum* are of concern to human health if consumed.

This helps establish the potential of *R. philippinarum* as a widely available bioindicator species. The risk of metal pollution to humans through ingestion of seafood from Poole Harbour is also considered.

2.10.3 Chapter 5: Metallothionein responses in sympatric populations of *Ruditapes philippinarum*, *Ruditapes decussatus* and *Venerupis corrugata* using active sampling

This study aimed to assess differences in metal accumulation patterns and MT response of native and introduced species of clams in the natural environment, which as yet has not been addressed thoroughly in the literature. The causes of MT variability are investigated and the limits of use for MT as a biomarker in each species are evaluated in two locations with different levels of environmental quality and contamination status. Poole Harbour presents an opportunity to address this issue, as it is one of the northernmost locations where *R. philippinarum* has naturalised, and lives in sympatry with two native species of clam (*R. decussatus* and *V. corrugata*).

The objectives to achieve this were to:

- Actively sample *R. philippinarum*, *R. decussatus*, and *V. corrugata* in two sites of contrasting contamination in Poole Harbour, sampled after 4 weeks of exposure.
- Measure MT and metal concentrations within tissue and determine if there are significant differences in response to metal contamination.

This further evaluates the potential and limits of *R. philippinarum* as a widely available bioindicator species. It also provides a useful assessment of alternative native bioindicator clam species.

2.10.4 Chapter 6: Metal accumulation and metallothionein response in *Fucus spiralis*

Despite the extensive use of seaweeds as a bioindicator for metal pollution, there is a lack of literature on seaweeds as a MT biomarker species. There is potential for species such as spiral wrack (*Fucus spiralis*) to be a sentinel species for MT biomarker studies due to its wide availability and ease of sampling.

This chapter aimed to investigate the potential of *F. spiralis* as a valuable MT biomarker species.

The objectives to achieve this were to:

- Sample *F. spiralis* from sites with varying degrees of metal contamination within Poole Harbour throughout a period of one year.
- Measure tissue MT and metal concentrations and analyse the relationship between them.

F. spiralis could offer a cosmopolitan bioindicator species for dissolved metal pollution if MT is shown to be a reliable biomarker in this species.

2.10.5 Chapter 7: Is metallothionein a worthwhile biomarker of metal pollution?

The aim of this chapter was to reflect upon the reliability of MT as a biomarker for metal pollution by assessing its agreement with biomarker criteria, and to determine if it is a worthwhile component of biomonitoring studies. The contribution of the work in this thesis in refining the use of MT, and its applicability to international biomonitoring protocols, is also discussed.

Chapter 3: Effects of organism preparation in metallothionein and metal analysis in marine invertebrates for biomonitoring of marine pollution

3.1 Introduction

Anthropogenic metal pollution is prevalent throughout much of the world's coastal and estuarine environments, due to urbanisation and the industries that frequently surround them (Boldina-Cosqueric et al., 2010). Concern for marine metal pollution is reflected in international legislation such as the European Water Framework Directive (WFD; 2000/60/EC) and the European Marine Strategy Framework Directive (MSFD; 2008/56/EC). Their ultimate objectives are to protect and enhance the quality of all water bodies including coastal waters and estuaries, achieving 'good ecological and chemical status' for all European water bodies by 2015 (Marin-Guirao et al., 2005, Solaun et al., 2013). This is to be achieved, in part, by eliminating 'priority hazardous substances', and contributing to achieving concentrations of naturally occurring substances in the marine environment near to background values (Tueros et al., 2008). Thirty-three priority substances are listed under an amendment to the WFD (Environmental Quality Standards Directive 2008/105/EC), including Cd, Hg, tributyltin (TBT), Ni, and Pb. Other relevant legislation addressing water quality and pollution includes the Bathing Waters (76/160/EEC) and Fish (78/659/EEC) Directives, as well as those based on substances or sources of pollution such as Urban Wastewater (91/271/EEC) and Integrated Pollution Prevention Control (IPPC) (96/61/EC) Directives (Allan et al., 2006). The Shellfish Waters (79/923/EEC) and Dangerous Substances (76/464/EC) Directives have been repealed and requirements transferred to the WFD in 2013 (Boyes and Elliott, 2014).

The monitoring of xenobiotics (foreign substances) in biological tissue, or biomonitoring, provides a synoptic measure of exposure to elements of direct ecotoxicological relevance, and allows spatial and temporal derivations to be examined more readily than traditional techniques (Rainbow, 1995a). Many species are well known bio-accumulators and are characterised by efficient detoxification methods, such as intracellular compartmentalisation, or metal inactivation by binding to protein molecules (Demuynck et al., 2004). This can be complemented with the use of biomarkers. An ecotoxicological biomarker is: 'a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical

pollutants (and/or radiations)' (Depledge, 1994). Biomarkers are regarded as early warning signals of pollution as changes at molecular level occur at a threshold less toxic than levels that can be detected by monitoring change in a species, population or community (Won et al., 2008). Metallothionein-like proteins (MTLPs) are non-enzymatic proteins with a low molecular weight, high cysteine content, and good heat stability that can be used as biomarkers (Langston et al., 1998). They consist of thiol groups (sulphur-hydrogen) that bind to metals, preventing oxidative stress to the organism (Amiard et al., 2006). Metallothionein (MT) induction as a response to metal exposure is well documented in many species and is known to play a role in the detoxification of toxic metals (Amiard et al., 2006). It is included as part of a core suite of biomarkers recognized at European level in the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM), and the Mediterranean Action Plan for the Barcelona Convention (MED POL) (Amiard et al., 2006). It is also part of Natural England's suite of assays (Galloway et al., 2008). However, many factors, such as animal size and weight, life stage, and environmental conditions can affect quantitative relationships between metal exposure and MT response. The results delivered can be ambiguous and usually advised cautiously as indicators of exposure rather than predictors of risks (Forbes et al., 2006, Aly et al., 2014).

Inconsistencies exist for the treatment of organisms before analysis of MT and metals (hereby referred to as pre-treatment) at almost every stage, from collection and transportation to the laboratory, to initial preparation of the samples and storage (Table 5). Close examination of Table 5 highlights the lack of comparability across studies and sites, as no studies use the exact same combination of pre-treatments (or they are not specified). This could contribute to ambiguity of MT results. Davies and Vethaak (2012), on behalf of the International Council for the Exploration of the Sea (ICES), offered guidance on methodologies for biological sampling. It is explained that care must be taken when sampling and transporting specimens, a 24-hour time frame of preparation of organisms (dissection etc.) before analysis should be adhered to, and that specimens should be frozen in liquid nitrogen before analysis. However, the effects and testing of these pre-treatment procedures on MT is still lacking.

Within the field of biomonitoring, the issue of dissection and tissue selection remains. The 'biomarker response index' (BRI), described by Hagger et al. (2008), aimed to create a comparative format for MT, yet used different tissues for MT analysis. UNEP/RAMOGE (1999) recommend using the digestive gland for measuring MT and its use is generally preferred as it contains high basal levels of MT due to the storage of metals (Geffard et al., 2001). However, seasonal mass changes of digestive gland, due to food availability and sexual maturation (gametogenesis), may cause concentrations of MT (and metals) to vary independently of metal

Table 5 Metallothionein studies highlighting inconsistencies in pre-treatments of organisms before analysis.

Organism	Study	Transport	Depuration	Storage	Storage Time	Tissue	Reference
Cerastoderma edule	Field	Not specified	No	-80°C	Not specified	Whole tissue	Galloway et al., (2004b)
Cerastoderma edule, Haliclona oculata	Field	Ice	No	-20°C	Not specified	Whole tissue	Aly et al. (2014)
Crassostrea gigas	Field	Ice	No	-20°C	Not specified	Gills	Geffard et al. (2002)
Dreissena ploymorpha	Laboratory	N/A	N/A	N/A	N/A	Whole tissue	Lecoeur et al. (2004)
Mytilus edulis	Field	Not specified	No	Not specified	Not specified	Digestive gland, gills	Geffard et al. (2005)
Mytilus edulis	Field	Seawater	Yes (local seawater)	Analysed within 24h	N/A	Gills	Hagger et al. (2008)
Mytilus edulis, Patella vulgata	Laboratory	N/A	N/A	-80°C	Not specified	Whole tissue	Brown et al. (2004)
Mytilus galloprovincialis	Field	Not specified	No	Liquid nitrogen	Not specified	Digestive gland, gills	Bodin et al. (2004)
Mytilus galloprovincialis	Field	Not specified	Yes (24h)	-80°C	Not specified	Digestive gland	Raspor et al. (2004)
Mytilus galloprovincialis	Field	Dissected, liquid nitrogen	No	-80°C	Not specified	Digestive gland	Ivankovic et al. (2005)
Mytilus galloprovincialis	Field	Dissected within 3h	No	Not specified	Not specified	Digestive gland	Pytharopoulou et al. (2006)
Mytilus galloprovincialis	Field	Dissected, liquid nitrogen	No	Liquid nitrogen	Not specified	Digestive gland	Zorita et al. (2005)
Mytilus galloprovincialis	Field	Not specified	No	Not specified	Not specified	Digestive gland, gills, whole tissue	Mourgaud et al. (2002)
Mytilus galloprovincialis	Field	Ice	No	-80°C	Not specified	Digestive gland	Damiens et al. (2007)
Mytilus galloprovincialis	Field	Not specified	No	Not specified	Not specified	Digestive gland	Serafim et al. (2011)
Mytilus galloprovincialis	Laboratory	N/A	N/A	Not specified	Not specified	Digestive gland, gills, mantle	Zorita et al. (2007b)
Mytilus galloprovincialis	Laboratory	N/A	N/A	N/A	N/A	Gills	Serafim et al. (2002)
Mytilus galloprovincialis	Laboratory	N/A	N/A	-80°C	Not specified	Gills	Hamer et al. (2008)
Mytilus galloprovincialis, Adamussium colbecki	Laboratory	N/A	N/A	70°C (typographical error in paper)	Not specified	Digestive gland, gills	Viarengo et al. (1997)
Mytilus galloprovincialis, Ruditapes philippinarum	Field	Dissected, liquid nitrogen	No	-80°C	Not specified	Digestive gland	Bocchetti et al. (2008)
Ruditapes decussatus	Field	Not specified	Yes (24h)	-20°C	Not specified	Gills, digestive gland	Geret et al. (2003)
Ruditapes decussatus	Field	Not specified	No	-20°C	Not specified	Gills	Smaoui-Damak et al. (2004)
Ruditapes decussatus	Field	Not specified	No	-75°C	Not specified	Digestive gland	Hamza-Chaffai et al. (2000)
Ruditapes philippinarum, Ruditapes decussatus	Field/ Laboratory	Ice	Both	-80°C	Not specified	Whole tissue	Freitas et al. (2012)
Scrobicularia plana	Field	Ice	Yes (24h)	-80°C	Not specified	Whole tissue	Boldina-Cosqueric et al. (2010)

exposure (Raspor et al., 2004). Depuration is also often cited as a sensible step when measuring metal tissue residues in organisms in order to purge any metals residing in the gut of the organism. Freitas et al. (2012) tested the effects of depuration on clam species and after 2 days element concentrations reduced significantly. MT response after depuration was also measured and showed reductions, though non-significant. Serafim and Bebianno (2007b) found similar results. However, it remains unclear if depuration can disrupt the relationship between metals and MT, which is important for biomonitoring purposes.

Transporting organisms from the study site to the laboratory may have an impact on MT as a result of normal physiological processes (Izagirre et al., 2008, Vidal-Linan et al., 2010). The most widely-used method of keeping organisms during transportation to the laboratory is in an isothermic container, on ice. Other methods include transportation in local seawater, and to dissect the animal in the field immediately after sampling, preventing MT concentration from altering (Marigomez et al., 2013). Freezing organisms with liquid nitrogen in the field has been used on some occasions (Table 5); however it will not be addressed in this study as it is an expensive and has relatively limited availability, and therefore is not feasible to implement as standard in monitoring studies. The majority of studies will require storage of organisms before the tissue can be analysed. The temperature at which MT degradation is prevented is not explicitly defined, however, most studies keep samples at, or close to, -80°C. There are exceptions to this, for example, Geffard et al. (2002), Geret et al. (2003), and Smaoui-Damak et al. (2004) kept samples at -20°C. Storing samples at higher temperatures has obvious advantages, such as less energy cost and use of unspecialized, easily available equipment.

This study aims to determine if different pre-treatments, such as, transportation method, depuration, and storage temperature are significant enough to affect measures of MT and metal accumulation response, and to further address the issue of tissue dissection. It also aims to recommend the most appropriate pre-treatments to identify a consistent approach in order for MT to be a more useful biomarker.

3.2 Methodology

3.2.1 Active sampling and pre-treatment

Seven hundred, common mussels (*Mytilus edulis*), each 45 to 50mm long, from Poole Harbour, United Kingdom, were actively sampled from the National Oceanography Centre Southampton (NOCS), in early February 2014. *M. edulis* were used as they are one of the most widely studied organisms in MT studies. They were collected from permanently submerged cages secured from a

pontoon on two occasions, four and eight weeks after deployment, over the winter-spring transition, in March and April. A number of different pre-treatments were applied to each sample. Two groups were transported from field to laboratory on ice in an isothermic container, or submersed in seawater without aeration at ambient temperature, a process that took approximately three hours. A group of organisms was also left to depurate in filtered, UV treated seawater for 24 hours, as this is the time period used by previous studies (Table 5), before transportation on ice. A final group was dissected in the field before transportation to the laboratory on ice in an isothermic container. Once in the laboratory, *M. edulis* from each sample were divided into three replicates with a minimum of nine individuals per replicate. Collective whole weight, including shell, was first measured, except for organisms dissected in the field. The digestive glands and remaining tissue were then dissected using a ceramic blade, blotted dry, and weighed. They were then stored at either -20℃ or -80°C for ten weeks before analysis.

3.2.2 Metallothionein analysis

A modified spectrophotometric method, as described by Viarengo et al. (1997) with modifications by Aly et al. (2014), was used to measure MTs in *M. edulis*. This is a sensitive, time saving, and low cost technique that has been inter-calibrated and standardized by a number of laboratories (UNEP/RAMOGE, 1999, Zorita et al., 2005). Frozen samples were first homogenised using a ceramic pestle and mortar. An accurately weighed sample of approximately 1g was taken from each replicate. Tissue samples were further homogenised with 3 ml homogenising buffer of 0.5M sucrose, 20mM Tris-HCl (pH 8.6), 0.006mM leupeptin, 0.5mM phenylmethylsulfonyl fluoride (PMSF), and 0.01% B-mercaptoethanol, then centrifuged for 30 minutes at 24,600 x g (times gravity) at 4°C. The supernatant solution was purified with 1.05 ml annular grade cold ethanol (-20°C) and 80 µl chloroform (per 1 ml of supernatant) and centrifuged for 10 minutes at 6000 x g at 4°C. Supernatant solution was then collected and 40 µl of 35% hydrochloric acid (HCl) and three times the supernatant volume of annular grade cold ethanol was added and left to allow the proteins to denature for at least one hour at -20°C. This was centrifuged at 6000 x g for 10 minutes and the pellet saved. The pellet was washed with the previously described buffer (without B-mercaptoethanol, leupeptin, and PMSF), annular grade cold ethanol and 80 µl chloroform (87:12:1 v/v) and centrifuged for 10 minutes at 6000 x g. The supernatant was discarded and the pellet dried with nitrogen gas. The pellet was resuspended with 150 µl 0.25M sodium chloride (NaCl) and 150 µl 1N HCl with 4 mM EDTA. After resuspension, 4.2 ml of a solution containing 2M NaCl, 0.43mM DTNB and 0.2M sodium phosphate (NaH_2PO_2) (pH 8.0) was added, and mixed by centrifugation at 3,000 x g for 5 minutes. The absorbance was read at 412 nm against standard solutions of reduced glutathione (GSH) using a UV-visible

spectrophotometer. Whole tissue concentrations were calculated from a combination of digestive gland and remaining tissue concentration based on the ratio of component weights.

3.2.3 Metals in *M. edulis*

The metals determined were Cr, Fe, Ni, Cu, Zn, the metalloid As, Silver (Ag), Cd, Tin (Sn), and Pb due to a combination of their presence in Southampton Water and the potential for bioaccumulation. Frozen homogenised *M. edulis* replicates (as per first step in MT analysis) were freeze-dried for three days. Accurately weighed samples of approximately 10 mg of dried, ground sample were placed in 5 ml Teflon sealable pots. For digestive gland samples, 1 ml of a 3:1 v/v solution of trace metal grade, redistilled, 37% HCl and 68% nitric acid (HNO_3) (*Aqua Regia*) was added. For the remaining tissue, 1 ml of trace metal grade, redistilled, 68% HNO_3 was added. These different digestion procedures were found to have little influence on detected metal concentrations (Enamorado-Báez et al., 2013). Blank samples consisting of empty Teflon pots were also prepared. Sealed pots were then heated at 60°C for 4 hours, and then heated at 120°C for a further 12 hours on a hot plate. Hydrogen peroxide (H_2O_2) was then added to samples in 50 µl increments, and heated unsealed at 120°C until fully reacted (approximately 5 minutes). Samples were then dried on the hot plate at 120°C and resuspended with 3% HNO_3, containing 5 ppb In/Re and 20 ppb Be as internal standards to correct for matrix effects (which are a suppression or enhancement of analyte signal caused by concentrations of matrix elements) and instrument drift. Samples were then emptied and washed into vials, and completed to a 4,000-fold dilution with 3% HNO_3 containing 5 ppb In/Re and 20 ppb Be, via a two-step process. At each stage of dilution and completion, empty and full vials were weighed to calculate precise dilution factors. Analysis by inductively coupled plasma mass spectrometry (ICP-MS) was carried out. Five, matrix matched, standard solutions containing Cr, Fe, Ni, Cu, Zn, As, Ag, Cd, Sn, and Pb at known concentrations were used to calibrate and quantify metal concentrations of the samples. Results are reported as µg/g dry weight relative to original sample weight taken.

3.2.4 Statistical analysis

All statistical analysis was completed using IBM SPSS Statistic v21. Data were tested for normality (Shapiro-Wilk) and for homogeneity of variances (Levene's Test), and were tested parametrically (Paired samples T-Test, One-way ANOVA, Pearson's correlation) or non-parametrically (Wilcoxon Signed Rank test, Kruskall-Wallis test, Spearman's rank) accordingly. Statistical significance was established at $P = 0.05$. Appendix A presents additional statistical data associated with metal concentrations. Statistical data associated with MT concentrations are presented in the text.

3.3 Results

3.3.1 Metallothionein

Results are presented in Figure 5a. No significant differences existed for mean MT concentrations between samples stored at -20°C and -80°C. However, samples stored at -80°C had less variability between replicates, evidenced by coefficient of variation (Table 6). MT was affected by methods for transportation and depuration. Specimens transported in seawater and depurated before analysis revealed slightly lower concentrations of MT. For example, after four weeks, average MT concentrations in the digestive gland when transported on ice were 74.23 μg/g, whereas MT concentrations were 60.72 μg/g after being transported in seawater. Depurated specimens had MT concentrations of 65.10 μg/g in the digestive gland, and specimens that were rapidly dissected had concentrations of 67.74 μg/g. In whole tissue, the highest average MT concentrations were in specimens that were depurated at 36.41 μg/g after four weeks, unlike the digestive gland. Average MT concentrations for samples transported on ice, in seawater, and dissected rapidly in the field were 30.46 μg/g, 31.52 μg/g, and 31.89 μg/g respectively. In the digestive gland, MT concentrations in samples transported on ice were significantly different from samples transported in seawater, and samples that were depurated (One-way ANOVA, post-hoc LSD, $P = 0.004$, $P = 0.047$, respectively, based on average concentrations). Samples that were rapidly dissected were also significantly different from samples that were transported in seawater (One-way ANOVA, post-hoc LSD, $P = 0.045$, based on average concentrations). The same relationship was not observed in whole tissue, with no significant differences.

MT concentration was higher in the digestive gland than in the whole tissue and was significantly different (Paired samples T-Test, $P < 0.001$). There was a significant positive correlation between MT in the digestive gland and MT in whole tissue (Pearson's correlation = 0.386, $P = 0.013$) (Figure 6a). A linear equation was applied to this relationship (Equation 1). However, the relationship between digestive gland MT and whole tissue MT varied depending on the pre-treatment applied. When depuration was applied, no correlation existed. When transportation in seawater was applied, weak insignificant positive correlations existed. However, samples that underwent transportation on ice exhibited a stronger significant positive correlation between digestive gland MT and whole tissue MT (Pearson's correlation = 0.647, $P = 0.031$) (Figure 6b). A linear equation was applied to this relationship (Equation 2). Samples that were rapidly dissected in the field also showed positive correlations, though non-significant.

$$\text{MT (whole tissue)} = 0.25*\text{MT (digestive gland)} + 17.71 \ (R^2 = 0.149) \qquad \text{(Equation 1)}$$

$$\text{MT (whole tissue)} = 0.34*\text{MT (digestive gland)} + 8.91 \ (R^2 = 0.216) \qquad \text{(Equation 2)}$$

3.3.2 Metals

Results are presented in Figure 5b-k. Similarly to MT, depuration or transportation in seawater resulted in generally lower concentrations of most metals in specimens, compared to transportation on ice or rapid dissection (Appendix A, Table 18 and 19). In the digestive gland, Pb concentrations in specimens transported in seawater and depurated were significantly lower than specimens transported on ice and rapidly dissected. This was also the case for Cr and Fe in the digestive gland, though Fe was not significantly lower in specimens transported in seawater compared with transportation on ice. For Ni in the digestive gland, rapid dissection in the field resulted in significantly higher concentrations in specimens than after depuration and transportation in seawater. For Cu in the digestive gland, depuration and transportation in seawater resulted in significantly lower concentrations than transportation on ice and rapid dissection. Depurated specimens were also significantly lower in Cu than specimens transported in seawater. Depurated specimens were significantly less concentrated than all other pre-treatments for As. Ag was significantly less concentrated in the digestive gland following transportation in seawater compared to all other treatments. For Sn, transportation in seawater and depuration resulted in significantly lower concentrations in the digestive gland compared to transportation on ice. Zn and Cd had no significant differences between pre-treatments in the digestive gland. In whole tissue, every metal correlated with digestive gland metal concentrations (Pearson's correlation, Spearman's rank). However, Ni, Zn, As, and Cd had relatively weak positive correlations.

Similarly to MT, metals were more concentrated in the digestive gland compared to whole tissue, and were significantly different for all metals, except Zn that exhibited little difference in concentrations between the digestive gland and whole tissue (Appendix A, Table 20 and 21).

3.3.3 Metallothionein and metals

Relationships between MT and some metals were tested to establish which metals were promoting MT production (Table 7). Cr, and Sn in the digestive gland and whole tissue had significant positive correlations with MT in the digestive gland. Ni, Cu (Figure 6c), Zn, Ag, Cd, and Pb all had significant positive correlations with MT in the digestive gland, and metals in whole tissue. Fewer metals correlated with MT in whole tissue. Ag in both the digestive gland and whole tissue positively correlated with MT in whole tissue. Cd and As in the digestive gland also correlated with MT in whole tissue, positively and negatively, respectively.

Correlations between MT and metals between different pre-treatments were analysed to obtain which exhibited the most, or strongest, correlations and therefore which was the most

appropriate pre-treatment (Table 8-10). Non-parametric (Spearman's rank) positive correlations were observed for Cr and Ag in the digestive gland when samples were depurated. For samples transported on ice, significant positive correlations existed between Cu (Figure 6d), and Pb in whole tissue and MT in the digestive gland. For MT in whole tissue Ag in both the digestive gland and whole tissue positively correlated. Significant negative correlations existed with As in the digestive gland and MT in both the digestive gland and whole tissue. For samples rapidly dissected in the field, Cr and Fe in whole tissue significantly positively correlated with MT in the whole tissue. Pb in the digestive gland also had a significant positive correlation with MT in whole tissue. Digestive gland As had a significant negative correlation with MT in whole tissue.

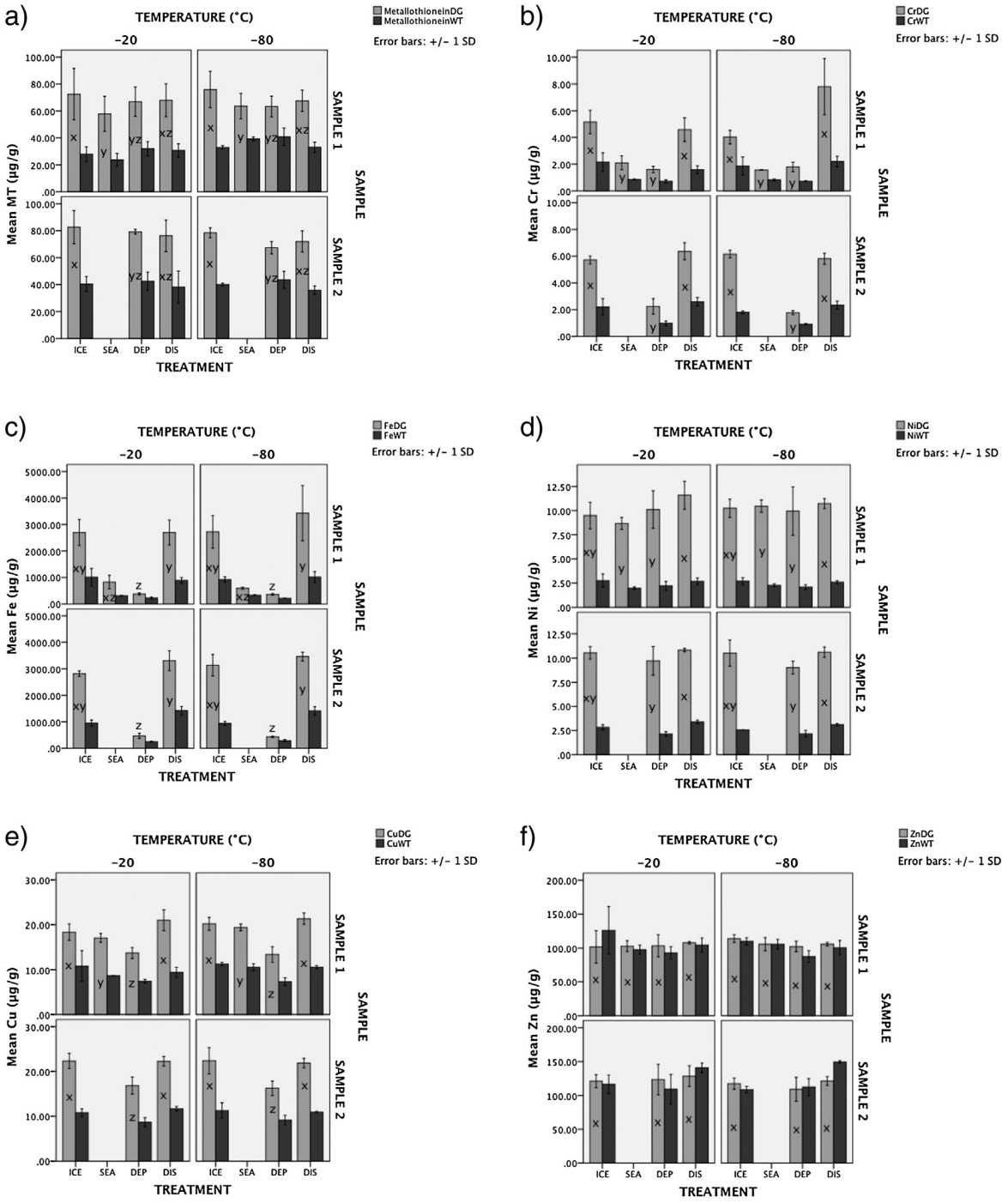


Figure 5 Mean concentrations (µg/g) for different pre-treatments (non-matching letters denote significant differences in digestive gland concentrations averaged over storage temperatures and sample date (P = 0.05)), sample dates and storage temperatures with standard deviation (n = 3) for a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn and k) Pb. NB: ICE, SEA, DEP and DIS is samples transported on ice, in seawater, depurated, and rapidly dissected, respectively, DG is digestive gland, WT is whole tissue, SAMPLE 1 is after 4 weeks, SAMPLE 2 is after 8 weeks.

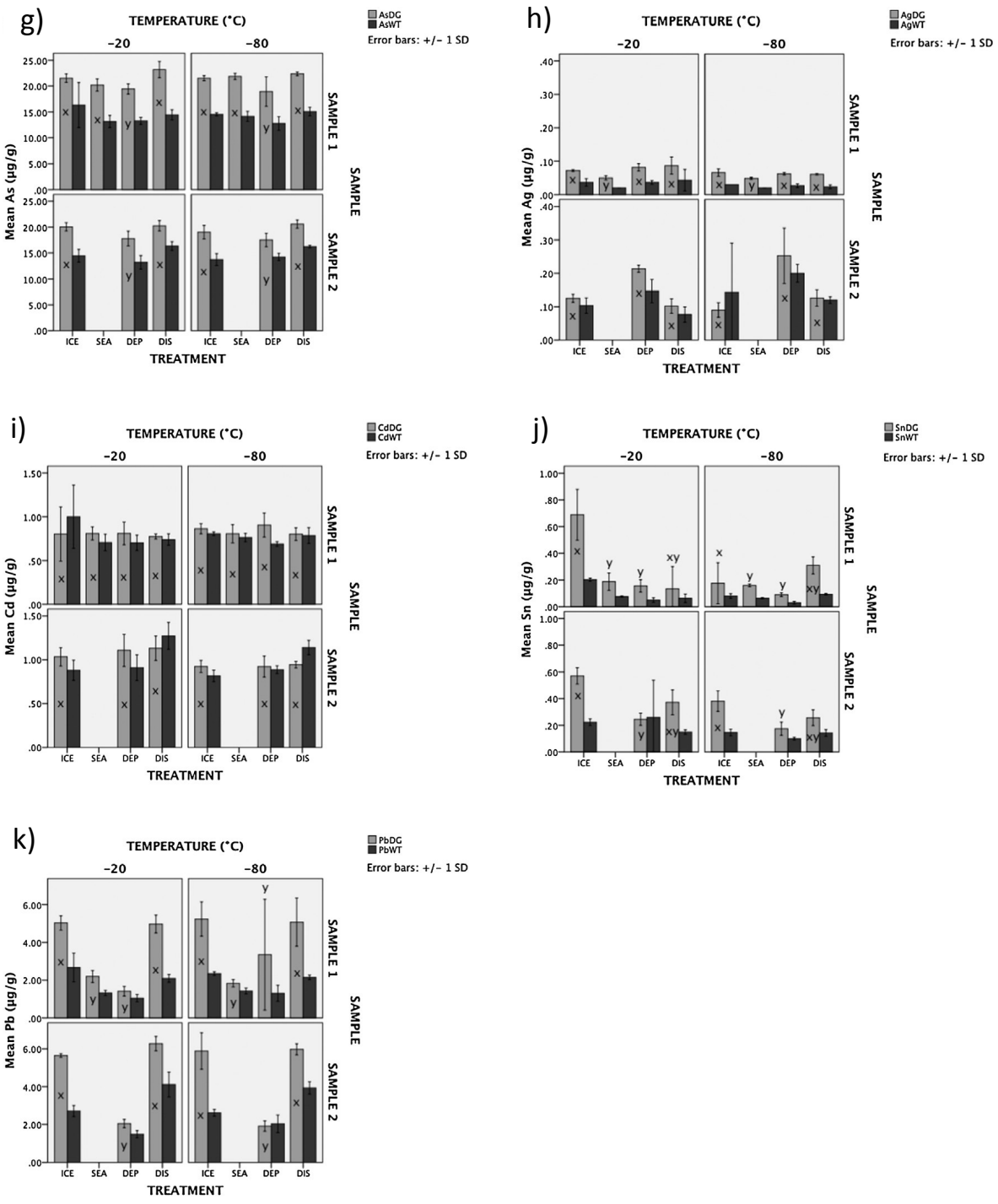


Figure 5 cont. Mean concentrations (µg/g) for different pre-treatments (non-matching letters denote significant differences in digestive gland concentrations averaged over storage temperatures and sample date (P = 0.05)), sample dates and storage temperatures with standard deviation (n = 3) for a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn and k) Pb. NB: ICE, SEA, DEP and DIS is samples transported on ice, in seawater, depurated, and rapidly dissected, respectively, DG is digestive gland, WT is whole tissue, SAMPLE 1 is after 4 weeks, SAMPLE 2 is after 8 weeks.

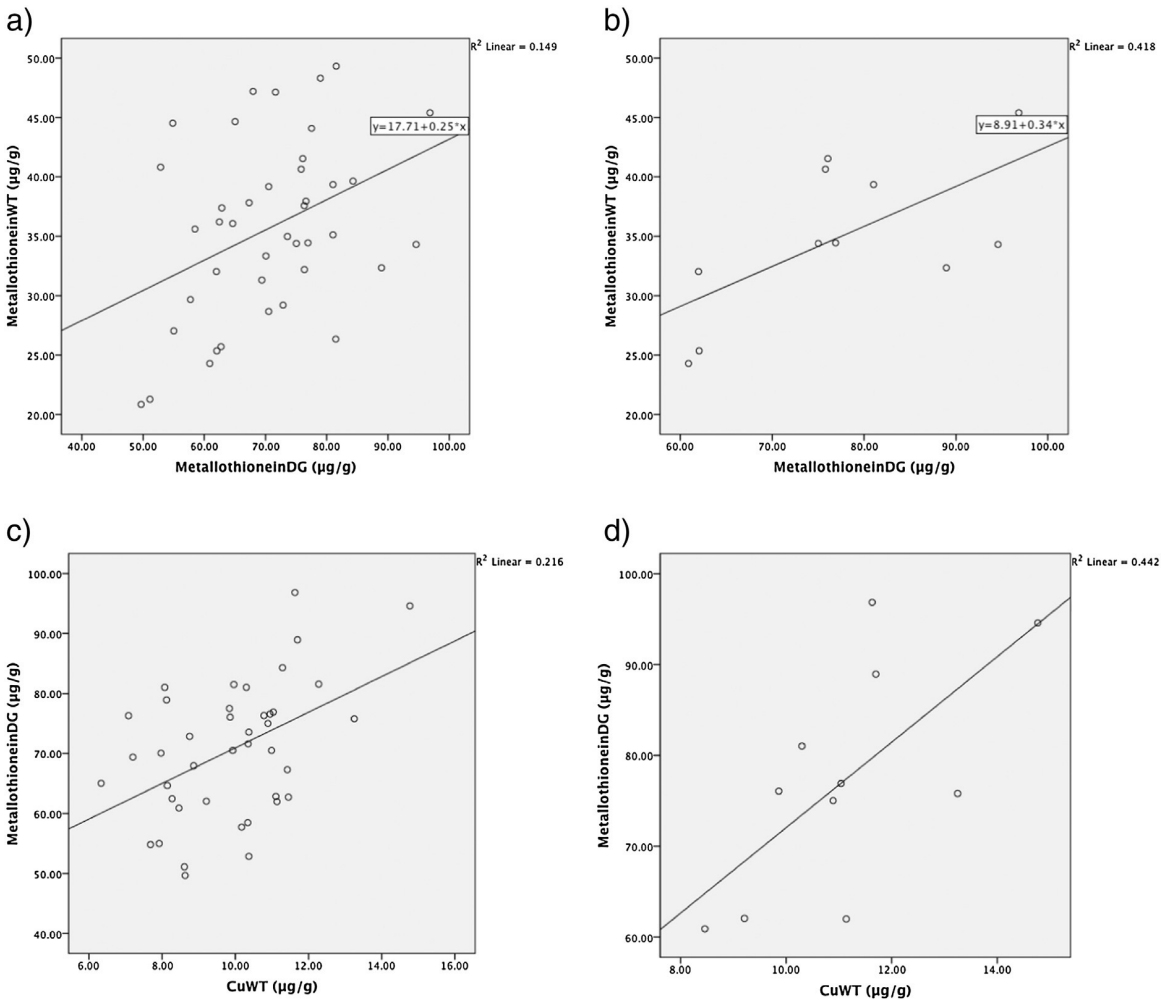


Figure 6 Linear relationships between a) MT in digestive gland and whole tissue for all samples with linear equation, b) MT in digestive gland and whole tissue for samples transported on ice with correction equation, c) Cu in whole tissue and MT in digestive gland for all samples, and d) Cu in whole tissue and MT in digestive gland for samples transported on ice. NB: DG is digestive gland, WT is whole tissue.

Table 6 Coefficient of variance (%) for MT concentrations between storage temperatures for each pre-treatment. NB: ICE, SEA, DEP, DIS is samples transported on ice, in seawater, depurated, and rapidly dissected, respectively, DG is digestive gland, WT is whole tissue.

Temperature	ICE DG	ICE WT	SEA DG	SEA WT	DEP DG	DEP WT	DIS DG	DIS WT
-20 °C	26.38	19.68	22.44	19.80	16.25	16.60	17.98	16.13
-80 °C	17.78	3.99	14.81	3.81	12.26	15.91	11.81	11.59

Table 7 Correlations (Pearson's correlation coefficient, or Spearman's rank correlation coefficient) between MT and metal concentrations in digestive gland (DG) and whole tissue (WT) for all samples (P = 0.05, significant results are bold and marked with *).

Metal concentration		MT concentration DG		MT concentration WT	
		Correlation	Significance	Correlation	Significance
Cr	DG (Spearman's rank)	0.376*	**0.016***	-0.015	0.924
	WT (Spearman's rank)	0.320*	**0.041***	-0.043	0.789
Fe	DG (Spearman's rank)	0.215	0.177	-0.101	0.530
	WT (Spearman's rank)	0.274	0.083	-0.044	0.783
Ni	DG (Pearson's correlation)	0.170	0.288	-0.107	0.504
	WT (Pearson's correlation)	0.394*	**0.011***	0.036	0.823
Cu	DG (Pearson's correlation)	0.292	0.062	-0.099	0.540
	WT (Pearson's correlation)	0.456*	**0.002***	0.093	0.562
Zn	DG (Pearson's correlation)	0.288	0.067	0.150	0.348
	WT (Spearman's rank)	0.454*	**0.003***	0.097	0.548
As	DG (Pearson's correlation)	-0.086	0.593	-0.522*	**<0.001***
	WT (Spearman's rank)	0.281	0.075	-0.050	0.755
Ag	DG (Spearman's rank)	0.295	0.061	0.395*	**0.021***
	WT (Spearman's rank)	0.361*	**0.020***	0.396*	**0.010***
Cd	DG (Pearson's correlation)	0.264	0.095	0.340*	**0.030***
	WT (Spearman's rank)	0.467*	**0.002***	0.219	0.169
Sn	DG (Spearman's rank)	0.382*	**0.014***	-0.022	0.891
	WT (Spearman's rank)	0.425*	**0.006***	0.123	0.443
Pb	DG (Spearman's rank)	0.291	0.065	-0.033	0.837
	WT (Spearman's rank)	0.367*	**0.018***	0.049	0.763

Table 8 Correlations (Pearson's correlation coefficient, or Spearman's rank correlation coefficient) between MT and metal concentrations in digestive gland (DG) and whole tissue (WT) for samples transported on ice (P = 0.05, significant results are bold and marked with *).

Metal concentration		MT concentration DG		MT concentration WT	
		Correlation	Significance	Correlation	Significance
Cr	DG (Spearman's rank)	0.255	0.450	0.573	0.066
	WT (Spearman's rank)	0.173	0.612	0.164	0.631
Fe	DG (Pearson's correlation)	0.093	0.787	0.480	0.135
	WT (Pearson's correlation)	0.516	0.104	0.276	0.412
Ni	DG (Pearson's correlation)	0.044	0.897	0.478	0.137
	WT (Pearson's correlation)	0.457	0.158	0.413	0.206
Cu	DG (Pearson's correlation)	0.006	0.985	0.553	0.078
	WT (Pearson's correlation)	0.665*	**0.026***	0.419	0.200
Zn	DG (Pearson's correlation)	0.052	0.879	0.372	0.259
	WT (Spearman's rank)	0.555	0.077	0.009	0.979
As	DG (Pearson's correlation)	-0.667*	**0.025***	-0.915*	**<0.001***
	WT (Spearman's rank)	0.482	0.133	-0.155	0.650
Ag	DG (Pearson's correlation)	0.178	0.600	0.620*	**0.042***
	WT (Spearman's rank)	0.351	0.290	0.727*	**0.011***
Cd	DG (Spearman's rank)	-0.155	0.650	0.318	0.340
	WT (Spearman's rank)	0.436	0.180	0.045	0.894
Sn	DG (Pearson's correlation)	-0.294	0.381	-0.244	0.470
	WT (Pearson's correlation)	0.016	0.963	0.111	0.746
Pb	DG (Spearman's rank)	-0.018	0.958	0.527	0.096
	WT (Pearson's correlation)	0.710*	**0.014***	0.446	0.169

Table 9 Correlations (Pearson's correlation coefficient, or Spearman's rank correlation coefficient) between MT and metal concentrations in digestive gland (DG) and whole tissue (WT) for depurated specimens (P = 0.05, significant results are bold and marked with *).

Metal concentration		MT concentration DG		MT concentration WT	
		Correlation	Significance	Correlation	Significance
Cr	DG (Spearman's rank)	0.746*	**0.005***	0.000	1.000
	WT (Pearson's correlation)	0.360	0.250	0.075	0.817
Fe	DG (Pearson's correlation)	0.446	0.146	-0.080	0.805
	WT (Pearson's correlation)	0.020	0.951	0.291	0.359
Ni	DG (Pearson's correlation)	-0.005	0.987	-0.587*	**0.045***
	WT (Spearman's rank)	-0.014	0.966	-0.375	0.230
Cu	DG (Pearson's correlation)	0.453	0.139	0.096	0.766
	WT (Pearson's correlation)	0.243	0.447	0.360	0.251
Zn	DG (Pearson's correlation)	0.258	0.418	0.185	0.566
	WT (Pearson's correlation)	0.291	0.358	0.370	0.236
As	DG (Pearson's correlation)	-0.088	0.786	-0.534	0.074
	WT (Pearson's correlation)	-0.071	0.826	0.039	0.905
Ag	DG (Spearman's rank)	0.613*	**0.034***	0.294	0.353
	WT (Spearman's rank)	0.363	0.246	0.328	0.298
Cd	DG (Pearson's correlation)	0.205	0.524	0.464	0.128
	WT (Pearson's correlation)	0.290	0.360	0.454	0.138
Sn	DG (Pearson's correlation)	0.568	0.054	0.090	0.780
	WT (Spearman's rank)	0.395	0.204	0.420	0.174
Pb	DG (Spearman's rank)	0.287	0.366	0.196	0.542
	WT (Pearson's correlation)	0.028	0.931	0.162	0.614

Table 10 Correlations (Pearson's correlation coefficient, or Spearman's rank correlation coefficient) between MT and metal concentrations in digestive gland (DG) and whole tissue (WT) for samples rapidly dissected in the field (P = 0.05, significant results are bold and marked with *).

Metal concentration		MT concentration DG		MT concentration WT	
		Correlation	Significance	Correlation	Significance
Cr	DG (Pearson's correlation)	-0.230	0.472	0.364	0.245
	WT (Pearson's correlation)	0.171	0.596	0.653*	**0.021***
Fe	DG (Pearson's correlation)	-0.214	0.504	0.566	0.055
	WT (Pearson's correlation)	0.242	0.449	0.614*	**0.034***
Ni	DG (Spearman's rank)	-0.364	0.245	-0.392	0.208
	WT (Pearson's correlation)	0.319	0.312	0.392	0.207
Cu	DG (Pearson's correlation)	0.365	0.243	-0.364	0.245
	WT (Spearman's rank)	0.308	0.331	0.315	0.319
Zn	DG (Spearman's rank)	0.357	0.255	0.049	0.880
	WT (Pearson's correlation)	0.468	0.125	0.321	0.310
As	DG (Pearson's correlation)	-0.251	0.431	-0.720*	**0.008***
	WT (Pearson's correlation)	0.271	0.394	0.146	0.650
Ag	DG (Pearson's correlation)	-0.086	0.791	0.152	0.637
	WT (Spearman's rank)	0.060	0.853	0.254	0.425
Cd	DG (Spearman's rank)	0.510	0.090	0.231	0.471
	WT (Pearson's correlation)	0.515	0.087	0.503	0.096
Sn	DG (Spearman's rank)	0.308	0.331	0.014	0.966
	WT (Pearson's correlation)	0.456	0.136	0.242	0.449
Pb	DG (Pearson's correlation)	0.017	0.959	0.580*	**0.048***
	WT (Pearson's correlation)	0.389	0.212	0.480	0.114

3.4 Discussion

3.4.1 Metallothionein and metals

3.4.1.1 Storage temperatures

Samples stored at -20℃ or -80℃ showed no significant differences in mean MT concentrations after ten weeks of storage and therefore it is not necessary to store samples in -80℃ temperature freezers or liquid nitrogen, which may be difficult to source, expensive to run and fuel intensive. As well as this, previous studies with differing storage measures can now be directly compared with confidence, providing other pre-treatments that were applied are similar. However, inter-sample variability of MT increases when samples are stored at -20°C compared with -80°C, probably due to protein degradation, but this does not cause a significant difference to overall mean concentrations after storage periods less than ten weeks. If other biomarkers are to be measured in conjunction with MT, such as oxidative stress markers, storage at -80°C may be appropriate.

3.4.1.2 Digestive gland vs. whole tissue

A relationship was apparent between MT concentrations in the digestive gland compared with whole tissue, highlighted by a significant positive correlation. Equation (1) can be used to relate each matrix and is correct to a fairly low accuracy of ±45%, due to a low correlation coefficient (Pearson's correlation coefficient = 0.386) from which the equation is derived. However, a stronger positive relationship was found between MT in each biological matrix when specimens were transported on ice, and Equation (2) can be used to calculate MT concentrations to an accuracy of ±22%, due to a higher correlation coefficient (Pearson's correlation coefficient = 0.647) from which the equation is derived. In order to increase comparability between studies, these formulae can be applied to previous results that use varying biological matrices. This concept was tested on a study that recorded both digestive gland and whole tissue MT concentrations (Mourgaud et al., 2002). Equation (1) calculated MT concentrations from each biological matrix to an accuracy of ±37%, while Equation (2) calculated MT concentrations to a higher accuracy of ±17%. This study did not specify which pre-treatment was used. These equations can be improved, or tailored, by follow-up studies in different environments and levels of contamination. In general, metals followed a very similar pattern in whole tissue compared to digestive gland, with significant positive correlations. With the exception of Zn, metal concentrations in the digestive gland were significantly higher. This is expected as metals are accumulated via ingestion and are stored in the digestive gland before absorption into the rest of

the tissue. Zn however may accumulate more in the gills according to Zorita et al. (2007b), which may explain the similar concentrations between whole tissue and digestive gland.

3.4.1.3 Pre-treatments

MT concentrations were not uniform across pre-treatments. The highest concentrations in the digestive gland were found in specimens that were transported on ice, and lowest were found in specimens that were transported in seawater or depurated. This is in agreement with a study on depurating clam species by Freitas et al. (2012). It is likely that transportation in seawater effectively acted to depurate specimens as they were left in seawater before dissection. This allowed the gut contents of mussels to be purged. These processes may have simultaneously caused soluble elements to reduce, leaving high levels of elements in the insoluble fraction (metal-rich granules, cellular debris and organelles) that plays a role in detoxification, leaving an unavailability of elements for MT stimulation (Freitas et al., 2012). It may also be possible that reduced metal exposure during transportation, and 24h depuration period, was sufficient for MT induction to be reduced. As well as this, oxidative stress in depuration conditions may be lower than in the environment, and as MTs are known to assist in the reduction of oxidative stress by acting as reactive oxygen species (ROS) scavengers, this could explain their reduction (Freitas et al., 2012). Metal concentrations reacted in a similar way to MT to the various pre-treatments, with decreases observed after depuration and transportation in seawater. This can be explained as metals present in the gut are expelled as clean seawater flushed through the organism. Within the whole tissue, MT concentrations did not differ significantly between pre-treatments. This is probably due to depuration and transportation in seawater purging the gut of the mussels and therefore less important when considering the whole tissue. The effect of depuration may have also been too small when whole tissue concentrations were considered. Consequently, the relationship between digestive gland and whole tissue MT broke down when specimens were depurated, and no correlations existed. Specimens transported on ice had the highest MT concentrations, and it may be the case that MT increased due to stress and shock of the organisms from drastically altered environmental conditions. This is difficult to verify and it would be necessary to quantify the speed of response of MT to altering conditions. However this study suggests, within the time scale for transportation from field to laboratory (approximately three hours), MT concentrations did not increase significantly, compared to rapid dissection in the field.

3.4.2 Metals and metallothionein induction

MT seems to be induced by almost all metals including Cu, Zn, Cd and Cr. These metals are all known inducers of MT. However, Pb correlated with MT despite it not being known to induce MT

(Serafim et al., 2011). Therefore, it is likely Pb is merely following similar changes in concentration to other metals that are inducing MT (Cundy et al., 2003, Aly et al., 2013). MT in the digestive gland exhibited more correlations with metals (in both digestive gland and whole tissue) than MT in whole tissue. This may suggest it is more appropriate to measure MT in the digestive gland as whole tissue concentrations may be masking responses to metals. However, Raspor et al. (2004) did not consider the digestive gland of *Mytilus galloprovincialis* a tissue of choice for estimating sub-lethal metal exposure by means of MT. This is because it was found that processes of gametogenesis and food availability cause the digestive gland to engorge, as it supplies substances needed for gonad development (glycogen, lipids, and proteins), and gonadic tissue penetrates into the digestive gland and cannot be effectively separated (Regoli and Orlando, 1994b). This in turn causes minimal levels of MT and trace metals due to biological dilution. On the other hand, Geffard et al. (2001) found weight and MT to be positively correlated, but still concluded the digestive gland to be vulnerable to natural changes that affect the relationship between MT and metals. In this study, weight appears not to influence MT or metal concentrations despite changes over the sampling period, probably because the sampling time was not long enough to observe significant mass changes. Therefore, it may be sensible to measure MT and metals in whole tissue rather than digestive gland to avoid influences of mass changes for long-term studies.

Correlations between MT and metals did not stay consistent when considering the pre-treatments separately. Specimens transported on ice had the greatest amount of correlations followed by specimens that were rapidly dissected. It is possible that depuration disrupts the relationship between MT and metals, as evidenced by the relatively small number of positive correlations, which were only significant when tested non-parametrically and so less meaningful and non-linear correlations. Consequently, we suggest that depuration should not be used when examining MT response to metal exposure until further research clarifying its utility is reported.

3.5 Conclusion

MT is established as a useful biomarker for monitoring aquatic pollution. This paper has addressed widespread inconsistencies in pre-treatments applied to organisms before MT and metal analysis in biomarker studies, which have hampered comparability and implementation of this valuable monitoring approach. It is recommended that: i) samples are transported to the laboratory on ice and dissected as soon as possible thereafter and ii) depuration should not be used when examining MT response to metal exposure until further research clarifying its utility is reported. A positive relationship between biological matrices was found for MT and metal concentrations. It is therefore recommended that iii) using both digestive gland and whole tissue

is acceptable to measure in MT biomarker studies. The equations derived from this study can convert MT concentrations between whole tissue and digestive gland concentrations, so facilitating comparison between studies. Stronger correlations were found with metals when MT was measured in the digestive gland and it may be the case that whole tissue concentrations are masking MT responses. However, for long-term studies, it may be advisable to avoid measuring MT in the digestive gland, as it is susceptible to mass changes, which alter MT concentrations separately to metal exposure. It is also recommended that iv) storage temperatures of -20°C can be used and do not reduce MT concentrations via protein degradation on time-scales on the order of ten weeks. These practices can be applied to future biomonitoring studies and will improve the comparability and repeatability of using MT as a biomarker.

Chapter 4: Seasonal effects to metallothionein responses to metal exposure in a naturalised population of *Ruditapes philippinarum* in a semi-enclosed estuarine environment

4.1 Introduction

Estuarine environments are often subjected to metal pollution, posing a potentially significant environmental concern. Anthropogenic sources of metals include mining, urbanisation, fuel combustion, and industrial and domestic waste (solid and liquid) disposal. Contaminants entering semi-enclosed systems, such as some estuaries, tend to remain close to their sources due to a lack of flushing, therefore posing a long-term risk (Dassenakis et al., 2003). Effects of metal exposure can occur at low concentrations, causing environmental deterioration and can affect the organisms inhabiting these areas (Figueira et al., 2012). Marine organisms, particularly bivalves, can be used as bioindicator species due to the accumulation of metals that reflect concentrations present in the environment. This can be accompanied by the use of biomarkers, such as metallothionein (MT). MTs are non-enzymatic proteins, consisting of thiol groups (sulphur-hydrogen) that bind to metals, preventing oxidative stress to the organism (Amiard et al., 2006). This acts as a detoxification mechanism. An increase in MT concentrations in response to metal exposure has been reported in bivalves by numerous studies (Bebianno and Langston, 1991, Roesijadi, 1994, Hamza-Chaffai et al., 2000, Amiard et al., 2006, Serafim and Bebianno, 2007b). Therefore, MT is commonly included in biomonitoring studies and monitoring programmes, such as the Biological Effects and Quality Assurance in Monitoring Programmes (BEQUALM) (Amiard et al., 2006), and in the Natural England suite of assays (Galloway et al., 2008).

Questions remain about the use of MT as a biomarker for metal exposure, as MT may alter independently of metal exposure. This relates to the multifunctional role of MT, such as metabolism of essential metals, and the interaction with natural factors, which may vary seasonally (Geffard et al., 2005). Biotic effects on MT concentrations include mass increases to the digestive gland that may dilute MT concentrations, interferences relating to gametogenesis, physiological condition, and genetic adaptation (Baudrimont et al., 1997, Raspor et al., 2004, Paul-Pont et al., 2010b). Abiotic effects on MT concentrations encompass physical parameters such as salinity and temperature (Legras et al., 2000, Bocchetti et al., 2008, Hamer et al., 2008). It is therefore important to establish the influence of non-metallic controls (biotic and abiotic factors)

on MT concentrations in bivalves, and seasonal variation, before they are used for biomonitoring purposes.

The Manila clam (*Ruditapes philippinarum*) was introduced to Poole Harbour, England, in 1988 for aquaculture and is now naturalised (Jensen et al., 2004). Humphreys et al. (2015) documents its spread and naturalisation in at least 11 estuaries in southern England, including estuaries with no history of licensed introduction. It is likely *R. philippinarum* will continue to spread throughout the UK, via mechanisms of dispersal such as accidental introduction through fishing from other sites (as well as illegal introductions to establish new fisheries), and if sea surface temperature continues to rise as predicted (supporting breeding and recruitment).

R. philippinarum is tolerant of physical stress and pathogens (Tanguy et al., 2008). This is an attribute that has allowed it to spread from the Indian-Pacific region to Atlantic and Mediterranean coasts (Delgado and Perez-Camacho, 2007). Its wide-ranging presence offers an opportunity to evaluate its potential as a widely available bioindicator species. However, seasonal effects on MT concentration in species at their limit of distribution are unknown. MT response to metal exposure in *R. philippinarum* has not been studied as far north as the UK, or northern Europe, nor as a general bioindicator species for metal contamination. Studies have found that *R. philippinarum* produces MT in response to metal exposure (Wang et al., 2011, Figueira et al., 2012, Won et al., 2012). There is also evidence that seasonal effects, such as reproductive cycle or temperature, can influence the apparent MT response to metal exposure in *R. philippinarum* (Bocchetti et al., 2008, Moschino et al., 2012). Further analysis of seasonal effects on MT response in this species is needed before it can be implemented in monitoring programmes as a reliable biomarker.

Metal accumulation is particularly relevant in this species as it is an important shellfishery resource in Poole Harbour, and in much of Europe, and is consumed by the human population. Holes Bay in Poole Harbour is a closed fishery due to environmental pollution (Aly et al., 2013). Although regulatory enforcement largely prevents illegal fishing, there is still a risk of human consumption of shellfish from this area. Metal contamination is not always quantified in shellfish intended for consumption despite other contaminant analysis, such as microbial contaminants (Freitas et al., 2012). Hence, currently, the amount of metals within the organisms from closed fishery areas of Poole Harbour, and indeed other areas fished commercially, is unknown. It is important to continually monitor metals in marine invertebrates in order to manage the risk to human health from seafood consumption (Figueira and Freitas, 2013). Furthermore, Poole Harbour holds a number of statutory designations to protect the natural environment as it is recognised as being of international importance for protected habitats and species, such as

overwintering waterbirds on intertidal mud-flats (Natural England, 2015). Designations include a Special Protection Area (SPA), a Ramsar site, and a Site of Special Scientific Interest (SSSI), which include Holes Bay.

This study aimed to evaluate the potential of *R. philippinarum* as a MT biomarker species at the northernmost extent of its distribution for the first time, accounting for seasonal effects on MT responses to metal exposure and consequent reliability of MT in this species. It also aimed to evaluate the risk of its consumption relating to tissue metal concentrations.

4.2 Methodology

4.2.1 Field sampling

Four sites within Poole Harbour were studied: Wareham Channel, Arne Bay, Holes Bay, and Holes Bay (north) (Figure 7). *R. philippinarum* of length between 32 mm and 45 mm were dredged from the seabed in each of these sites, using a pump scoop (steel scooped cage designed to be dragged along seabed, sifting mud and collecting dwelling invertebrates) from a small survey vessel. Four samplings were conducted throughout 2015 in January, April, August, and October. These are referred to as winter, spring, summer, and autumn samples respectively. Sea surface temperature, pH, dissolved oxygen and conductivity were also measured at each site during sampling, to ascertain prevailing conditions. Three replicate sediment samples were collected to test metal concentrations from each site using plastic piping (to prevent metal contamination) during the autumn sample (October). The upper layer of approximately 10 cm was sampled, where clams reside. The pre-treatment of organisms before analysis was as advised by Oaten et al. (2015). Care was taken to prevent trace metal contamination by using a ceramic blade for dissection, where possible. Three replicates, containing at least six individuals, were dissected into digestive gland, gills, and remaining tissue for each sample. During dissection, whole weights (including shell), and tissue weights were measured, to investigate their effects on metal and MT concentrations, as was condition index (soft tissue weight (g) / whole weight (g) including shell and pallial liquid) (Mourgaud et al., 2002). Whole tissue concentrations (of MT and metals) were calculated from digestive gland, gill, and remaining tissue concentration based on the ratio of component weights.

4.2.2 Metallothionein analysis

A modified spectrophotometric method, as described by Viarengo et al. (1997) with modifications by Aly et al. (2014), was used to measure MTs in *R. philippinarum*. This is a sensitive, time saving,

and low cost technique that has been inter-calibrated and standardized by a number of laboratories (UNEP/RAMOGE, 1999, Zorita et al., 2005). Frozen samples of digestive gland, gill, and remaining tissue were first homogenised using a ceramic pestle and mortar. An accurately weighed sample of approximately 1 g was taken from three replicates. Tissue samples were further homogenised with 3 ml homogenising buffer of 0.5 M sucrose, 20 mM Tris–HCl (pH 8.6), 0.006 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.01% B-mercaptoethanol, then centrifuged for 30 min at 24,600 ×*g* (times gravity) at 4 °C. The supernatant solution was purified with 1.05 ml annular grade cold ethanol (−20°C) and 80 µl chloroform (per 1 ml of supernatant) and centrifuged for 10 min at 6000 ×*g* at 4°C. Supernatant solution was then collected and 40 µl of 35% hydrochloric acid (HCl) and three times the supernatant volume of annular grade cold ethanol was added and left to allow the proteins to denature for at least 1 h at −20°C. This was centrifuged at 6000 ×*g* for 10 min and the pellet saved. The pellet was washed with the previously described buffer (without B-mercaptoethanol, leupeptin, and PMSF), annular grade cold ethanol and 80 µl chloroform (87:12:1 v/v) and centrifuged for 10 min at 6000×*g*. The supernatant was discarded and the pellet dried with nitrogen gas. The pellet was resuspended with 150 µl 0.25 M sodium chloride (NaCl) and 150 µl 1N HCl with 4 mM EDTA. After resuspension, 4.2 ml of a solution containing 2M NaCl, 0.43 mM DTNB and 0.2M sodium phosphate (NaH_2PO_4) (pH 8.0) was added, and mixed by centrifugation at 3000 ×*g* for 5 min. The absorbance was read at 412nm against standard solutions of reduced glutathione (GSH) using a UV–visible spectrophotometer (Cecil 3000 series). Results are reported as µg/g (wet weight).

4.2.3 Tissue metal analysis

The metals determined were Cr, Fe, Ni, Cu, Zn, Ag, Cd, Sn, Pb, and the metalloid As, due to a combination of their presence in Poole Harbour and the potential for bioaccumulation (Aly et al., 2013). Frozen homogenised *R. philippinarum* replicates (as per first step in MT analysis) were freeze-dried for three days. Accurately weighed samples of approximately 10 mg of dried, ground sample were placed in 7 ml Teflon sealable pots. Blank samples consisting of empty Teflon pots were also prepared for analytical quality assurance. 1 ml of *Aqua Regia* (3:1 v/v solution of trace metal grade, redistilled 37% HCl and 68% nitric acid (HNO_3)) was added. Sealed pots were then heated at 60°C for 4 h, and then heated at 120°C for a further 12 h on a hot plate. Samples were dried and 1 ml of HNO_3 was added and heated to 120°C for 12 h. Samples were then dried again and 300 µl of trace metal grade hydrogen peroxide (H_2O_2) was added to samples, and heated unsealed at 120°C until fully reacted. Samples were dried and resuspended with 3% HNO_3, containing 5 ppb In/Re and 20 ppb Be as internal standards to correct for matrix effects (which

are a suppression or enhancement of analyte signal caused by concentrations of matrix elements) and instrument drift for analytical quality assurance. Samples were subsequently emptied and washed into vials, and completed to a 4000-fold dilution with 3% HNO_3 containing 5 ppb In/Re and 20 ppb Be, via a two-step process. At each stage of dilution and completion, empty and full vials were weighed to calculate accurate dilution factors. Analysis by inductively coupled plasma mass spectrometry (ICP–MS) was carried out. Five, matrix matched, standard solutions containing Cr, Fe, Ni, Cu, Zn, As, Ag, Cd, Sn, and Pb at known concentrations were used to calibrate and quantify metal concentrations of the samples. A mussel reference material (European Reference Materials – CE278k) was measured as a bivalve comparator and concentrations were adjusted according to the recovery rate (Table 11). Limits of detection (LOD) were also calculated as three times the standard deviation of 20 blank sample concentrations above the mean blank sample concentration (Analytical Methods Committee, 1987, Armbruster and Pry, 2008) (Table 11).

4.2.4 Sediment metal analysis

The same metals as analysed in organism tissue were analysed in sediments. Samples were freeze-dried and sieved using a 0.25 mm, non-metallic sieve, to remove large stones or shells. This was as advised by Hubner and Haslam (2011), and based on a particle size distribution analysis confirming all sediments had similar grain sizes, and less than 1 % of grains were greater than 0.25 mm in diameter. Accurately weighed samples of approximately 50 mg were placed in 20 ml Teflon sealable pots. Blank samples consisting of empty Teflon pots were also prepared. 1 ml of trace metal grade, redistilled HNO_3 was added to the samples, followed by 2 ml of trace metal grade, redistilled hydrofluoric acid (HF). Sealed pots were heated at 130°C for 12 h on a hot plate. Samples were then dried, 2 ml of trace metal grade, redistilled 6M HCl was added, and left at 130°C for 12 h on a hot plate. Samples were subsequently dried down; 2 ml of HNO_3 was added, followed by 1 ml of H_2O_2, which was repeated until the samples were clear. Samples were then dried and resuspended with 3% HNO_3, containing 5 ppb In/Re and 20 ppb Be as internal standards to correct for matrix effects and instrument drift, and completed and measured as per tissue analysis. Results for both sediment and tissue metal concentrations are reported as µg/g (dry weight) relative to original sample weight taken.

Canadian sediment quality guidelines (CSQGs), which are cautiously recommended by the Centre for Environment Fisheries Aquaculture Science (CEFAS) in the absence of statutory standards in the UK (Langston et al., 2003b), were used to assess the magnitude of sediment metal pollution in Poole Harbour. CSQGs consist of threshold effect levels (TELs) at which effects may be observed in some sensitive species, and probable effect levels (PELs) at which adverse effects are likely in a wide range of organisms (Hubner et al., 2009).

4.2.5 Statistical analysis

All statistical analysis was completed using IBM SPSS Statistic v21. Tests for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test) were completed and data were tested parametrically (one-way ANOVA) or non-parametrically (Kruskall-Wallis test), accordingly. Linear regression was used to determine the effects of metal exposure and biotic and abiotic variables on MT concentrations in *R. philippinarum*. Statistical significance was established at P = 0.05.

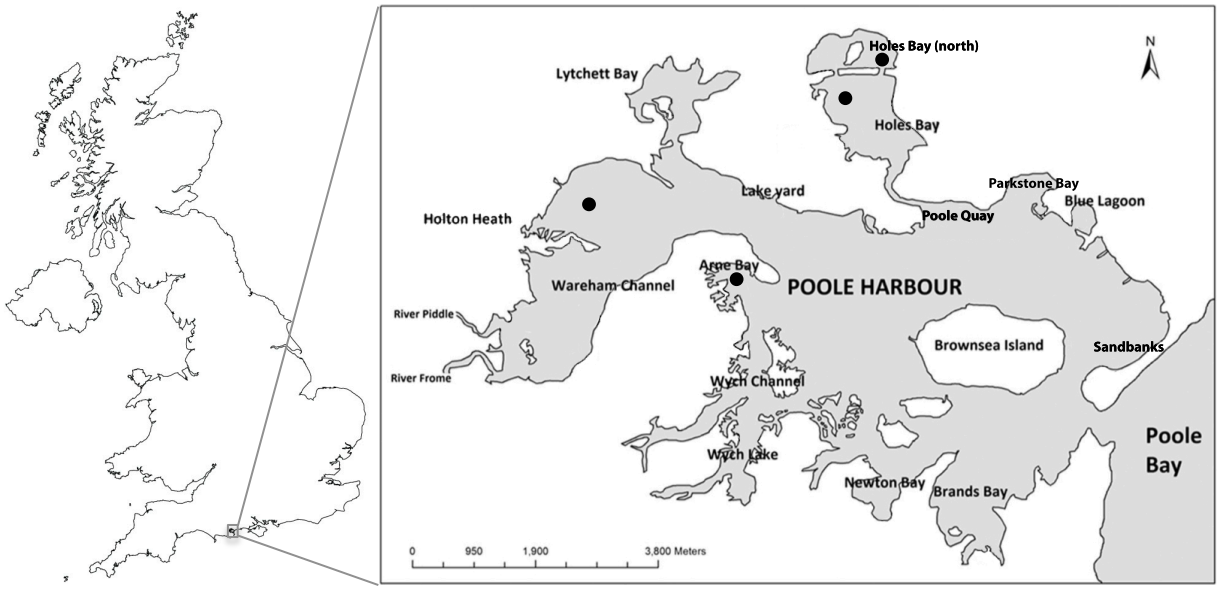


Figure 7 Site map of Poole Harbour, UK, and sampling locations.

Table 11 Recovery values (%) for metals in mussel reference material (European Reference Materials – CE278k) averaged across seasons, and limits of detection (µg/g) (calculated as three times the standard deviation of 20 blank sample concentrations above the mean blank sample concentration).

Metal	Cr	Fe	Ni	Cu	Zn	As	Ag	Cd	Sn	Pb
Recovery rate (%)	98.1	93.85	86.32	85.86	98.40	78.49	-	91.56	-	89.56
Limits of detection (µg/g)	0.076	3.817	0.039	0.038	2.453	0.078	0.009	0.002	0.019	0.023

4.3 Results

4.3.1 Spatial variation of metallothionein and metal concentrations, by season

In **winter**, MT concentrations were highest in *R. philippinarum* collected from Holes Bay (north), closely followed by Holes Bay (Figure 8a). *R. philippinarum* from Arne Bay on the west side of Poole Harbour had the lowest concentration of MT. Digestive gland MT concentrations were significantly higher in Holes Bay (north) compared to the Wareham Channel and Arne Bay (*post-hoc* Scheffe, P = 0.024, P = 0.019, respectively). Gill MT concentrations were significantly higher in Holes Bay (north) compared to Arne Bay (Kruskall-Wallis, pairwise comparison, P = 0.028). Whole tissue MT concentrations were significantly higher in Holes Bay (north) compared to the Wareham Channel and Arne Bay (*post-hoc* Scheffe, P = 0.033, P = 0.006, respectively).

In **spring**, MT concentrations in *R. philippinarum* from Holes Bay (north) decreased whilst concentrations in *R. philippinarum* from Wareham, Arne Bay, and Holes Bay increased, leaving Holes Bay MT concentrations to be highest (Figure 8a). MT concentrations in the digestive gland were significantly lower in Arne Bay than in the Wareham Channel, Holes Bay, and Holes Bay (north), and MT concentrations in Holes Bay were significantly higher than in the Wareham Channel (*post-hoc* Scheffe, P = 0.041, P = 0.001, P = 0.013, P = 0.029, respectively). Gill MT concentrations were significantly higher in Holes Bay compared to Arne Bay and Holes Bay (north) (*post-hoc* Scheffe, P = 0.006, P = 0.011, respectively). Whole tissue MT concentrations were significantly lower in Arne Bay compared to the Wareham Channel and Holes Bay (Kruskall-Wallis, pairwise comparison, P = 0.013, P = 0.009, respectively).

In **summer**, *R. philippinarum* from Holes Bay (north) had the lowest concentrations of MT, whilst Holes Bay MT concentrations remained the highest (Figure 8a). Whole tissue MT concentrations were significantly higher in Holes Bay compared to the Wareham Channel, Arne Bay, and the Holes Bay (north) (*post-hoc* Scheffe, P = 0.003, P = 0.005, P < 0.001, respectively).

This pattern remains similar in **autumn** (Figure 8a); however MT concentrations increased in *R. philippinarum* from Holes Bay (north), and were significantly higher than in the Wareham Channel and Arne Bay in whole tissue (*post-hoc* Scheffe, P = 0.003, P = 0.05, respectively). MT concentrations in gills were significantly lower in the Wareham Channel compared to Arne Bay and Holes Bay (north) (*post-hoc* Scheffe, P = 0.023, P = 0.012, respectively).

For tissue metal concentrations in *R. philippinarum* (Figure 8b – k), Cu, Zn, Ag, Sn, and Pb exhibited a similar pattern in each season: highest concentrations in Holes Bay (north) and Holes Bay, and lowest concentrations in Wareham Channel and Arne Bay. This pattern also generally

existed for Cr, Fe, Ni, As, and Cd during winter and spring. However, during summer and autumn, patterns of these metal concentrations altered across sites.

Sediment metal concentrations were significantly higher in Holes Bay (north), followed by Holes Bay, Arne Bay, and Wareham Channel for Cr, Cu, Zn, Ag, Cd, Sn, and Pb (Figure 9). For Fe and Ni, sediment concentrations were highest in Holes Bay. For As, sediment concentrations were highest in Arne Bay, followed by Holes Bay, Holes Bay (north), and Wareham Channel.

4.3.2 Between season variation in metallothionein response to metal exposure

MT concentrations in *R. philippinarum* varied between each season with generally higher concentrations in spring, apart from in Holes Bay (north) where MT concentrations were lowest in summer. **Wareham Channel** digestive gland MT was significantly higher in spring compared to winter (Kruskall-Wallis, pairwise comparison, P = 0.017) and summer (Kruskall-Wallis, pairwise comparison, P = 0.013). Gill MT was significantly higher in spring compared to winter (*post-hoc* Scheffe, P = 0.017) and autumn (*post-hoc* Scheffe, P = 0.002). **Arne Bay** gill MT was significantly lower in winter compared to autumn (*post-hoc* Scheffe, P = 0.035). **Holes Bay** digestive gland MT was significantly higher in spring compared to winter (*post-hoc* Scheffe, P = 0.028). Gill MT in spring was significantly higher than winter, summer, and autumn (*post-hoc* Scheffe, P = 0.019, P = 0.023, P = 0.006, respectively). Whole tissue MT in winter was significantly lower than in spring (Kruskall-Wallis, pairwise comparisons, P = 0.007), and summer (Kruskall-Wallis, pairwise comparisons, P = 0.017). **Holes Bay (north)** digestive gland MT in summer was significantly lower than in winter (Kruskall-Wallis, pairwise comparisons, P = 0.007), and spring (Kruskall-Wallis, pairwise comparisons, P = 0.017). Similarly, gill MT was significantly lower in summer compared to winter (Kruskall-Wallis, pairwise comparisons, P = 0.009), and autumn (Kruskall-Wallis, pairwise comparisons, P = 0.031).

Linear regression analysis was performed to determine the importance of metal exposure to MT induction across seasons, which was the focus of this study. In winter many significant positive relationships existed between MT and metal concentrations in each tissue, though fewer existed in gills compared to digestive gland and whole tissue (Table 12). However in spring, summer, and autumn, fewer significant positive relationships existed in whole tissue (Figure 10). This was also the case in digestive gland and gill tissue, and between sediment metal concentrations and tissue MT concentrations (analyses not reported).

Linear regression analyses with other biotic variables were examined to determine their influence. Statistical data and the strength of relationships are noted within parentheses. Tissue weight had significant positive relationships with MT concentrations during spring in digestive gland (R^2 =

0.519 (strong), P = 0.008), and whole tissue (R^2 = 0.577 (strong), P = 0.004), and during autumn in whole tissue (R^2 = 0.830 (very strong), P < 0.001) (Figure 11). Whole weight also had some significant positive relationships with MT concentrations during spring in digestive gland (R^2 = 0.723 (strong), P < 0.001), and whole tissue (R^2 = 0.491 (strong), P = 0.011), and during autumn in whole tissue (R^2 = 0.736 (strong), P < 0.001) (Figure 12). Condition index showed a positive relationship with MT concentrations during summer in digestive gland (R^2 = 0.374 (moderate), P = 0.035), and whole tissue (R^2 = 0.368 (moderate), P = 0.037) (Figure 13). Gill MT also showed significant positive relationships between: tissue weight during spring (R^2 = 0.477 (moderate), P = 0.013) and autumn (R^2 = 0.453 (moderate), P = 0.017); whole weight during spring (R^2 = 0.520 (strong), P = 0.008), and autumn (R^2 = 0.347 (moderate), P = 0.044); and condition index during spring (R^2 = 0.349 (moderate), P = 0.043).

4.3.3 Localised variation in metallothionein response

Variation in MT response between sites was analysed for completeness. Linear regression analysis showed significant positive relationships between MT and metal concentrations at all sites in *R. philippinarum*, except for the **Wareham Channel.** Here, MT concentrations were also not affected by tissue weight, whole weight, or condition index.

R. philippinarum from **Arne Bay** showed a negative relationship between whole weight and MT concentration in gills (R^2 = 0.498, P = 0.01), and whole tissue (R^2 = 0.547 (strong), P = 0.006). A positive relationship existed between MT and condition index in gills (R^2 = 0.496 (strong), P = 0.011).

Unlike Arne Bay, *R. philippinarum* from **Holes Bay** showed positive relationships between whole weight and MT concentration in gills (R^2 = 0.641 (strong), P = 0.002), and in whole tissue (R^2 = 0.448 (moderate), P = 0.017). There were also positive relationships between MT concentration and tissue weight in gills (R^2 = 0.507 (strong), P = 0.009), and whole tissue (R^2 = 0.645 (strong), P = 0.002).

R. philippinarum from **Holes Bay (north)** showed negative relationships between MT concentration in the digestive gland and whole weight (R^2 = 0.763 (strong), P < 0.001), and tissue weight (R^2 = 0.593 (strong), P = 0.003).

The relationship between abiotic factors and MT concentration was also examined at each site using linear regression analysis (Table 13 and 14). Consistent relationships were not observed across sites. Unlike all other sites, negative relationships existed between temperature and MT concentrations in the digestive gland (R^2 = 0.592 (strong), P = 0.003), gills (R^2 = 0.532 (strong), P =

0.007), and whole tissue (R^2 = 0.452 (moderate), P = 0.017), in *R. philippinarum* from Holes Bay (north).

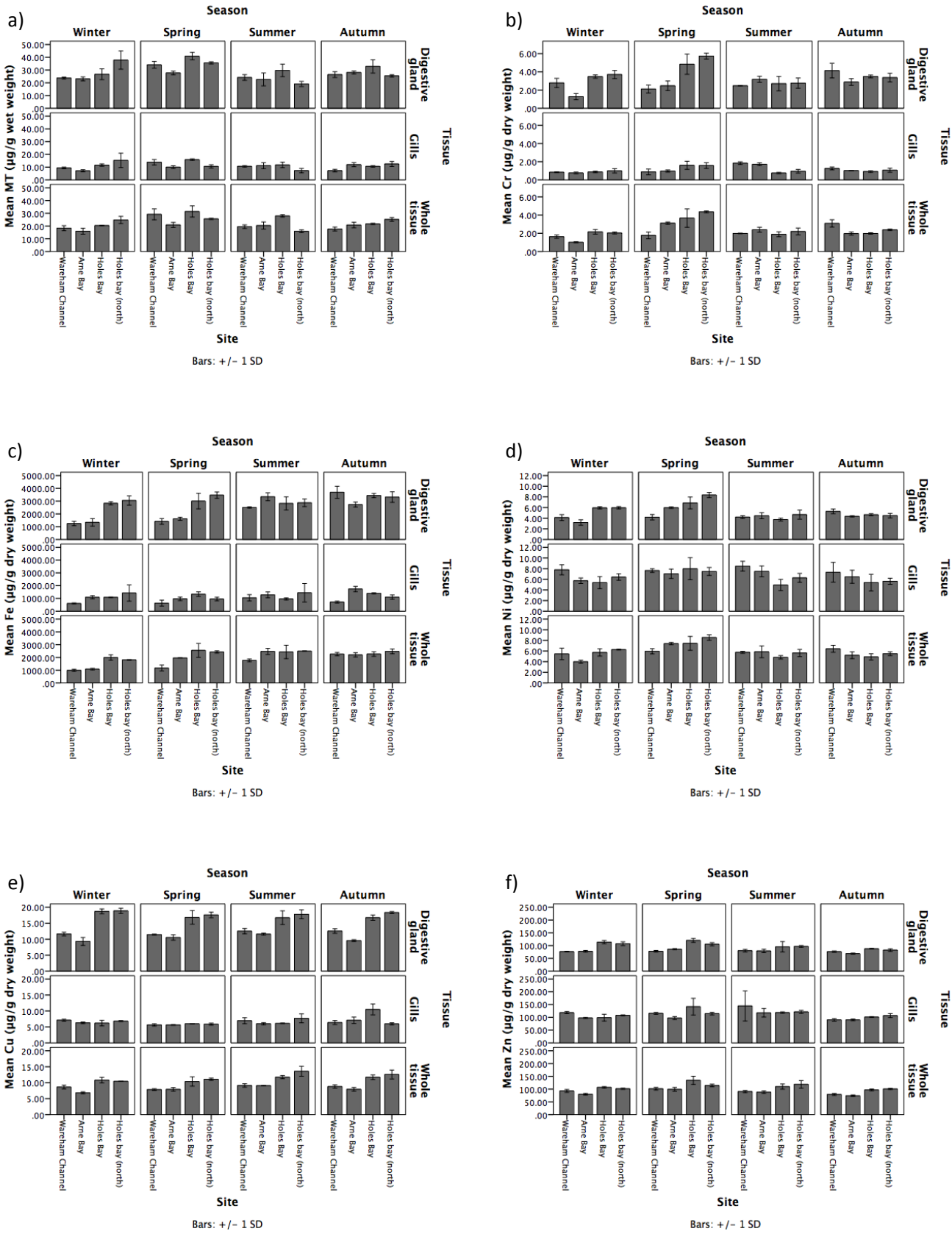
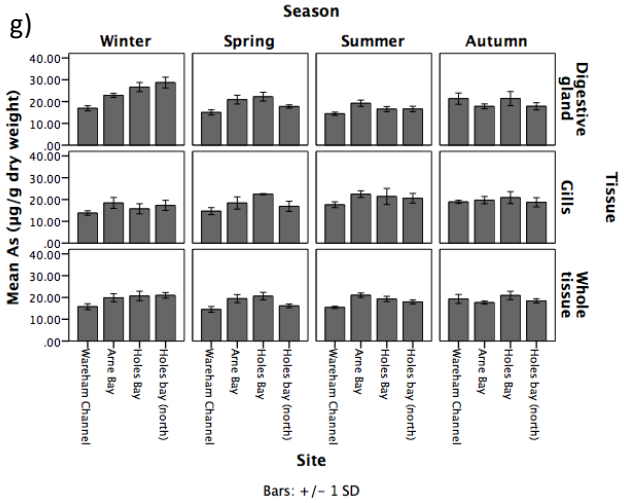
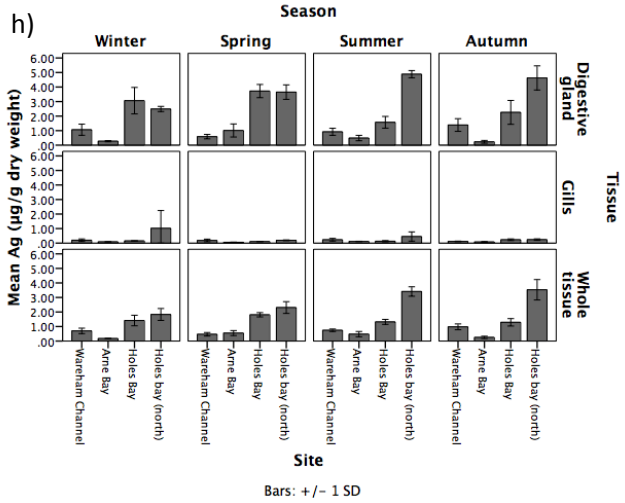
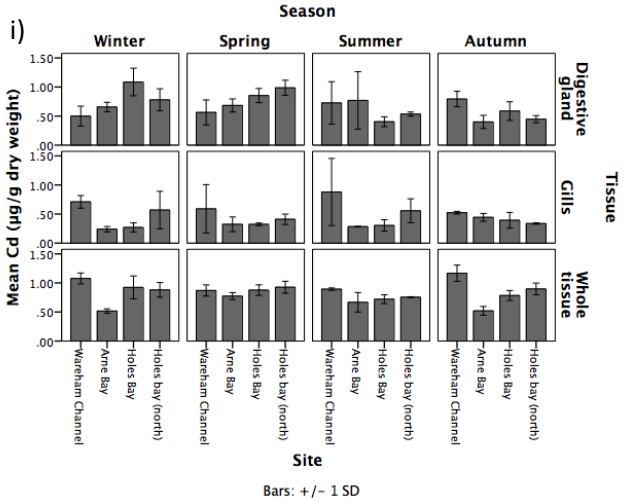
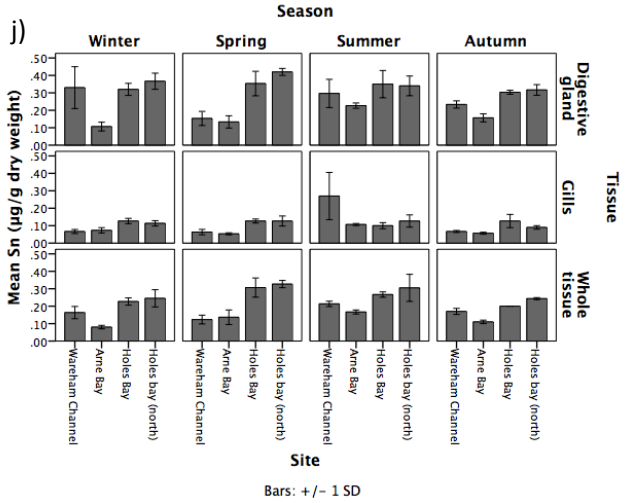


Figure 8 Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn and k) Pb in different tissues from *R. philippinarum* from Poole Harbour throughout each season in 2015, with standard deviation (SD) (n = 3).









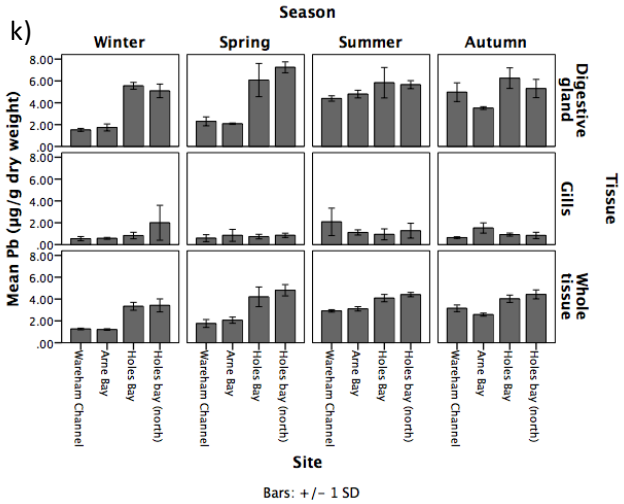


Figure 8 cont. Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn and k) Pb in different tissues from *R. philippinarum* from Poole Harbour throughout each season in 2015, with standard deviation (SD) (n = 3).

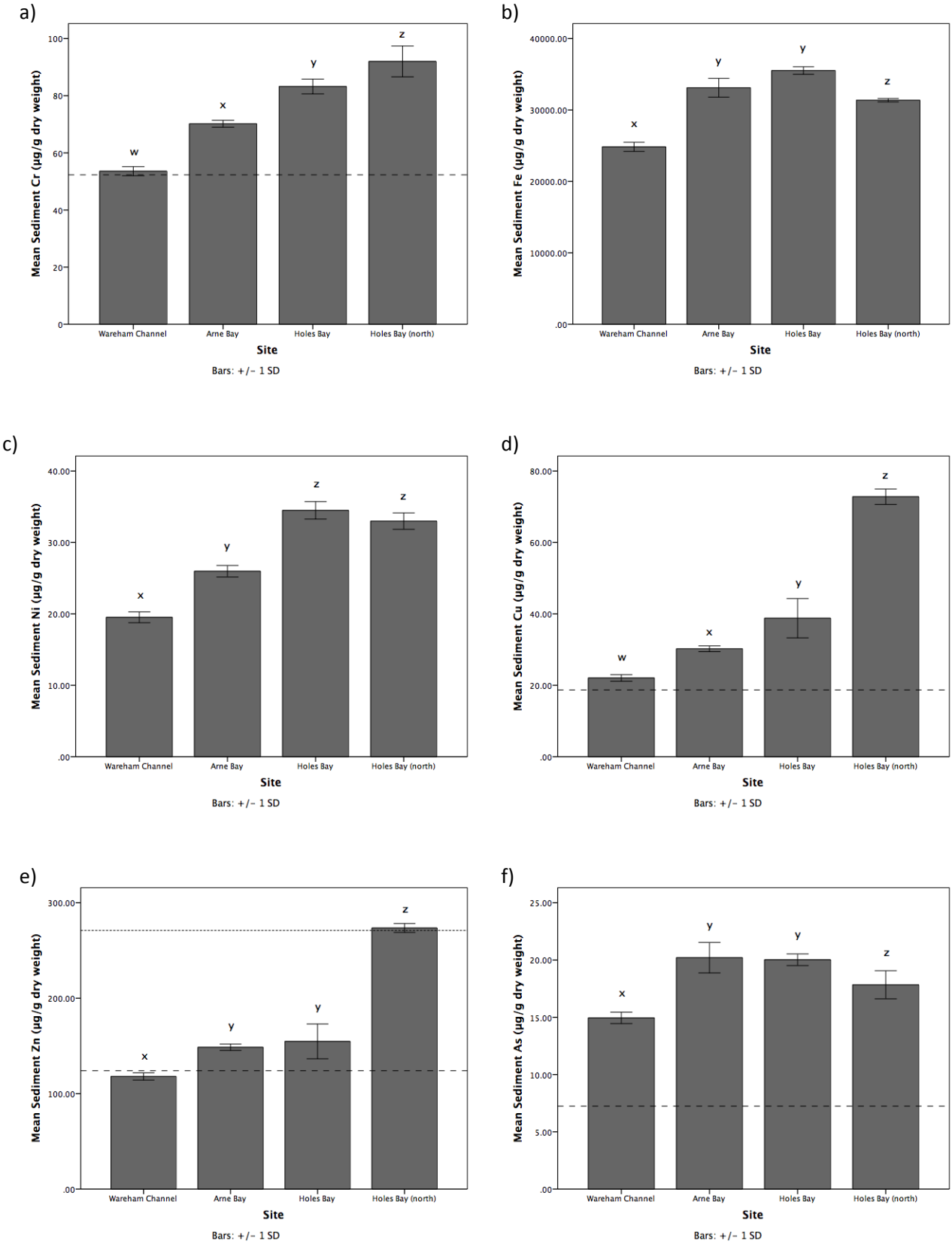


Figure 9 Mean concentrations (µg/g) of a) Cr, b) Fe, c) Ni, d) Cu, e) Zn, f) As, g) Ag, h) Cd, i) Sn, j) Pb in sediments from Poole Harbour, with standard deviation (SD) (n = 3) (dashed lines indicate the Threshold Effect Level (TEL), dotted line indicates the Probable Effect Level (PEL) (Hubner et al., 2009)). Different letters indicate significant differences (P = 0.05).

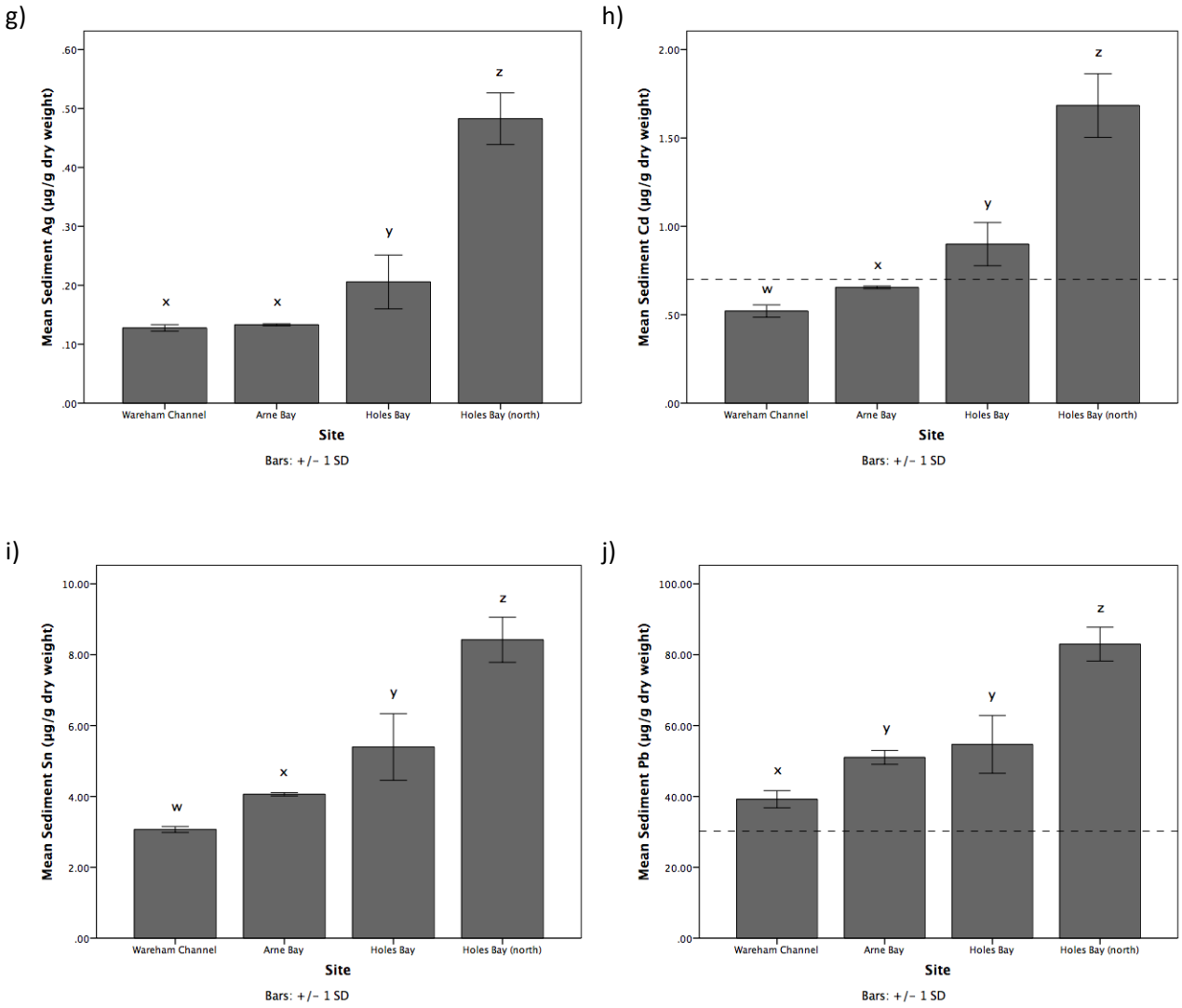


Figure 9 cont. Mean concentrations (µg/g) of a) Cr, b) Fe, c) Ni, d) Cu, e) Zn, f) As, g) Ag, h) Cd, i) Sn, j) Pb in sediments from Poole Harbour, with standard deviation (SD) (n = 3) (dashed lines indicate the Threshold Effect Level (TEL), dotted line indicates the Probable Effect Level (PEL) (Hubner et al., 2009)). Different letters indicate significant differences (P = 0.05).

Table 12 Linear regression between tissue metal concentrations and MT concentrations in *R. philippinarum* in each tissue, during winter (P = 0.05, significant results are bold and marked with *).

Metal concentration	Digestive gland MT concentration		Gill MT concentration		Whole tissue MT concentration	
	R^2	Sig.	R^2	Sig.	R^2	Sig.
Cr	0.391	**0.03***	0.728	**<0.001***	0.608	**0.005***
Fe	0.528	**0.007***	0.564	**0.005***	0.447	**0.024***
Ni	0.391	**0.03***	0.031	0.583	0.384	**0.042***
Cu	0.426	**0.021***	0.004	0.846	0.63	**0.004***
Zn	0.398	**0.028***	0	0.963	0.545	**0.009***
As	0.284	0.074	0.05	0.486	0.116	0.306
Ag	0.243	0.104	0.787	**<0.001***	0.581	**0.006***
Cd	0.147	0.219	0.261	0.089	0.146	0.246
Sn	0.203	0.141	0.446	**0.018***	0.774	**<0.001***
Pb	0.422	**0.022***	0.81	**<0.001***	0.523	**0.012***

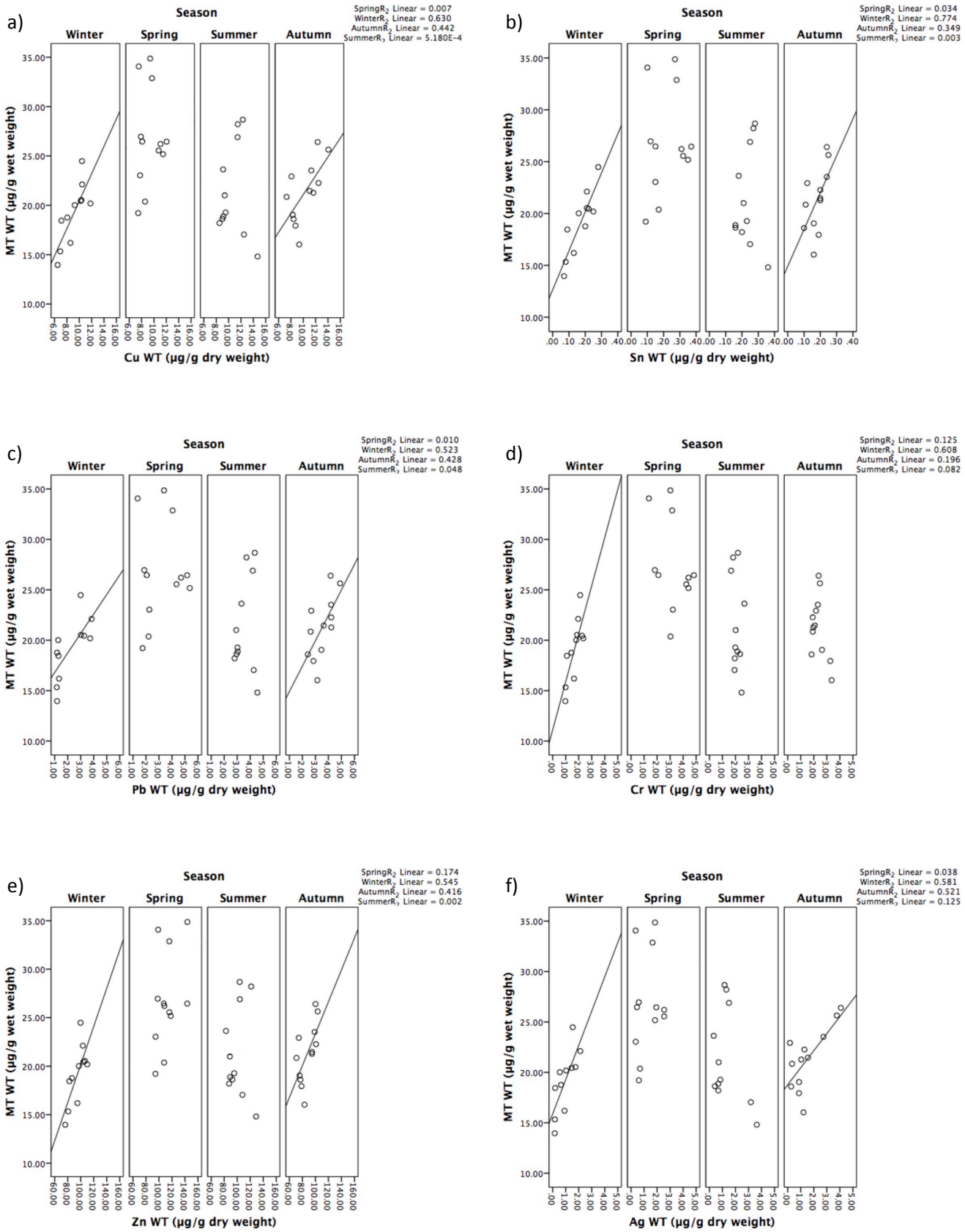


Figure 10 Linear regression between concentrations of MT and a) Cu, b) Sn, c) Pb, d) Cr, e) Zn, and f) Ag in whole tissue (WT) from *R. philippinarum* from Poole Harbour across seasons in 2015.

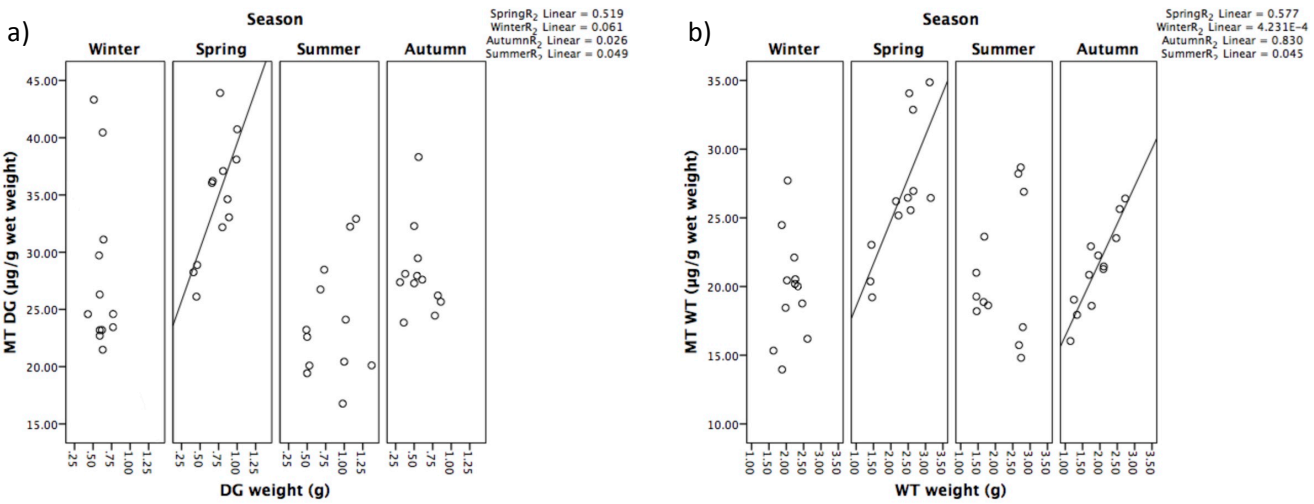


Figure 11 Linear regression between MT concentrations in *R. philippinarum* from Poole Harbour and tissue weight in a) digestive gland (DG), and b) whole tissue (WT), across seasons in 2015.

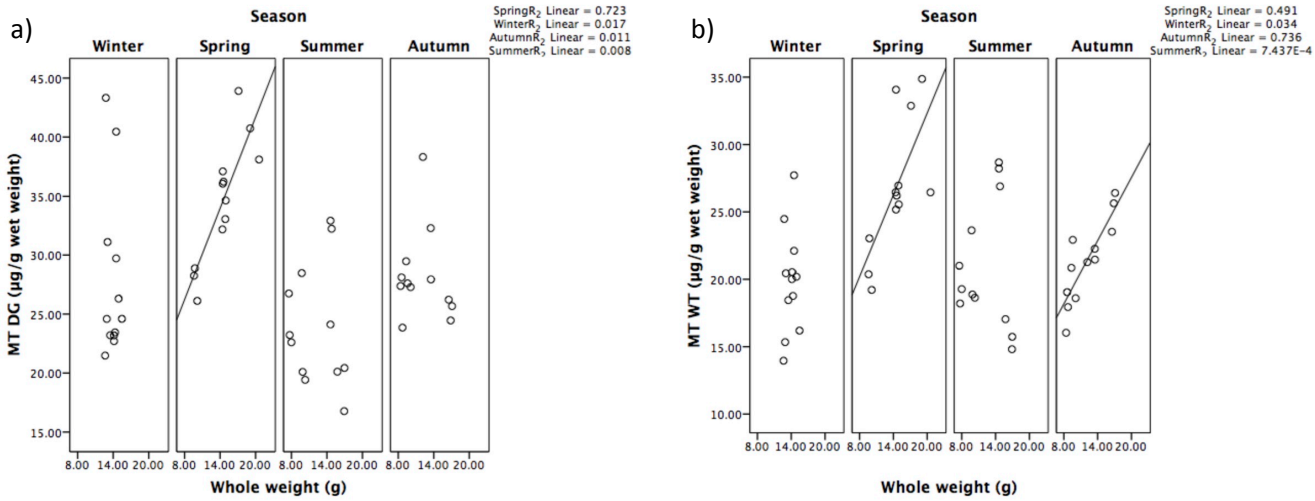


Figure 12 Linear regression between MT concentrations in *R. philippinarum* from Poole Harbour and whole weight in a) digestive gland (DG), and b) whole tissue (WT), across seasons in 2015.

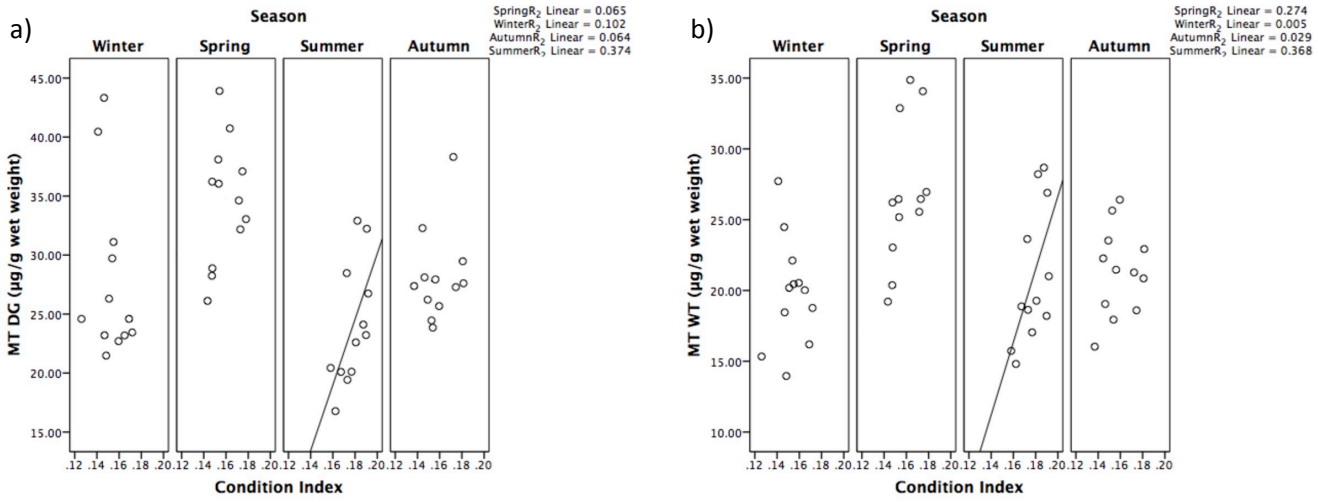


Figure 13 Linear regression between MT concentrations in *R. philippinarum* from Poole Harbour and condition index in a) digestive gland (DG), and b) whole tissue (WT), across seasons in 2015.

Table 13 Abiotic variables measured during sampling at each site (NB: DO is dissolved oxygen).

	Wareham Channel				Arne Bay				Holes Bay				Holes Bay (north)			
	W	Sp	Su	A	W	Sp	Su	A	W	Sp	Su	A	W	Sp	Su	A
Temp (°C)	7.85	12.8	18.3	11.6	3.97	12.9	18.8	12.7	4.90	13.4	19.2	13.1	8.65	14.9	19.2	14.4
pH	8.45	8.7	8.11	8.27	8.7	8.7	8.31	8.15	8.7	8.65	8.04	8.36	8.4	8.5	8.08	8.50
Salinity (psu)	19.0	20.0	-	20.1	27.3	21.2	-	26.9	27.1	21.8	-	30	24	20.2	-	21.6
DO (mg/l)	9.50	7.05	-	7.95	7.30	7.40	-	8.79	7.56	6.28	-	8.29	7.01	7.22	-	8.37

Table 14 Significant linear regression analysis between MT in the digestive gland (DG), gills (G), and whole tissue (WT) and abiotic variables at each site across seasons (NB: DO is dissolved oxygen, insignificant results indicated by –).

		Wareham Channel			Arne Bay			Holes Bay			Holes Bay (north)		
		DG	G	WT	DG	G	WT	DG	G	WT	DG	G	WT
Temp (°C)	R^2	-	-	-	-	0.471 +ve	0.385 +ve	-	-	0.346 +ve	0.592 -ve	0.532 -ve	0.452 -ve
	P	-	-	-	-	0.014	0.031	-	-	0.044	0.003	0.007	0.017
pH	R^2	0.572 +ve	0.337 +ve	0.508 +ve	-	0.465 -ve	-	-	-	-	0.390 +ve	-	0.866 +ve
	P	0.008	0.048	0.009	-	0.015	-	-	-	-	0.03	-	< 0.001
Salinity (ppt)	R^2	-	-	-	-	-	-	-	-	-	-	-	-
	P	-	-	-	-	-	-	-	-	-	-	-	-
DO (mg/l)	R^2	-	-	-	-	-	-	-	-	0.671 –ve	-	-	-
	P	-	-	-	-	-	-	-	-	0.007	-	-	-

4.4 Discussion

4.4.1 Metal contamination in Poole Harbour

Sediment metal concentrations were mostly highest in Holes Bay (north), although this only represents a 'spot' measurement of contamination in autumn 2015. However, tissue metal concentrations in *R. philippinarum* were also generally highest in Holes Bay (north) throughout the year, representing an integrated measure of contamination in Poole Harbour, but some metal concentrations varied seasonally. The magnitude of metal contamination found in the present study is consistent with previous work (Langston et al., 2003b, Aly et al., 2013). Metal contamination in Holes Bay is probably partly due to historic contamination that resides within the sediments due to low water circulation in this system. This occurrence may be more prevalent in Holes Bay (north), where flushing is even more restricted. A coal-fired power station, which was closed and demolished in 1993, was situated on the southwest shore of Holes Bay. This may have been a source of various metals such as Pb, Sn, and Ni. Another historic source of metals, which may still be present in the environment, is the Merck chemical plant and electroplating works, which stopped operations in 1998 (Langston et al., 2003b). Cr, As, Cu, Cd, Pb, Hg, Ni, and Zn were discharged into Holes Bay from the plant during its operation (Langston et al., 2003b). There was also a major fire at this site in 1988, which may have led to contaminated water entering Holes Bay (Hansard, 1988). The largest sources of contamination to the area, both historically and currently, include a large concentration of combined sewer overflows (CSOs) and a sewage treatment works (STW), owned by Wessex Water, that discharge into the northeast corner of Holes Bay (north) (Langston et al., 2003b).

Most sites in Poole Harbour exceed CSQGs. Sediment concentrations of Cr, Cu, As, and Pb exceed TELs in all sites in Poole Harbour. Zn and Cd sediment concentrations exceed TELs in all but the Wareham Channel, and the Wareham Channel and Arne Bay, respectively. Zn concentrations also exceed PELs in Holes Bay (north). However, European Commission (EC) Regulation 1881/2006 sets maximum levels for certain contaminants in foodstuffs. The maximum concentration of Pb and Cd permitted in bivalve molluscs is 1.5 μg/g (wet weight) and 1 μg/g (wet weight), respectively. These can be converted from wet weight to dry weight concentrations using a conversion factor of 7, assuming moisture content of 85% (Dincer, 2006, Pan and Wang, 2012, Zhao et al., 2013). These equate to 10 μg/g (dry weight) and 7 μg/g (dry weight) limits for Pb and Cd, respectively. The highest mean concentration of Pb was found in *R. philippinarum* from Holes Bay (north) at 4.34 μg/g (maximum of 5.37 μg/g). The highest mean concentration of Cd was found in *R. philippinarum* from the Wareham Channel at 1 μg/g (maximum of 1.28 μg/g). Therefore, concentrations of these metals found in *R. philippinarum* in Poole Harbour are below limits

deemed safe for human consumption. Additionally, this suggests that the magnitude of metal contamination in Poole Harbour is not severe, and may be below a threshold at which MT is not induced reliably. This may be what is occurring in the Wareham Channel, which exhibited insignificant relationships with metal and MT concentrations in *R. philippinarum*. For comparison, the magnitude of metal contamination in the Fal Estuary, in the southwest of the UK, which received mining waste in 1989, is much higher. Sediment metal concentrations in the most polluted part of the estuary, Restronguet Creek, are approximately two orders of magnitude higher than Poole Harbour for As and Cu, and two times more concentrated in Fe (Langston et al., 2003a).

During winter, the pattern of tissue concentration of most metals in *R. philippinarum* across sites seems to be in accordance with sediment metal concentrations. This is also true of Cu, Zn, Ag, Sn, and Pb throughout each season, suggesting that the bioavailabilities of these metals to clams are relatively unaffected seasonally. However, Cr, Fe, Ni, As, and Cd vary in tissue concentration in summer and autumn seasons, suggesting the bioavailability of these metals to clams change seasonally. An explanation for this may be that the speciations of some metals (Cd, Cr, As) are particularly sensitive to redox potential (Bryan and Langston, 1992). They tend to be complexed with sulphide minerals in anoxic conditions, which have very low solubility (Morse, 1994). Moreover, reduced dissolved oxygen concentrations in summer due to eutrophic conditions may reduce the oxidation of metals from Fe and Mn oxides, further reducing metal bioavailability (Mubiana et al., 2005). This may explain the generally lower As, Cr and Cd tissue concentrations in *R. philippinarum* in summer, as eutrophic conditions deplete dissolved oxygen in the water column (Humphreys et al., 2007). Cd bioavailability can increase with lower salinity due to lower complexation with the ion chloride (Langston et al., 1998, Ivankovic et al., 2005, Smaoui-Damak et al., 2009). This may explain the higher Cd accumulation in the Wareham Channel compared to other sites, as the salinity was lower. Furthermore, Ng and Wang (2004) have reported that the interaction of the metals could affect their uptake, accumulation, and toxicity by aquatic organisms.

4.4.2 Biotic seasonal effects to metallothionein

Metal exposure seems to have a large influence on MT induction during winter, evidenced by highest MT concentrations in Holes Bay (north) and significant positive relationships between tissue metal and MT concentrations in *R. philippinarum*. However during spring, summer, and (to some extent) autumn, relationships between tissue metal and MT concentrations break down. Moschino et al. (2012) reported similar findings when assessing MT response during summer and autumn in the Venice lagoon, Italy, suggesting a cautionary approach to using *R. philippinarum* for

biomonitoring. Other physiological processes such as mass increase, condition, and gametogenesis appear to override the affect of metal exposure on MT concentration. Some studies on mussels (*Mytilus galloprovincialis*) show MT concentrations to decrease during spring periods, which coincide with maximum digestive gland mass (Raspor et al., 2004, Raspor et al., 2005). This is due to gametogenesis, where the gonads develop and encroach on digestive gland tissue. This swells the digestive gland and dilutes the concentration of MT. Clams have similar physiology to mussels, in that gonadal tissue is indiscrete from digestive gland tissue in clams. However, the present study shows significant positive relationships between digestive gland tissue mass and MT concentrations in spring. In addition, maximum MT concentrations occurred in spring, when digestive gland mass was not at a minimum. Therefore, the data presented here suggest that biological dilution is not causing MT concentrations to differ independently of metal exposure.

Maximum concentrations of MT have been reported to occur in spring in *R. philippinarum* (Bocchetti et al., 2008). Some studies suggest MT is highest during gametogenesis and decreases after spawning (Paul-Pont et al., 2010b). This is likely due to the onset of gametogenesis, and the associated hormonal secretion that can induce MT (Baudrimont et al., 1997, Baudrimont et al., 2006, Mao et al., 2012). Furthermore, a study by Meistertzheim et al. (2009) found MT in gonadal tissue to be in high concentrations in *Crassostrea gigas*. It suggests that oocytes within the gonads produce MT, which provides a protective role against metals (and other harmful agents) in a variable environment during embryo-larval stages, and that MTs may also play a role during meiosis. Consequently, as gonads develop, MT concentrations can increase. *R. philippinarum* in Poole Harbour undergo gametogenesis during spring, and regularly exhibit two spawning events, given the right thermal conditions, in around June and October (Humphreys et al., 2007). Therefore, it is probable that the increase in MT in spring in the present study is related to gametogenesis and the development of the gonads.

Although tissue concentrations for metals stayed relatively constant seasonally, metal body burden (tissue concentrations multiplied by tissue weight) increased during spring, concurrently with tissue weight. This is likely due to increased metabolic activity and increased food uptake to support growth, leading to increased dietary metal uptake (Rouane-Hacene et al., 2015). It can be argued that this caused MT concentrations to increase in spring, rather than gametogenesis. However, there were insignificant relationships between MT concentrations and metal body burden during spring. Therefore, it is still more likely that tissue weight (related to metal body burden but a proxy for gametogenesis) is causing MT response to be independent of metal exposure, from spring onwards.

MT concentrations demonstrated a significant positive relationship with condition index in summer (Figure 13). In summer, energy expended during gametogenesis and spawning may cause the health condition of clams to be poor, possibly causing mortality (Meistertzheim et al., 2009). This could reduce MT induction as energy resources are depleted. Increased temperatures of 25°C and anoxic conditions due to extensive algal cover caused mortality of *R. philippinarum* during summer in Poole Harbour in 2003 (Humphreys et al., 2007). It is therefore possible that the relatively poor physical condition of organisms (and by proxy weight) is reducing the capability of MT induction in summer.

The number of significant relationships between MT and metal concentrations are similar for digestive gland and whole tissue, during winter, but lower in gill tissue (Table 12). However, the strength of relationships were moderate to strong between MT and certain metals in gills. One reason for this may be that gills are in direct contact with the surrounding environment, and reflect short-term contaminant exposure and are the primary target for water contaminants (Bebianno et al., 2004, Cravo et al., 2013). Nevertheless, other studies advocate the use of MT in the digestive gland in *Ruditapes decussatus* as the most appropriate biomarker of metal contamination (Smaoui-Damak et al., 2009). The present study shows the most moderate to strong significant relationships in whole tissue between MT and metals during winter (Table 12). Therefore whole tissue MT may be advantageous to use as a biomarker due to the simplicity of dissection.

4.4.3 Abiotic seasonal effects to metallothionein

Consistent relationships between MT concentration and other abiotic variables such as, pH, salinity, and dissolved oxygen were not found across individual sites. It is important to note that these variables were measured at each sampling, and not throughout the year, so may not be representative. However, this may suggest abiotic factors do not influence MT concentrations in the same manner, but have specific localised effects. For example, organisms subjected to chronic metal exposure, or parasitic infections may have adapted detoxification mechanisms (Paul-Pont et al., 2010b). Smaoui-Damak et al. (2004) showed that location was an important contributor to MT concentrations rather than metal concentrations in *R. decussatus*. This may be due to variations in environmental conditions, reproductive process, or genetic factors (Tanguy et al., 2003). In this study, minimum concentrations of MT were found in Holes Bay (north) in summer, which exhibited a significant negative relationship with temperature. This could be due to increased temperature and light irradiance reducing total oxyradical scavenging capacities (Bocchetti et al., 2008). This could be exacerbated by extreme temperature fluctuations as benthic habitats are

exposed to air at low water at this site. However, negative relationships with temperature and MT concentrations were not evident in *R. philippinarum* from other sites.

4.5 Conclusion

In order for MT to present a useful tool in biomonitoring and relay the biological impact of metals to *R. philippinarum*, external seasonal influences on MT concentration must be taken into account. Here it is shown that gametogenesis and the associated increase in MT concentrations, and condition index, override the affect on MT concentration by metal exposure. It is consequently not a reliable biomarker for metal contamination during spring and summer, when clams are undergoing reproductive processes and may be in relatively poor body condition. It is therefore recommended that MT is only used as a biomonitoring tool during winter periods, when *R. philippinarum* is at a resting reproductive state. This study adds to the knowledge of *R. philippinarum* MT response at latitudes approaching the limit of its geographic extent, and constrains its potential as a cosmopolitan bioindicator species. In addition, the concentration of metals in *R. philippinarum* from the most contaminated site in Poole Harbour, Holes Bay (north), is unlikely to pose a risk to human health following consumption.

Chapter 5: Metallothionein responses in sympatric populations of *Ruditapes philippinarum*, *Ruditapes decussatus* and *Venerupis corrugata* using active sampling

5.1 Introduction

The European clam, or grooved carpet shell clam (*Ruditapes decussatus*) is native to Atlantic and Mediterranean coastlines (Usero et al., 1997). The pullet carpet shell clam (*Venerupis corrugata*) has a similar geographical extent. The Manila clam (*Ruditapes philippinarum*) is native to the Indian-Pacific region, but its range has expanded to northern European and Mediterranean waters as it was introduced to the region in the 1970s for commercial exploitation and cultivation (Delgado and Perez-Camacho, 2007). It has been able to naturalise in these environments due to its high growth rates and its ability to tolerate a range of environmental conditions (Delgado and Perez-Camacho, 2007, Tanguy et al., 2008). Because each clam species occupies a similar ecological niche they may compete partially in both natural media and on aquaculture farms (Usero et al., 1997).

R. philippinarum was introduced to Poole Harbour, England, in 1988 for aquaculture and naturalised (Jensen et al., 2004). It has since spread and naturalised in at least 11 estuaries in southern England, including estuaries with no history of licensed introduction, as described by Humphreys et al. (2015). It is not currently thought to be aggressively invasive and is not presenting a significant risk to indigenous species. However, within Poole Harbour it is the dominant species of clam, compared with native species of *R. decussatus* and *V. corrugata*. It is likely *R. philippinarum* will continue to spread throughout the UK, via mechanisms of dispersal such as accidental introduction through fishing from other sites (as well as illegal introductions to establish new fisheries), and if sea surface temperature continues to rise as predicted (supporting breeding and recruitment).

Bivalves, such as clams, have been used as bioindicators of aquatic metal pollution. They provide quantitative, time-integrated information on the portion of metals that are of ecotoxicological relevance (Moschino et al., 2012). Their use for evaluating contaminants in tissues is frequently combined with the use of biomarkers. Biomarkers are measures of biochemical, physiological or behavioural variations that relay the impact of a pollutant to the organism (Depledge, 1994).

Metallothionein (MT) is an example of a biomarker frequently used in metal biomonitoring. MTs are proteins characterised by low molecular weight and high cysteine content, and have a strong affinity to bind with metal cations (Hamza-Chaffai et al., 2000). The biological role of MTs is still debateable, but they are generally accepted to play a role in the detoxification of toxic metals, as well as homeostasis of essential metals, contributing to their use as a biomarker (Geffard et al., 2005). However, caveats are regularly attributed to the use of MTs as biomarkers due to potential interferences from natural factors, such as tissue weight, reproductive cycle, temperature, and pH that may disrupt the relationship between MT concentrations and metal exposure (Raspor et al., 2004, Geffard et al., 2005, Hamer et al., 2008, Smaoui-Damak et al., 2009). It is therefore important to establish the natural variation of MT concentrations in different species in the field, in order to interpret information correctly.

Previous studies have demonstrated the effects of metal contamination via MT induction in *R. decussatus* (Bebianno et al., 2000, Hamza-Chaffai et al., 2000, Bebianno and Serafim, 2003, Geret et al., 2003, Bebianno et al., 2004, Smaoui-Damak et al., 2004, Smaoui-Damak et al., 2009, Serafim and Bebianno, 2010, Cravo et al., 2013). Works performed on MT induction in *R. philippinarum* are less common, particularly in Europe (Ng and Wang, 2004, Paul-Pont et al., 2010a, Paul-Pont et al., 2010b, Wang et al., 2011, Won et al., 2012). MT concentrations in response to metal exposure in *V. corrugata* have only been examined once before (Velez et al., 2016). Furthermore, few studies have compared MT response to metal exposure between species. *R. decussatus* and *R. philippinarum* have been compared in the laboratory (Figueira et al., 2012, Freitas et al., 2012) and once in the field (Moraga et al., 2002). A comparison with *V. corrugata* has not been carried out.

Sympatric species of clam, *R. philippinarum*, *R. decussatus* and *V. corrugata,* offer a means to compare the contributing factors to natural variation in MT concentrations. Different sequestration systems are likely to be competing in each species and interfering with the response of MTs to metal exposure (George and Olsson, 1994). The role of MT and its potential to be used as a biomarker in each species for biomonitoring requires assessment. Furthermore, *R. philippinarum* is gaining more attention as a bioindicator due to its ecological and economic importance, as well as its increasingly wide occurrence (Ji et al., 2006, Moschino et al., 2012). It is therefore important to validate the use of MT in this species at higher latitudes (e.g. the UK) where natural effects on MT concentrations and levels of oxidative stress are likely to be different compared with other studies, and native species.

This study aimed to assess any differences in metal accumulation patterns and MT response of native and invasive species of clams in the natural environment. The causes of MT variability were

investigated and the limits of use for MT as a biomarker in each species were evaluated in two locations with different levels of environmental quality and contamination status. Poole Harbour presents a useful opportunity to address this issue, as it is one of the northernmost locations where *R. philippinarum* has naturalised and is living in sympatry with *R. decussatus* and *R. philippinarum*.

5.2 Methodology

5.2.1 Field sampling

Two sites within Poole Harbour were studied: Arne Bay and Holes Bay (north) (Figure 14). These locations were selected as they have contrasting severities of contamination, as described by Aly et al. (2013). Holes Bay (north) has had a historical input of metals and trade discharges that remain in sediments as the area is enclosed and has limited flushing. A sewage treatment works (STW) and combined sewer overflows (CSOs) also presently discharges into the northeast corner of Holes Bay (Langston et al., 2003b). This area is therefore the most contaminated site in Poole Harbour. Arne Bay does not receive direct anthropogenic contamination and is part of the Arne Nature Reserve of the Royal Society for the Protection of Birds (RSPB).

R. philippinarum, *R. decussatus* and *V. corrugata* were originally fished from the leased fishing beds in Poole Harbour situated in an open, well-flushed area in the centre of the harbour (Figure 14). They were then actively sampled, and placed in plastic cages laid on the seabed at each site in April 2016. Active sampling (collecting organisms from natural populations from a reference area and transplanting them to a study area for a certain period of time) was used, as wild species of clam were not abundant enough to be sampled directly. Samples were collected after four weeks. A further sample of *R. philippinarum* and *V. corrugata* was also collected after sixteen weeks from Arne Bay, to explore any possible seasonal change. *R. decussatus* was not subject to sixteen weeks exposure, as the number of organisms originally collected before active samples were deployed was too low to sustain a second sample. A sixteen-week exposure sample was not collected from Holes Bay (north) as samples went missing, likely moved by local fishermen. A pre-exposure sample of *R. philippinarum* and *V. corrugata* was also collected. Sea surface temperature, pH, dissolved oxygen and conductivity were measured at each site during sampling, but were not analysed as too few data could be obtained (one measurement during each sample). The pre-treatment of organisms before analysis was as advised by Oaten et al. (2015). Three replicates of pools of at least 5 individuals were dissected for each sample using a ceramic knife where possible, to prevent metal contamination. Digestive gland, gills, and remaining tissue were analysed. Tissue weight was recorded to determine biotic influences on MT concentrations.

5.2.2 Metallothionein analysis

MT was measured using a UV-spectrophotometric method devised by Viarengo et al. (1997), with modifications by Aly et al. (2014). Concentrations are expressed as µg/g (wet weight).

5.2.3 Tissue metal analysis

Metals were extracted from clam tissue using an *Aqua Regia* (3:1 v/v solution of trace metal grade, redistilled 37% hydrochloric acid (HCl) and 68% nitric acid (HNO_3)) digestion with hydrogen peroxide additions (H_2O_2). Inductively couple plasma mass spectrometry (ICP-MS) analysis was used to quantify metal concentrations from prepared solutions. Concentrations are expressed as µg/g (dry weight). A mussel reference material (European Reference Materials – CE278k) was measured as a bivalve comparator, and concentrations were adjusted according to recovery rate.

5.2.4 Statistical analysis

All statistical analysis was performed using IBM SPSS Statistic v21. Data were tested for homogeneity of variance (Levene's test) and for normality (Shapiro-Wilk test) and were tested parametrically or non-parametrically accordingly. Linear regression analysis was used to determine the effects of metal exposure on MT concentrations in clam species. Statistical significance was established at P = 0.05.

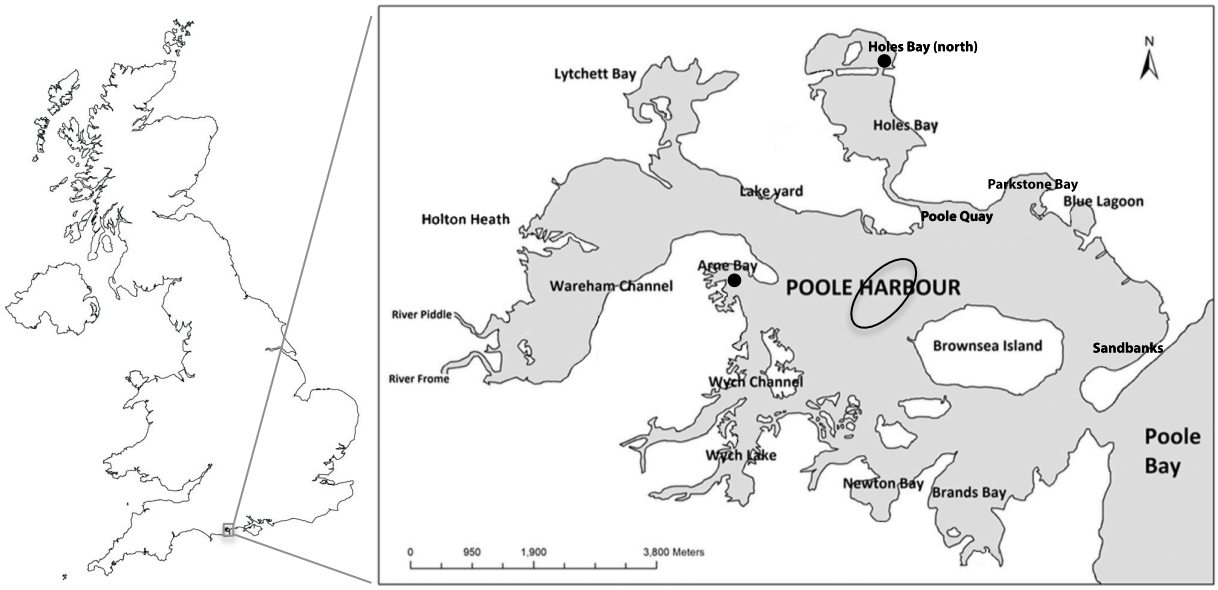


Figure 14 Site map of Poole Harbour, UK, showing sampling locations (black points), and the approximate location of the leased fishing beds where clams were originally collected (black circle).

5.3 Results

5.3.1 Species metallothionein concentrations

After four weeks of exposure, MT concentrations in *R. philippinarum* did not vary greatly between sites, or from pre-exposure (Figure 15a). Concentrations in the digestive gland and whole tissue did not show significant differences. However, gill concentrations were significantly lower in Holes Bay (north) compared with pre-exposure and Arne Bay (post-hoc Scheffe, P = 0.002, P = 0.036). MT concentrations in *R. decussatus* also showed little variation between sites; however, gill and whole tissue MT concentrations were significantly lower in Holes Bay (north) compared with Arne Bay (T = 7.360, T = 3.866, P = 0.002, P = 0.018, respectively). *V. corrugata* exhibited highest MT concentrations in Holes Bay (north) in the digestive gland and whole tissue; however, differences were insignificant. Gill concentrations were significantly lower in Holes Bay (north) compared to the pre-exposure sample and Arne Bay in this species (post-hoc Scheffe, P = 0.008, P = 0.005, respectively).

After sixteen weeks of exposure in Arne Bay, MT concentrations in *R. philippinarum* decreased slightly compared to four weeks of exposure (Figure 16a). This decrease was only significant in gills and whole tissue (T = 7.719, T = 3.259, P = 0.002, P = 0.031, respectively). In *V. corrugata* MT concentrations decreased significantly in digestive gland, gills, and whole tissue (T = 6.658, T = 4.394, T = 6.501, P = 0.003, P = 0.012, P = 0.003, respectively).

5.3.2 Species metal concentrations

After four weeks of exposure, concentrations of Cr, Fe, Ni, Cu, Zn, Sn, and Pb in *R. philippinarum* were higher in Holes Bay (north) compared with Arne Bay (Figure 15b – k). This was also predominantly the case for *R. decussatus* and *V. corrugata*. After sixteen weeks of exposure in Arne Bay, most metals increased in concentration in *R. philippinarum* (Figure 16b – k). This was also the case in *V. corrugata*, with the exception of Cu and Zn, which decreased.

5.3.3 Species metallothionein response to metal exposure

Linear regression analysis was performed to determine the importance of metal exposure to MT induction in each species (Figure 17). No significant positive relationships were observed between MT and metal concentrations in *R. philippinarum*. *V. corrugata* displayed significant positive correlations between MT and Cu and Zn in the digestive gland (R^2 = 0.424, R^2 = 0.490, P = 0.022, P = 0.011, respectively). *V. corrugata* also displayed significant positive relationships in whole tissue between MT and Zn (R^2 = 0.708, P = 0.001). In addition, in *R. decussatus,* MT concentrations in

gills showed positive relationships with Fe and Pb (R^2 = 0.719, R^2 = 0.789, P = 0.033, P = 0.018, respectively). When specifically analysing the change in concentrations from four weeks to sixteen weeks exposure in Arne Bay (Figure 18), *V. corrugata* exhibited significant positive relationships between MT and Cu and Zn concentrations, in the digestive gland (R^2 = 0.689, R^2 = 0.777, P = 0.041, P = 0.02, respectively), in the gills (R^2 = 0.693, R^2 = 0.871, P = 0.04, P = 0.007, respectively), and in whole tissue with Zn (R^2 = 0.937, P = 0.002, respectively).

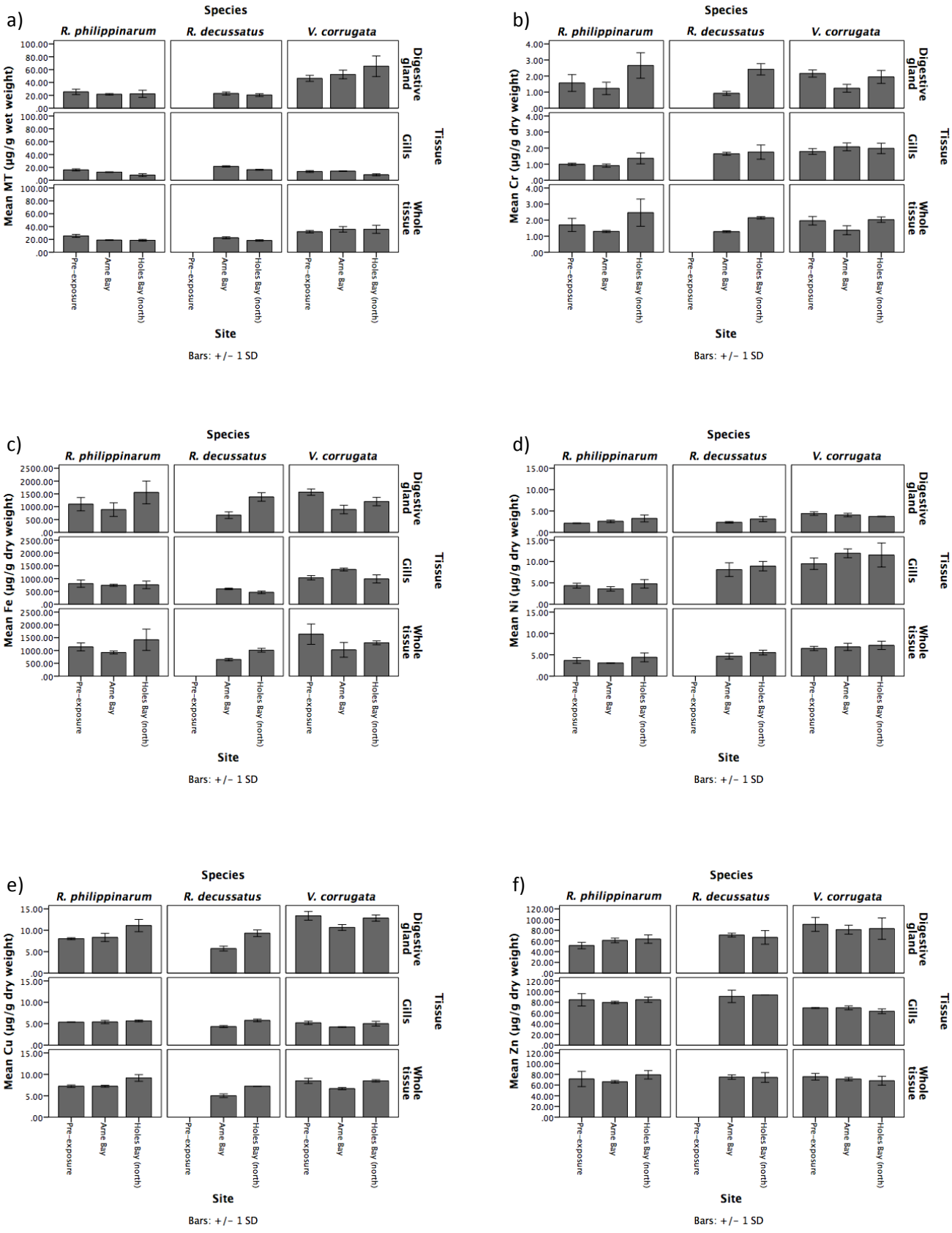
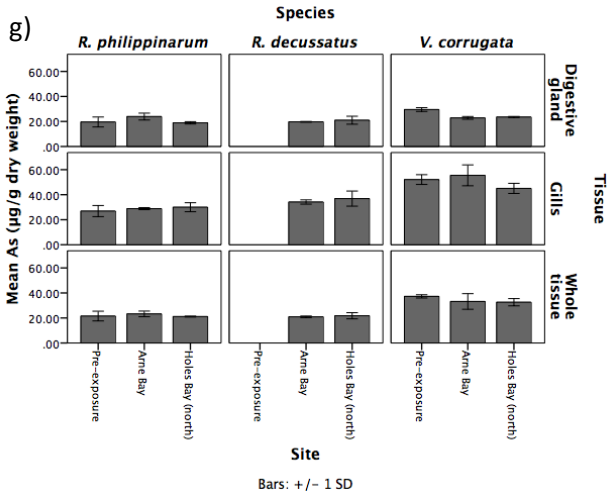
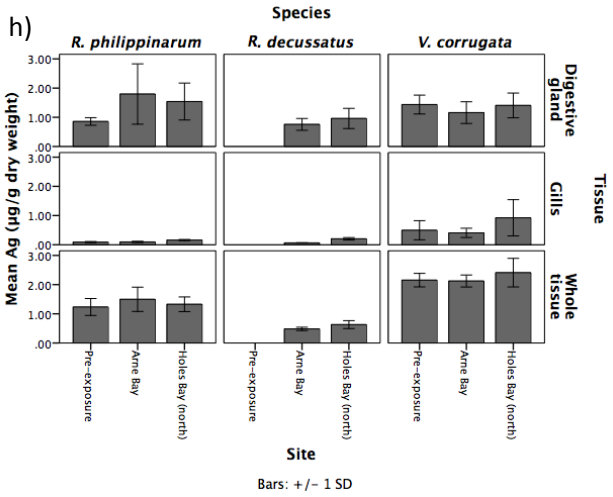
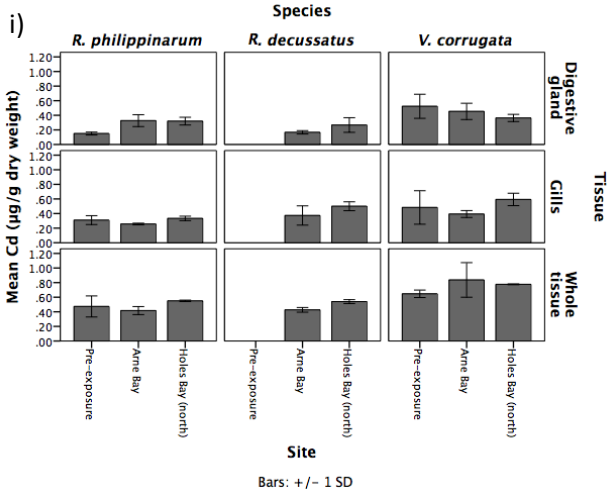
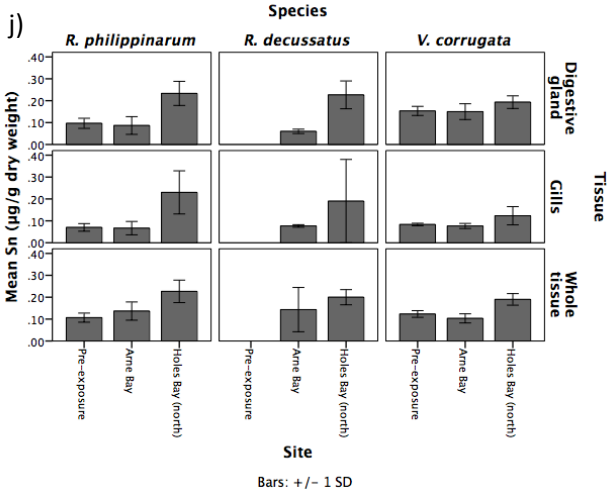


Figure 15 Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn, k) Pb in different tissues from *R. philippinarum, R. decussatus*, and *V. corrugata* from Poole Harbour after four weeks of exposure in each site, including a pre-exposure sample, with standard deviation (SD) (n = 3).









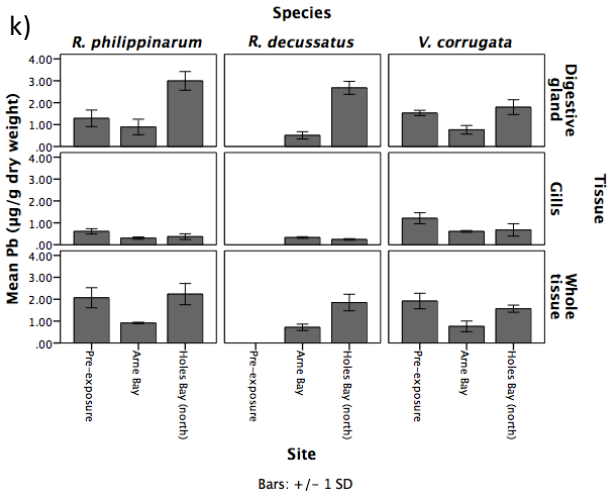


Figure 15 cont. Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn, k) Pb in different tissues from R. *philippinarum, R. decussatus*, and *V. corrugata* from Poole Harbour after four weeks of exposure in each site, including a pre-exposure sample, with standard deviation (SD) (n = 3).

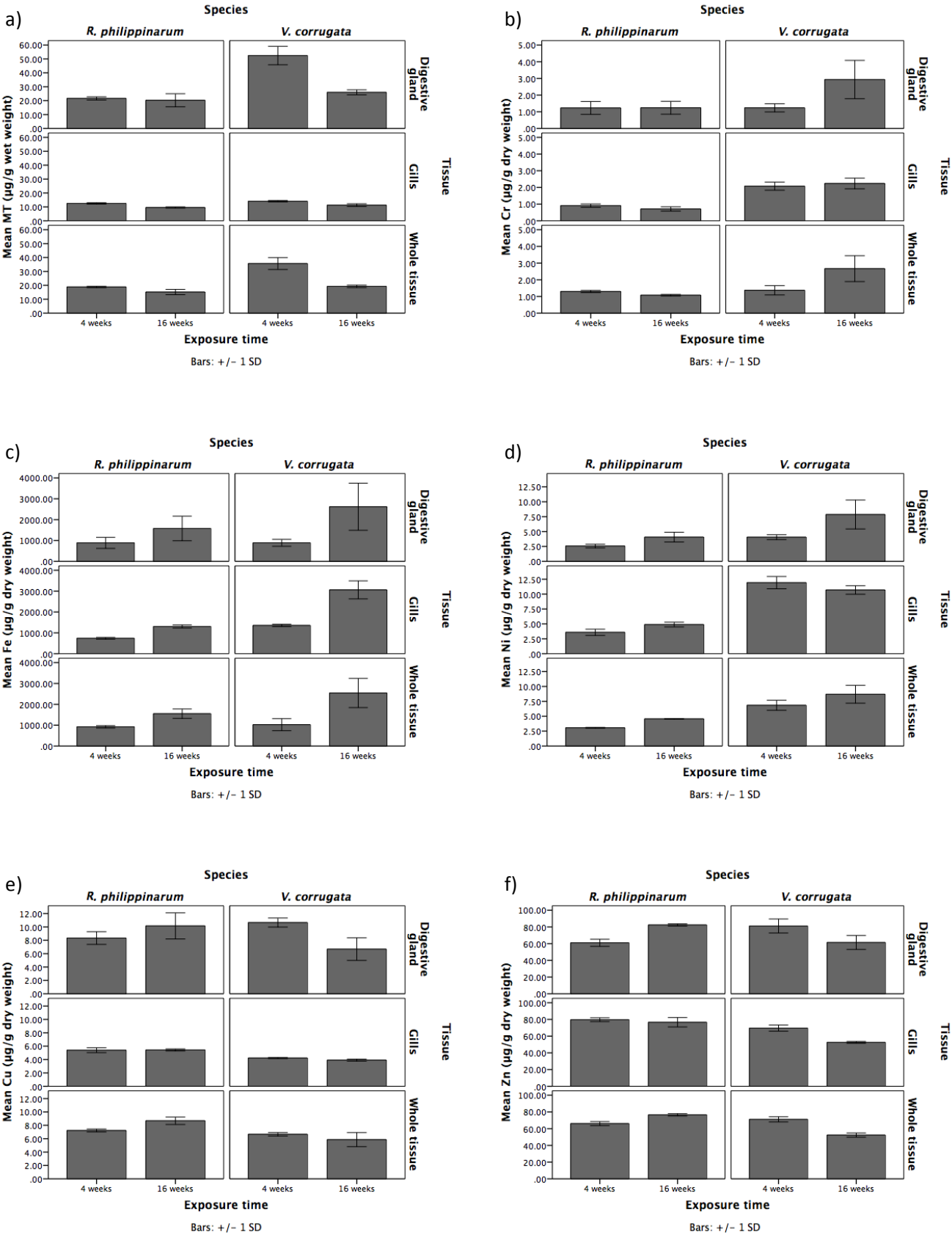
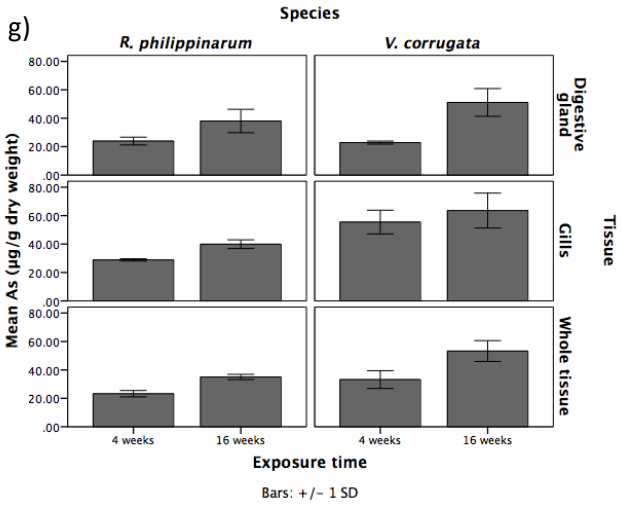
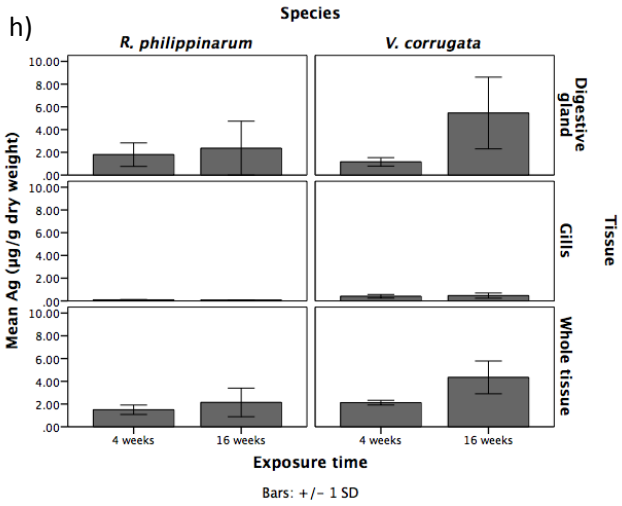
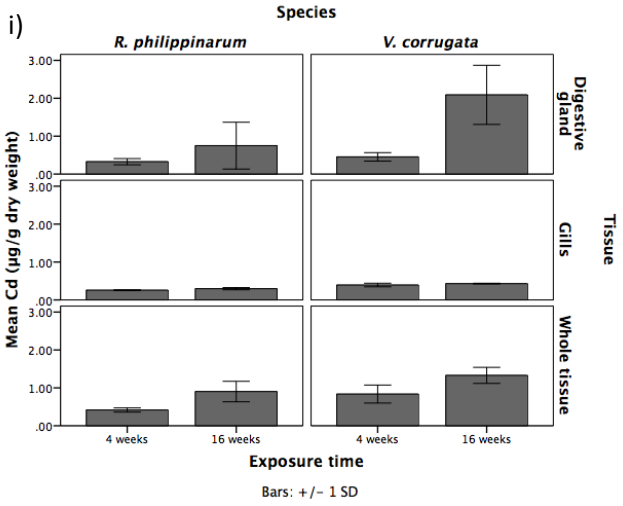
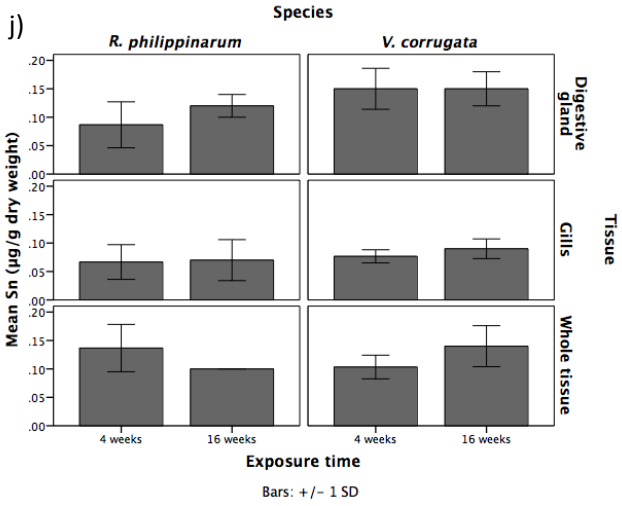


Figure 16 Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn, k) Pb in different tissues from *R. philippinarum* and *V. corrugata* from Arne Bay after four and sixteen weeks of exposure, with standard deviation (SD) (n = 3).









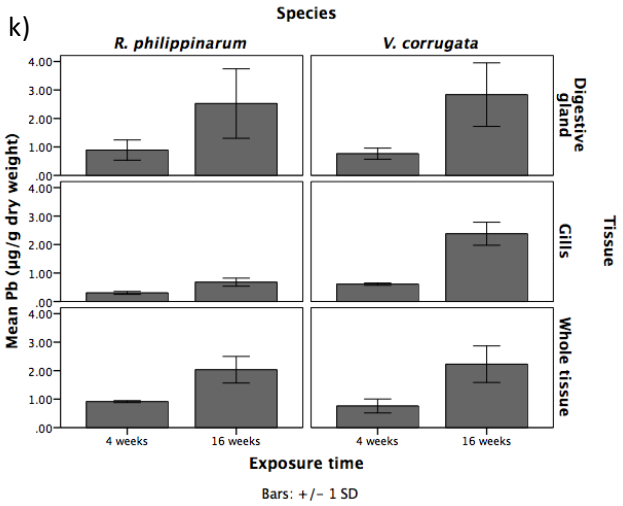


Figure 16 cont. Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn, k) Pb in different tissues from *R. philippinarum* and *V. corrugata* from Arne Bay after four and sixteen weeks of exposure, with standard deviation (SD) (n = 3).

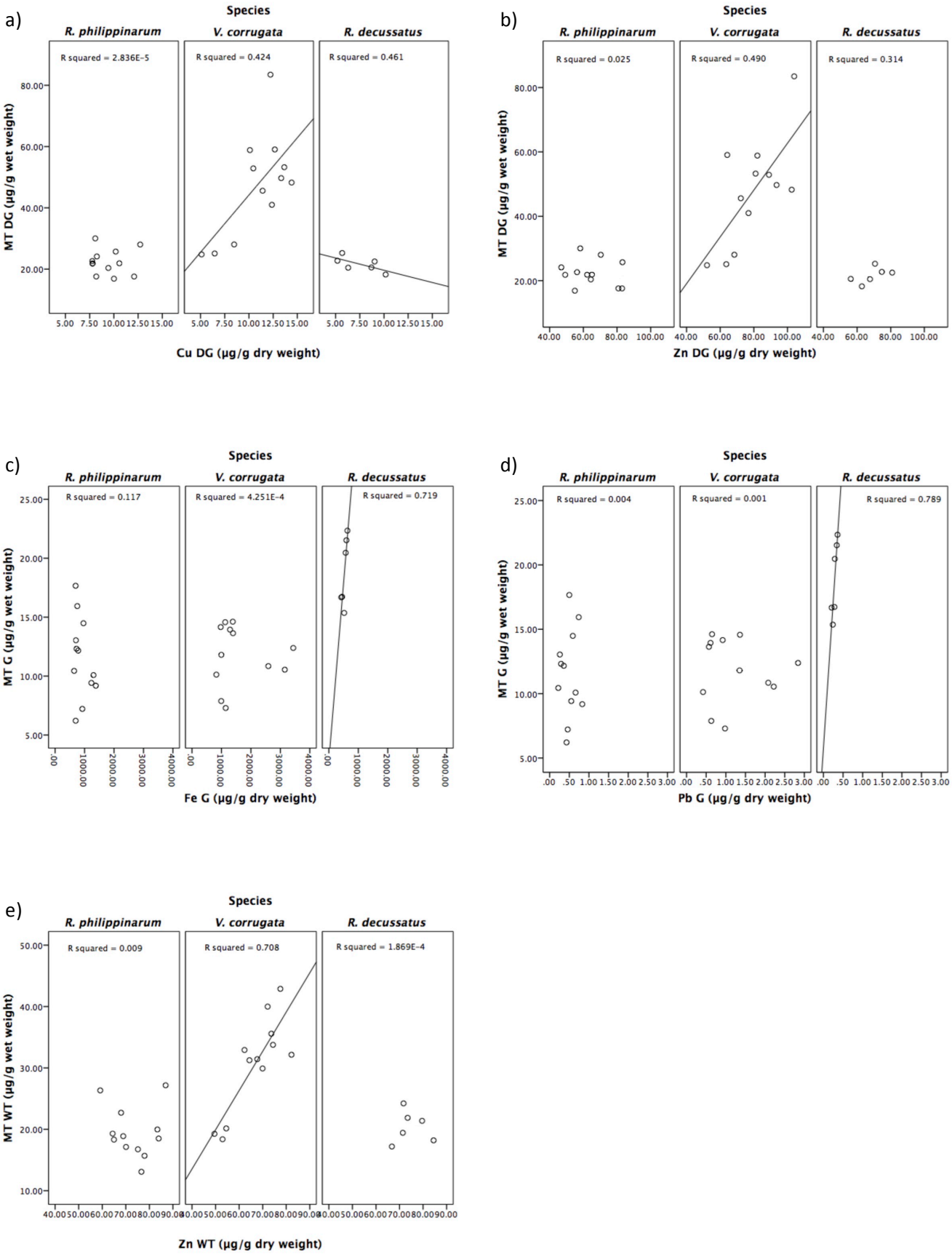


Figure 17 Linear regression between concentrations of MT and a) Cu and b) Zn in digestive gland (DG), c) Fe and d) Pb in gills (G), e) Zn in whole tissue (WT) from R. *philippinarum, V. corrugata*, and *R. decussatus* from Poole Harbour.

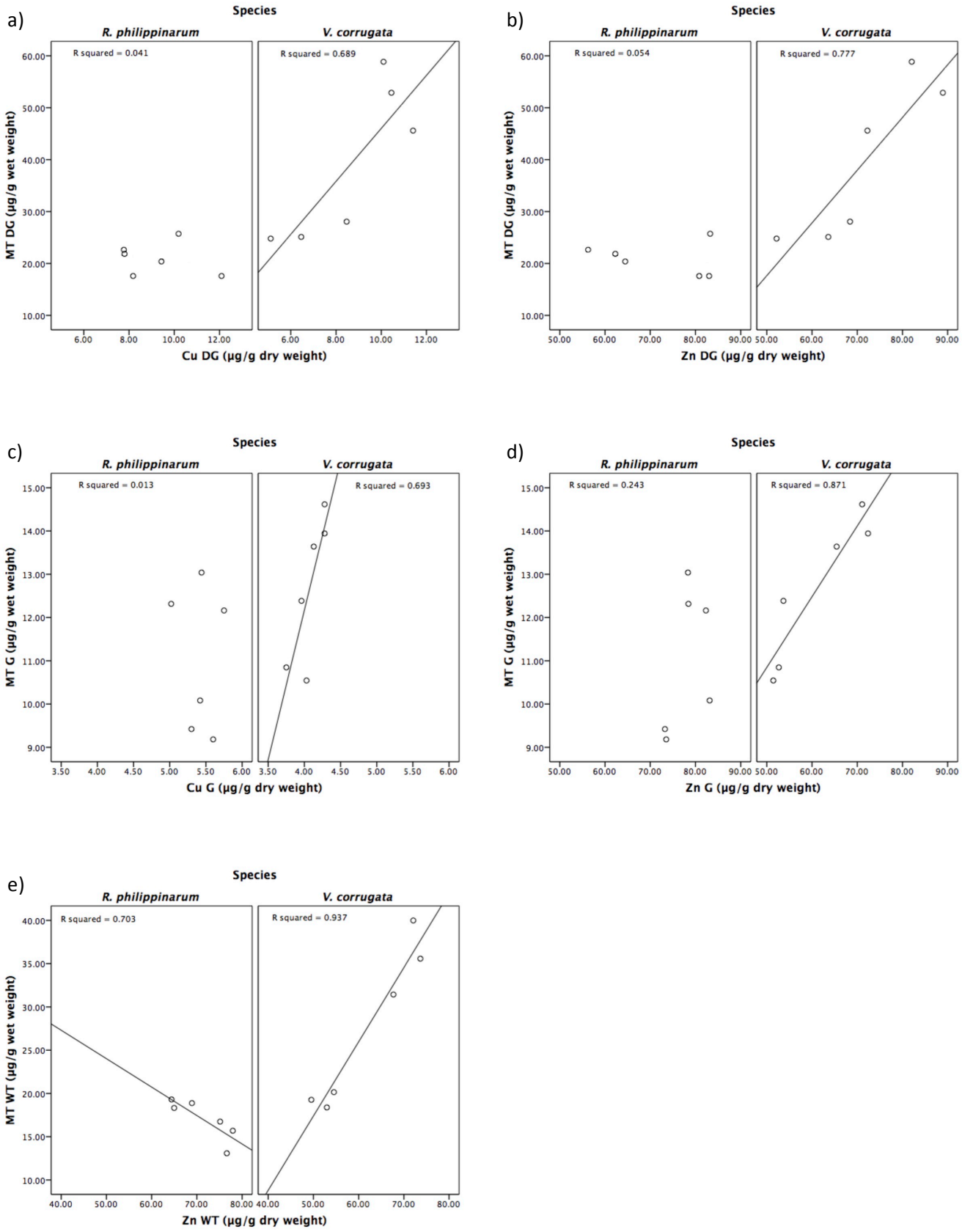


Figure 18 Linear regression between concentrations of MT and a) Cu and b) Zn in the digestive gland (DG), c) Cu and d) Zn in gills (G), e) Zn in whole tissue (WT) from *R. philippinarum* and *V. corrugata* from Arne Bay between four and sixteen weeks of exposure.

5.4 Discussion

After four weeks of exposure, tissue metal concentrations in each clam species indicate Holes Bay (north) had a greater magnitude of metal contamination compared with Arne Bay. This is in agreement with previous literature on Poole Harbour (Langston et al., 2003b, Aly et al., 2013). Furthermore, tissue metal concentrations in *R. philippinarum* and *V. corrugata* generally increased after sixteen weeks of exposure in Arne Bay, during summer. It is possible that higher temperatures increased the bioavailability of metals, through increases in free metal ion activity and other hydrolysed and carbonate metal species, as well as increasing the rate of metabolic activities and thus uptake (Mubiana and Blust, 2007, Rouane-Hacene et al., 2015).

Highest concentrations of MT were found in *V. corrugata* digestive gland and whole tissue sampled from Holes Bay (north), in conjunction with most metal concentrations. However, minimum concentrations of MT in all tissues were found in *R. philippinarum* and *R. decussatus* from Holes Bay (north). This suggests MT induction is not responding to metal exposure in the latter species. As well as this, despite most tissue metal concentrations increasing after sixteen weeks of exposure in *R. philippinarum* and *V. corrugata*, MT concentrations decreased in both species. However, in *V. corrugata*, this showed significant positive relationships with Cu and Zn. It is possible increased temperature and heat stress is causing MT to decrease irrespective of metal tissue concentrations (Bocchetti et al., 2008, Smaoui-Damak et al., 2009).

Previous studies have found an increase in MT following metal exposure in *R. decussatus* (Bebianno et al., 2000, Hamza-Chaffai et al., 2000, Bebianno and Serafim, 2003, Serafim and Bebianno, 2010) and *R. philippinarum* (Zhao et al., 2010, Wang et al., 2011, Won et al., 2012). Some studies suggest that MT in the gills, compared with the digestive gland, is a more sensitive biomarker of metal contamination in *R. decussatus* (Geret et al., 2003, Bebianno et al., 2004, Cravo et al., 2013). One stated reason for this is that gills are in direct contact with the surrounding environment, reflecting short-term contaminant exposure and are the primary receptor for water contaminants (Bebianno et al., 2004, Cravo et al., 2013). This may explain the significant positive relationship between gill MT concentrations and Pb and Fe in *R. decussatus*, and the lack of relationship between MT and metals in other tissues in this species. However, other studies advocate the use of MT in the digestive gland as opposed to gills in *R. decussatus* as the most appropriate biomarker of metal contamination (Smaoui-Damak et al., 2009).

Only one study, to our knowledge, has examined MT response to metal contamination in *V. corrugata* (Velez et al., 2016). This study concluded that MT concentrations in this species were highest in the most severely metal contaminated site in the Ria de Aveiro, Portugal. Strong correlations were also observed between MT and metal concentrations in sediments and tissue.

This suggests a capacity to regulate metal concentration in *V. corrugata*, with MT synthesis providing an important organism resistance against accumulated metals. These findings are in agreement with this study, as MT concentrations were highest in this species in Holes Bay (north), the most contaminated study site, and significant positive relationships between MT and some metals were evident in *V. corrugata*.

MT concentrations in *R. philippinarum* and *R. decussatus* were similar in this study. MT in each species also did not respond to metal exposure. This is contradictory to previous studies that have compared metal accumulation and MT in these species. Moraga et al. (2002), reported higher MT concentrations in *R. decussatus* in more contaminated sites, concluding that MTs could be used as a major specific indicator of the biological effects of metal pollution. *R. philippinarum* showed no differential responses according to the degree of metal contamination. The study suggested a differential response between the species in terms of detoxifying metals. Figueira et al. (2012) studied the toxicological effects and bioaccumulation patterns of both species exposed to Cd in the laboratory. It was found that *R. decussatus* had a higher capacity to increase the expression of MTs when exposed to Cd, compared to *R. philippinarum*. As a result, *R. philippinarum* presented higher oxidative stress, despite a much higher Cd accumulation in *R. decussatus* in the soluble fraction (cytosol). MT concentrations in response to metal contamination in *V. corrugata* have not been compared to other species previously.

There is evidence to suggest non-metallic influence over MT production in *R. philippinarum* and *R. decussatus*. Tissue weight (Smaoui-Damak et al., 2009, Moschino et al., 2012), reproductive cycle (Smaoui-Damak et al., 2009, Moschino et al., 2012), location (Smaoui-Damak et al., 2004), life history (Paul-Pont et al., 2010b), pathogens (Paul-Pont et al., 2010a), as well as environmental parameters such as pH, salinity, and temperature (Smaoui-Damak et al., 2009) have been found to be important in explaining the variation in MT concentrations in *R. decussatus* and *R. philippinarum*.

The potential role of MT in gametogenesis and reproduction, and consequent independent response of MT to metal exposure, has been reported in *R. decussatus* (Smaoui-Damak et al., 2009). The study found that at certain sites, physiological changes caused by gamete development contributed more to changes in MT concentrations than the bioavailable Cd concentrations. Studies on MT in *R. philippinarum* also confirm its sensitivity to biological factors such as the reproductive cycle in this species (Bocchetti et al., 2008, Moschino et al., 2012).

The timings of the reproductive cycle of *R. philippinarum* and *R. decussatus* are different to that of *V. corrugata,* as described by da Costa (2012). *R. philippinarum* adopts an opportunistic strategy for its reproduction, meaning gametogenesis is coupled with high abundances of food in order to

provide energy for producing gametes and for larval development (Lubet, 1986). This suggests gametogenesis in this species begins in spring, when temperatures begin to rise and food availability increases with the phytoplankton bloom. This is in agreement with a study on the recruitment of this species in Poole Harbour, which revealed two spawning events in June and October 2003 (Humphreys et al., 2007). However, *V. corrugata* is known to adopt a conservative strategy for its reproduction (Joaquim et al., 2011). This means it begins gametogenesis earlier when temperature and food availability is low, by utilising nutrient reserves (Sastry, 1979). This strategy avoids competition with other species during spawning (da Costa, 2012). *R. decussatus* is thought to adopt an intermediate strategy as both stored and recently assimilated nutrients are used for gametogenesis (Anibal et al., 2011). However, da Costa (2012) and the studies cited therein reveal spawning events at similar times to *R. philippinarum.* The differences in the timing of reproductive cycles in these species may explain why MT concentrations are not responding to metal exposure in *R. philippinarum* and *R. decussatus*, but are in *V. corrugata*. It is possible that *V. corrugata* had finished undergoing gametogenesis when this study was conducted in May and August, whereas *R. philippinarum* and *R. decussatus* were still undergoing this process. Therefore, gametogenesis may be influencing MT concentrations independently of metal exposure in these species.

5.5 Conclusion

Comparison of sympatric species of clam has enabled an assessment of the potential and limits of use of MT as a biomarker of metal pollution in these species. This study provides further evidence for the disruption of MT response to metal exposure during periods of gametogenesis. *R. philippinarum* and *R. decussatus* did not respond to differential exposure to metal contamination during spring and summer, when this study was undertaken. MT concentrations in *V. corrugata*, however, did show positive responses to metal exposure. This is possibly because *V. corrugata* adopts a conservative strategy for reproduction and begins gametogenesis earlier in the year to avoid competition with other clam species. Consequently, MT variation due to gametogenesis may be less influential in *V. corrugata* during spring periods, when MT concentrations in *R. philippinarum* and *R. decussatus* are altered independently of metal exposure. This study highlights the limits of use for MT as a biomarker in organisms that are undergoing gametogenesis, such as *R. philippinarum* and *R. decussatus*. Therefore, the use of MT in these species should be restricted to winter periods. Furthermore, we suggest the use of *V. corrugata,* a previously seldom studied organism, as an alternative MT biomarker species of clam when other species are redundant due to active stages in their reproduction.

Chapter 6: Metal accumulation and metallothionein response in *Fucus spiralis*

6.1 Introduction

Seaweeds are advocated bioindicators in temperate coastal waters due mainly to their high abundances and immobility (Rainbow, 1995a). They often dominate metal-contaminated habitats (Nielsen and Nielsen, 2005) and are resistant to metal pollution (Pawlik-Skowronska et al., 2007). They have an ability to accumulate dissolved metals from seawater so their intracellular concentrations reflect time-integrated contamination loads in the marine environment (Vasquez and Guerra, 1996). As a consequence, seaweeds are established sentinels for metal contamination and are exploited for biomonitoring (Owen et al., 2012).

Metallothionein (MT) is a protein of low molecular weight, high heat stability, and high cysteine content (Kagi and Kojima, 1987). The latter attribute lends itself to be used as a biomarker of metal pollution, as it has a high affinity to bond to metals due to sulphur containing thiol groups (Amiard et al., 2006). It is regarded to play a vital role in metal detoxification of metals within organisms (Klaassen et al., 1999, Amiard et al., 2006). This relays a biological response indicating the severity of metal pollution to the organism. Many organisms have been employed as a MT biomarker species, primarily bivalve species (Bebianno and Langston, 1991, Geffard et al., 2002, Serafim and Bebianno, 2007b, Frank et al., 2008, Geng et al., 2015). However, MT is also noted to have multiple roles such as maintaining homeostasis by regulating essential metals, and as a defence against reactive oxygen species (Viarengo et al., 2000, Le et al., 2016). There are also factors that contribute natural variation of MT including tissue weight (Raspor et al., 2004), reproductive stage (Moschino et al., 2012), temperature (Smaoui-Damak et al., 2009), and salinity (Legras et al., 2000). This limits the use of MT as a tool in biomonitoring, as it may alter concentrations independently of metal exposure, particularly in bivalve species.

Literature on MT response in marine algae to metal exposure is limited, compared with other organisms. MT in spiral wrack (*Fucus spiralis*) has never been reported. The MT gene has been identified in bladder wrack (*Fucus vesiculosus*) by Morris et al. (1999), which suggested that a protective mechanism against metal exposure exists for this species. Further studies suggest MT induction in this species following Cu exposure (Owen et al., 2012), as well as an ability to bind to As, Cd, and Zn (Merrifield et al., 2006, Ngu et al., 2009). This shows potential for MT to be developed as a biomarker in fucoids.

F. spiralis would offer a cosmopolitan bioindicator species for dissolved metal contamination, if MT is shown to be a reliable biomarker in this species. It is geographically widely available and easy to sample as they are sessile, suggesting it is a promising candidate. It may also be less susceptible to natural variation compared to traditional MT biomarker species. However, despite the potential for *F. spiralis* to be used as a MT biomarker species, its use has not been developed, and no study of its MT response to metals has been conducted in the field. Therefore, this study aimed to investigate the potential for MT in *F. spiralis* to be used a biomarker for metal pollution.

6.2 Methodology

6.2.1 Sample collection

Seaweed samples were collected from four sites in Poole Harbour, UK: Holes Bay (north), Holes Bay (south), Poole Quay, and Sandbanks (Figure 19). Sampling was carried out in January, April, August, and October 2015, which are referred to as winter, spring, summer, and autumn. Environmental variables could not be measured as samples were taken from shore. Samples were kept in storage at -20 °C before analysis, as advised by Oaten et al. (2015).

6.2.2 Metallothionein analysis

MT concentrations were measured using the UV-spectrophotometric method devised by Viarengo et al. (1997), with modifications by Aly et al. (2014). Before analysis, approximately 3 cm of the apical tips of seaweed were dissected, in order to select the tissue that reflected the most recent MT and metal concentrations in the surrounding water (Connan and Stengel, 2011). This was then homogenised using a ceramic blade and a pestle and mortar (to avoid metal contamination before metal analysis). Three replicates of each sample were measured. Concentrations are reported in µg/g (wet weight).

6.2.3 Metal analysis

Before analysis, previously homogenised samples (as per MT analysis) were freeze-dried for 72 hours. Accurately weighed samples of approximately 10 mg of dried, ground sample were placed in 7 ml Teflon sealable pots. Blank samples consisting of empty Teflon pots were also prepared. Samples were digested in *Aqua Regia* (3:1 v/v solution of trace metal grade, redistilled 37% hydrochloric acid (HCl) and 68% nitric acid (HNO_3)) on a hot plate. Additions of trace metal grade hydrogen peroxide (H_2O_2) were made to oxidize organic matter. Samples were dried, resuspended, and completed with 3% trace metal grade, redistilled, nitric acid (HNO_3), containing

5 ppb In/Re and 20 ppb Be as internal standards to correct for matrix effects (which are a suppression or enhancement of analyte signal caused by concentrations of matrix elements) and instrument drift. Analysis by inductively coupled plasma mass spectrometry (ICP–MS) was carried out. A mussel reference material (European Reference Materials – CE278k) was measured as a bivalve comparator and concentrations were adjusted according to the recovery rate. Concentrations are reported as µg/g (dry weight).

6.2.4 Statistical analysis

All statistical analysis was completed using IBM SPSS Statistic v21. Tests for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test) were completed and data were tested parametrically (one-way ANOVA) or non-parametrically (Kruskall-Wallis test), accordingly. Linear regression was used to determine the relationships between metal exposure and MT concentrations in *F. spiralis*. Statistical significance was established at P = 0.05.

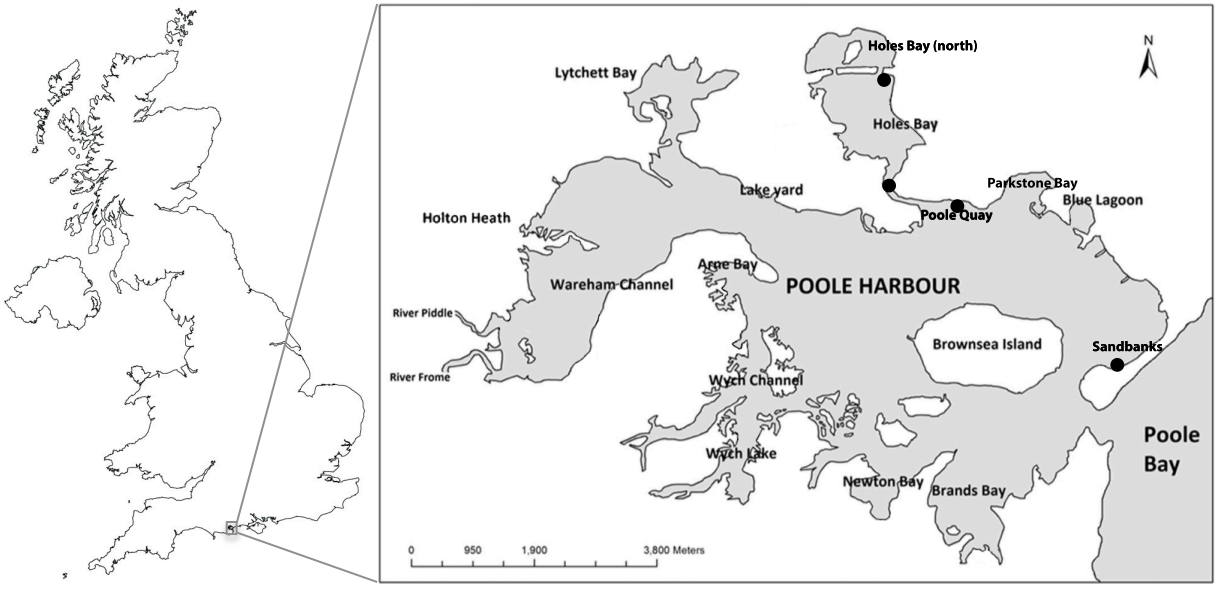


Figure 19 Site map of Poole Harbour, UK, and sampling locations.

6.3 Results

MT concentrations in *F. spiralis* varied greatly throughout the sampling year (Figure 20a, Table 15). In winter, MT concentrations were significantly higher in Holes Bay (north), compared with Holes Bay (south), Poole Quay, and Sandbanks (*post-hoc* Scheffe, P = 0.015, P < 0.001, P < 0.001, respectively). Holes Bay (south) was also significantly higher than Poole Quay and Sandbanks

(*post-hoc* Scheffe, P = 0.004, P = 0.048, respectively). Concentrations of MT in *F. spiralis* from Sandbanks increased in spring, and became highest in summer and autumn. In spring, MT concentrations were higher in Holes Bay (north) compared with Poole Quay (*post-hoc* Tukey, P = 0.04). In summer, the concentration of MT in *F. spiralis* from Sandbanks was significantly higher than Poole Quay (*post-hoc* Scheffe, P = 0.026). During autumn, significant differences in MT concentrations in *F. spiralis* did not exist between sites (F = 2.835, P = 0.106). Across seasons, mean MT concentrations in winter were significantly lower than concentrations in spring (post-hoc Scheffe, P = 0.047), summer (post-hoc Scheffe, P = 0.016), and autumn (post-hoc Scheffe, P = 0.015) (Table 15).

Metal concentrations in *F. spiralis* also varied greatly throughout the sampling year, and were inconsistent in each season (Figure 20b – k). For Cr, Fe, Ag, Cd, highest concentrations were generally found at Holes Bay (north) throughout the year. For Sn and Ni concentrations were highest at Sandbanks in winter and summer, and highest at Holes Bay (north) in spring and autumn. For Zn and As, highest concentrations were predominantly found at Holes Bay (south), and for Cu during winter and spring. Pb concentrations were highest in *F. spiralis* at Holes Bay (north), Poole Quay, and Sandbanks in winter, spring, and summer, respectively. Furthermore, concentrations of Cu, Zn, As, Ag, and Cd generally decrease from winter to autumn (Table 15). Across seasons, mean Zn, As, Ag, and Cd concentrations were significantly different (Table 15). Winter As concentrations were significantly higher than concentrations in spring (post-hoc Scheffe, P = 0.006), summer (post-hoc Scheffe, P < 0.001) and autumn (post-hoc Scheffe, P < 0.001). In winter, Zn and Ag concentrations were significantly higher than in summer (post-hoc Scheffe, P < 0.001, P = 0.003, respectively) and autumn (post-hoc Scheffe, P = 0.002, P = 0.004, respectively). Cd concentrations were significantly higher in winter than summer (post-hoc Scheffe, P = 0.002).

Linear regression analysis was used to assess the effect of metal exposure on MT concentration. During winter, only Fe tissue concentration showed a significant positive relationship with MT concentration (R^2 = 0.792, P < 0.001) (Figure 21a). In summer, significant positive relationships were evident, and existed between MT concentrations and Fe (R^2 = 0.631, P = 0.002) (Figure 21a), Ni (R^2 = 0.486, P = 0.012) (Figure 21b), Sn (R^2 = 0.579, P = 0.004) (Figure 21c), and Pb (R^2 = 0.415, P = 0.024) (Figure 21d). No significant positive relationships existed between MT and any other metals, during any season, and are therefore not shown graphically.

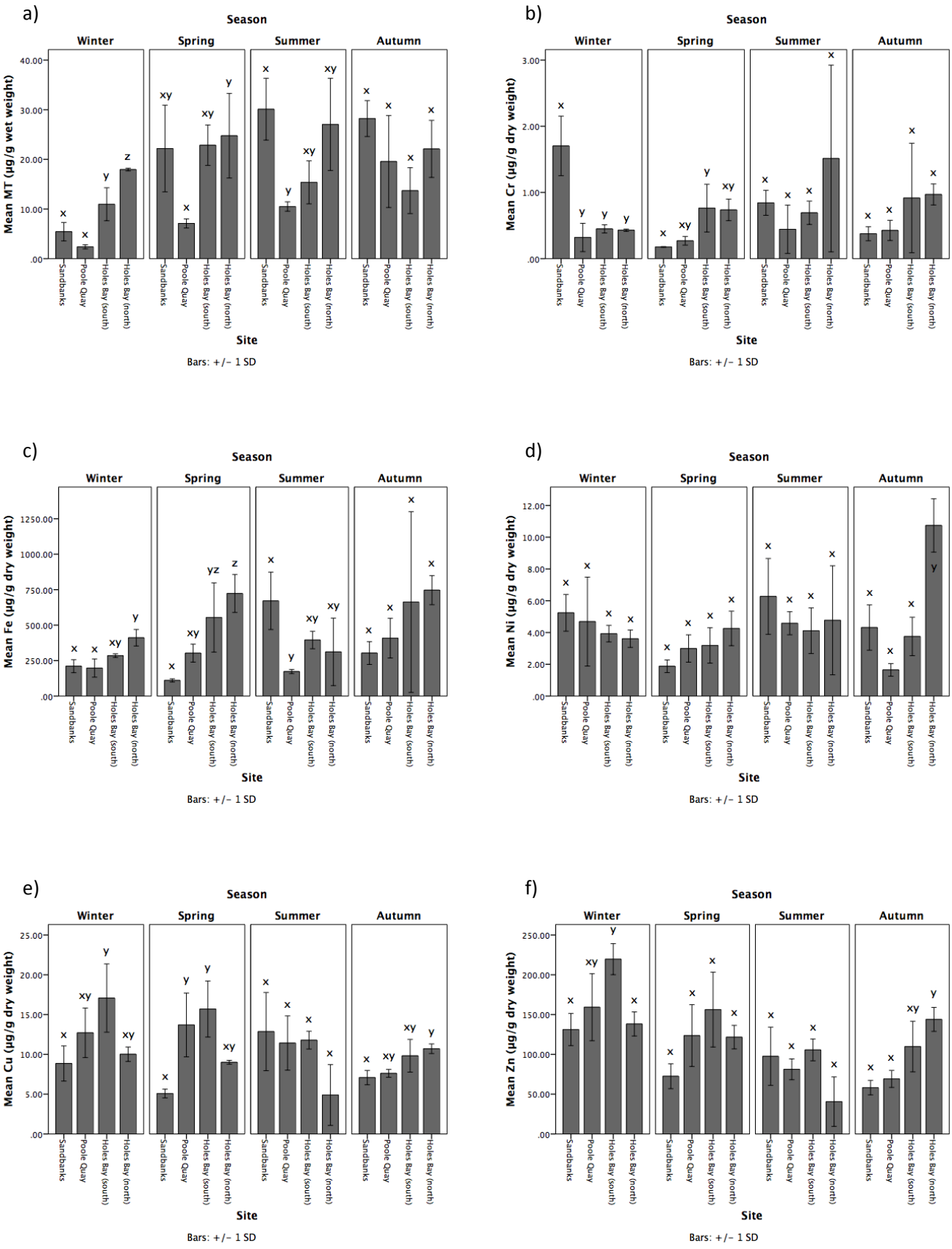


Figure 20 Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn, k) Pb in *F. spiralis* from Poole Harbour throughout each season in 2015, with standard deviation (SD) (n = 3). Different letters indicate significant differences (P = 0.05).

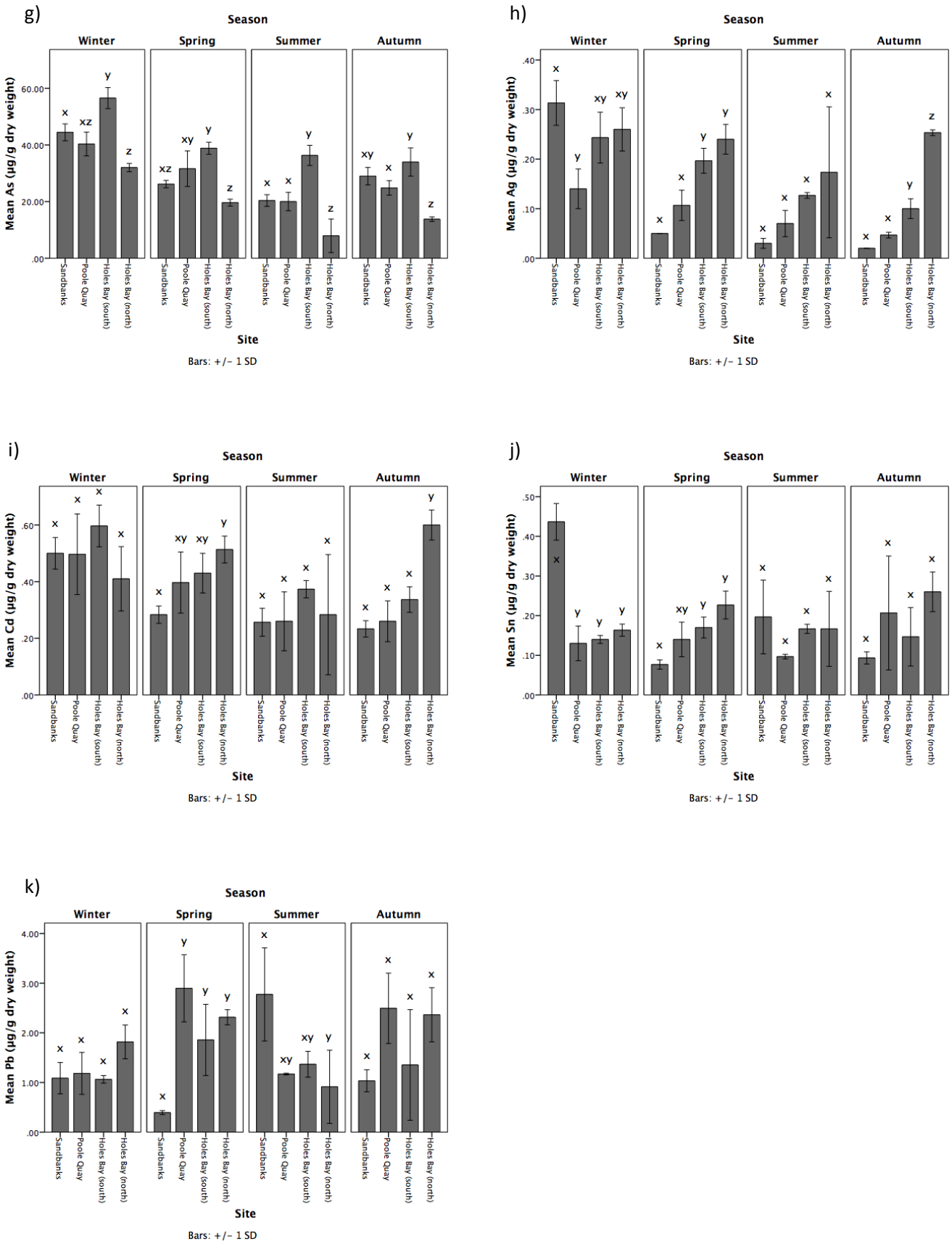


Figure 20 cont. Mean concentrations (µg/g) of a) MT, b) Cr, c) Fe, d) Ni, e) Cu, f) Zn, g) As, h) Ag, i) Cd, j) Sn, k) Pb in *F. spiralis* from Poole Harbour throughout each season in 2015, with standard deviation (SD) (n = 3). Different letters indicate significant differences (P = 0.05).

Table 15 Mean seasonal concentrations of MT (µg/g wet weight) and metals (µg/g dry weight) in *F. spiralis* sampled from Holes Bay (north), Holes Bay (south), Poole Quay, and Sandbanks in Poole Harbour in 2015. Different letters (x, y, z) indicate significant differences between seasons (P = 0.05).

		Winter	Spring	Summer	Autumn
MT (µg/g)	Mean	9.18^{x}	19.22^{y}	20.75^{y}	20.90^{y}
	SD	6.40	9.20	9.87	7.58
Fe (µg/g)	Mean	276.08^{x}	422.60^{x}	387.71^{x}	530.29^{x}
	SD	98.16	273.29	233.55	340.85
Ni (µg/g)	Mean	4.37^{x}	3.08^{x}	4.94^{x}	5.11^{x}
	SD	1.49	1.18	2.09	1.07
Cu (µg/g)	Mean	12.17^{x}	10.87^{x}	10.24^{x}	8.81^{x}
	SD	4.13	4.88	4.48	1.87
Zn (µg/g)	Mean	162.07^{x}	118.52^{xy}	81.28^{y}	95.29^{y}
	SD	42.75	41.66	34.20	39.05
As (µg/g)	Mean	43.34^{x}	29.07^{y}	21.15^{y}	25.40^{y}
	SD	9.64	7.93	11.06	8.23
Ag (µg/g)	Mean	0.24^{x}	0.15^{xy}	0.10^{y}	0.11^{y}
	SD	0.08	0.08	0.08	0.09
Cd (µg/g)	Mean	0.50^{x}	0.41^{xy}	0.29^{y}	0.36^{xy}
	SD	0.11	0.10	0.11	0.16
Sn (µg/g)	Mean	0.22^{x}	0.15^{x}	0.16^{x}	0.18^{x}
	SD	0.14	0.06	0.07	0.10
Pb (µg/g)	Mean	1.29^{x}	1.87^{x}	1.56^{x}	1.81^{x}
	SD	0.42	1.06	0.92	0.90

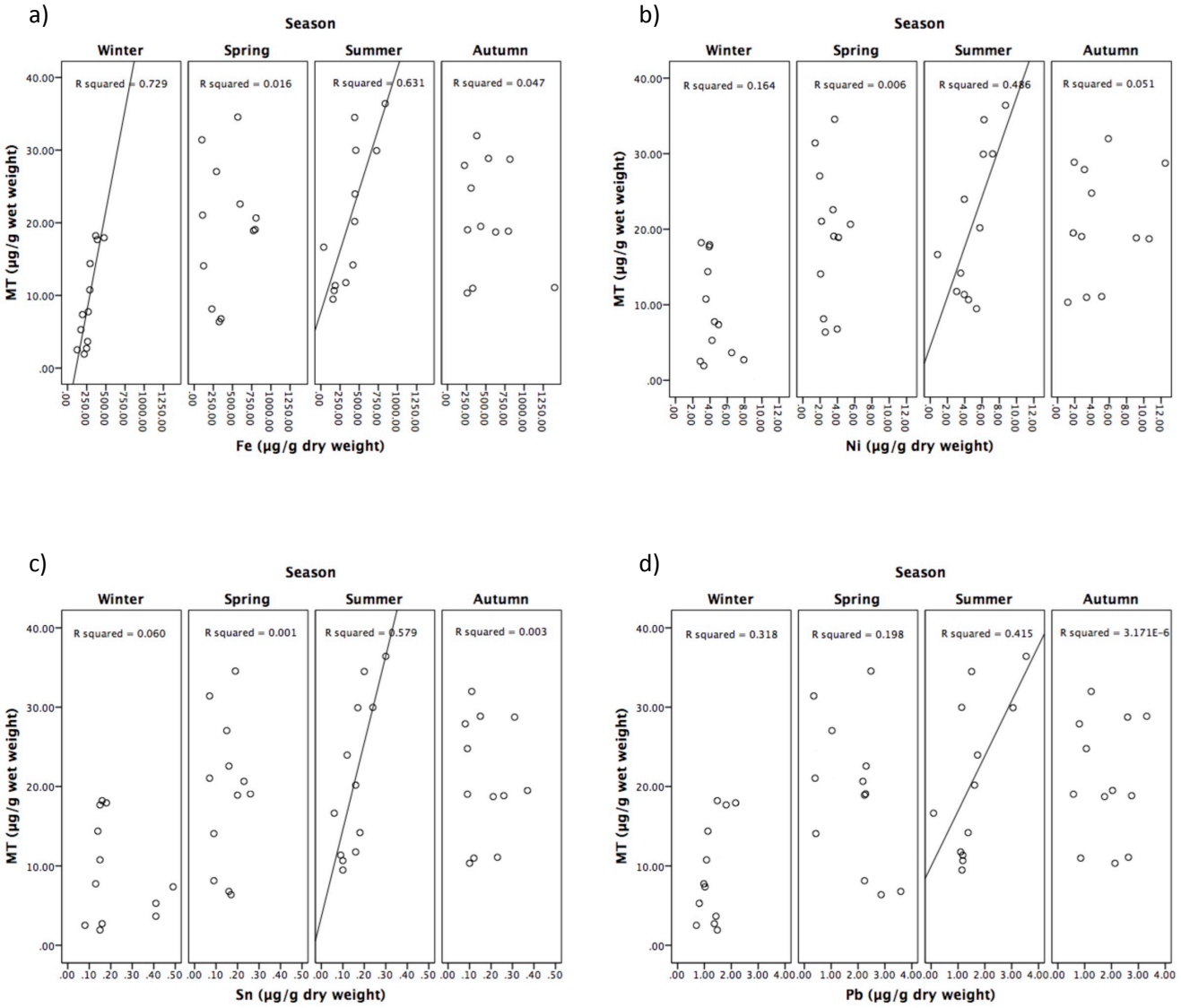


Figure 21 Linear regression between concentrations of MT and a) Fe, b) Ni, c) Sn, d) Pb in *F. spiralis* from Poole Harbour across seasons in 2015.

6.4 Discussion

6.4.1 Metal contamination and seasonal variation

Concentrations of metals in *F. spiralis* from Poole Harbour indicate the most contaminated area is Holes Bay (Figure 20b - k). This is in agreement with previous literature on metal contamination in Poole Harbour (Langston et al., 2003b, Aly et al., 2013), which reported generally higher metal concentrations within Holes Bay in sediment and seawater, as well as biota. *F. spiralis* metal concentrations at Sandbanks were also relatively high. This may be due to the sewage pumping station near to the site, which periodically discharges storm water. There are also yacht clubs in the vicinity, which may contribute to the metal burden in the area due to sources of metals such as anti-fouling paints on watercraft: higher Sn concentrations in winter could be related to boat maintenance in winter and the removal of old tributyltin (TBT) antifoulant.

Seasonal variation for some metal concentrations in *F. spiralis* is apparent, and metal concentrations tend to reduce from winter to autumn (Table 15). This could be explained by plant growth. Concentrations within seaweeds continue to mount through dormant periods during winter, and dilute as plants grow and reproduce in summer months (Fuge and James, 1974, Villares et al., 2002). Metal bioavailability may also influence seasonal variability of bioaccumulated metal concentrations. For example, dissolved Cd concentration tends to reduce during summer months due to uptake by phytoplankton (Pohl et al., 1993). Furthermore, higher salinity, which is observed during warmer months due to reduced rainfall and increased evaporation, also causes increased rates of Cd, Zn and Cu binding to chloride ions, thereby reducing dissolved metal bioavailability (Bryan and Langston, 1992, Rainbow, 1995b). Therefore, because seaweeds are predominantly exposed to dissolved metals concentrations within seawater, as they inhabit the water column, Cd, Cu and Zn exposure to seaweeds is reduced during summer. Seasonal variation in brown seaweed metal concentrations has been shown to be particularly evident for Cd and Zn, but less difference is observed for Pb and Ni (Miramand and Bentley, 1992, Riget et al., 1995). This may be explained by an ion-exchange process that actively takes up Pb and Ni, meaning the importance of bioavailability on bioaccumulation of these metals is less (Eide et al., 1980, Sanchez-Rodriguez et al., 2001, Besada et al., 2009). Furthermore, the process of phytoplankton metal uptake during summer, and salinity effects to dissolved metal bioavailability is rarely reported for Pb or Ni. This may explain the reduced seasonal variability in Pb and Ni *F. spiralis* concentrations observed in this study (Table 15).

6.4.2 Metal toxicity and metallothionein induction

Generally, the induction of MT is a physiological response to the insult caused by metal exposure (Morris et al., 1999, Merrifield et al., 2006). Therefore, toxic metals are more likely to cause MT induction. The order of toxicity of metals to seaweed species is generally Hg > Cu > Cd > Ag > Pb > Zn (Lobban and Harrison, 1994). Cu, despite being an essential metal, is the second most toxic metal to seaweeds, the effects of which have been extensively studied due to its use in antifouling paints (Lobban and Harrison, 1994, Coelho et al., 2000). It is often cited to inhibit photosynthetic processes and retard growth in seaweed species (Kupper et al., 2002, Connan and Stengel, 2011, Costa et al., 2016). In addition, inhibition of fertilization and reproduction resulting from Cu exposure has been identified in *F. spiralis* (Bond et al., 1999). Pb has also been shown to impact photosynthetic efficiency and growth of red and brown seaweeds, but is less toxic than Cu (Stewart, 1977, Costa et al., 2016). Cd can affect growth, pigment content, and carbon assimilation in seaweeds (Markham et al., 1980). Zn has also been shown to slow growth in seaweeds (Munda and Hudnik, 1988).

There is limited knowledge on MT response to metal exposure in seaweeds, though few studies exist on *F. vesiculosus*. Morris et al. (1999) noted the MT gene in *F. vesiculosus* to be induced by Cu exposure, and that MT can bind to both Cu and Cd. Further studies confirmed its role as a detoxification mechanism for metals, and reported MT binding abilities to Zn, and As (Merrifield et al., 2006, Ngu et al., 2009). However, only Owen et al. (2012) confirmed this role in the field *in vivo*. The study found MT to respond to Cu exposure, and this metal was found to be more important for MT induction, indicated by a stronger and more significant regression coefficient, compared with Zn and Fe. In this study, Cu, Zn and As seemed not to elicit a MT response in *F. spiralis*. Furthermore, during summer these metals were at minimal concentrations in *F. spiralis* when MT concentrations were at a maximum (Table 15). However, it is important to note that metal body burdens (total mass of metals within the organism) of these metals are likely to be less sensitive to change. This is due to metal uptake increases with increased metabolic rates in response to temperature and light increases, and subsequent growth rate increases, in spring and summer (Favero and Frigo, 2002, Besada et al., 2009). Fe, Ni, Sn and Pb showed a significant positive relationship with MT in *F. spiralis*, during summer. Previous studies have not reported MT induction in *Fucus* spp. following exposure from these metals, with the exception of Fe (Owen et al., 2012). However, these metals are known to induce MT in other species (Seregin and Ivanov, 2001, Amiard et al., 2008). Otherwise, it is possible these metals are contributing to a combination effect with other more toxic metals, such as Cu, and are cumulatively above a threshold for MT induction (Serafim and Bebianno, 2010). Another possibility is that these metals

are correlated with more toxic metals, not recorded here, that are eliciting a MT response in *F. spiralis*.

6.4.3 Influences of metallothionein response and variability

MT concentrations in *F. spiralis* were generally low for most of the year and were not related to tissue metal concentrations. This may be due to relatively low levels of metal exposure in Poole Harbour (Aly et al., 2013). Seaweed species are very tolerant of metal exposure (Pawlik-Skowronska et al., 2007). As such, the concentrations in Poole Harbour may not be high enough to elicit a MT response. A study by Owen et al. (2012) reported *F. vesiculosus* to begin exhibiting the gene for MT when exposed to a concentration of Cu of 30 µg/l. However, Cu concentrations in seawater in Poole Harbour do not exceed 3 µg/l (Aly et al., 2013). For comparison, Zn, Fe, and Pb concentrations in *F. vesiculosus* from the Fal Estuary, Cornwall, were as much as an order of magnitude higher, with Cu two orders of magnitudes higher, compared with this study (Bryan and Hummerstone, 1973). Owen et al. (2012) did not report tissue concentrations as high in *F. vesiculosus* from the Fal Estuary; perhaps indicating a recovery of contamination levels, but the most contaminated site studied was still approximately ten, five, and ten times higher for Cu, Zn and Fe concentrations, respectively, than in *F. spiralis* in this study.

Aside from low seawater concentrations, low accumulation of metals in seaweeds from Poole Harbour may be the product of low metal concentrations in the tips of fronds, with greater concentrations in the thallus (Bryan and Hummerstone, 1973). It has been suggested to dissect the frond at a pre-determined distance from the distal end (10 cm for *F. vesiculosus*) to allow time for new growth to equilibrate with the environment (Bryan et al., 1985, Rainbow et al., 2002). In this study, tips of seaweeds were analysed in order to select the tissue that reflected the most recent metal concentrations in the surrounding water (Connan and Stengel, 2011). However, it may be more suitable to analyse metal exposure and MT response in mature tissue in the thallus, due to potentially higher metal, and likely MT, concentrations. This may also explain the large degree of variation in metal and MT concentrations, evidenced by large standard deviations.

Biological processes may provide insight as to why metals only seem to elicit a MT response in this species during summer. It is known that prolonged exposure to metals can cause damage to growth rates and photosynthetic efficiency in seaweeds (Costa et al., 2016). This is likely due to the redirection of energy for defensive pathways to protect against metals, allowing less energy for growth (Costa et al., 2016). The oxidation of photosynthetic pigments by Cu, for example, may also cause a reduction in photosynthetic capability, which also causes production of reactive oxygen species leading to oxidative stress (Collen et al., 2003, Pinto et al., 2003). Furthermore, the

substitution of Mg by other metals within chlorophyll can inhibit photosystem II, the reaction centre for capturing photons for photosynthesis, leading to chloroplast dysfunction and photosynthetic processes to cease (Kupper et al., 2002). Pb, Zn, and Cu can also affect Photosystem II in plants, by inhibiting electron transport and altering the structure of the thylakoid membrane within chloroplasts, whilst Cd and Ni can interact with other metabolic processes (Szalontai et al., 1999). Both photosynthesis and growth rates are at a maximum during summer. Therefore, it is logical to suggest the affect of metals on these processes is exacerbated during summer, causing metal toxicity to seaweeds to increase. MT has been shown to protect against oxidative stress through oxyradical scavenging (Viarengo et al., 2000). MT may therefore follow metal concentrations more closely in summer, due to increased toxicity and oxyradical production caused by metal affects on photosynthesis and growth rates. This could explain the positive relationship between seaweed metal concentrations and MT during summer in this study (Figure 21).

Nutrient concentrations may also impact metal toxicity. Addition of nitrogen and phosphorus has been shown to reduce metal toxicity to Cd, allowing growth rates of red macroalga (*Gracilaria tenuistipitata*) to increase (Haglund et al., 1996, Collen et al., 2003). This may be caused by nutrient complexation with metals, competition of uptake sites with metals, and improved nutrient status of plant organisms (Haglund et al., 1996). Nutrient concentrations within Poole Harbour towards the end of summer are likely to be minimal (Franklin et al., 2012). This is due to reduced rainfall and consequent reduction in land based inputs of nitrogen and phosphorus (Bowes et al., 2005). Increased uptake of nutrients by primary production throughout spring and summer also further reduces nutrient concentrations in the water column (Boyle et al., 2004). Therefore, low nutrient concentrations in summer may further be increasing the vulnerability of *F. spirals* to metal toxicity, possibly resulting in a MT response.

6.5 Conclusion

The use of MT in *F. spiralis* as a sensitive biomarker of metal contamination at low concentrations, as subjected in Poole Harbour, is shown here to be limited, as MT does not appear to be consistently induced by metal exposure. It is possible that for most of the year, metal concentrations in Poole Harbour are not high enough to elicit a MT response in *F. spiralis,* as it is a metal tolerant species. However, during summer, concentrations of MT increase, and linear regression analysis reveals significant positive relationships with Ni, Pb, Sn, and Fe. This may be due to increased toxicity of metals, and vulnerability of seaweeds to metals, as they inhibit photosynthetic processes and growth, which becomes pertinent in summer months. This may have caused MT to relate to metal concentrations more closely, as it responds to the increased

effects of metals. This shows evidence for MT in *F. spiralis* to be able to relay the biological impact of metals at low metal concentrations during summer, when important physiological processes are taking place, such as photosynthesis. Therefore, the potential for using MT in *F. spiralis* as a biomarker for metal contamination may be restricted to summer months in temperate regions, at least at low metal concentrations. Further research is required to fully evaluate MT response in *Fucus* spp., addressing uncertainties in frond selection, and seasonal variation and effects. MT response in *Fucus* spp. in severely contaminated environments should also be examined in order to establish its relevance as a cosmopolitan bioindicator species.

Chapter 7: Is metallothionein a worthwhile biomarker of metal pollution within biomonitoring programmes?

7.1 Summary of thesis findings

The literature review, chapter 2 of this thesis, highlighted the current shortfalls in using metallothionein as biomarker of metal pollution. The preceding chapters in this thesis aimed to evaluate the potential of MT in various species, and to constrain and improve its use for maximum benefit. The aim of this chapter was to reflect upon the reliability of MT as a biomarker for metal pollution, assessing its alignment with criteria for a reliable biomarker, and evaluate if it is a worthwhile component of biomonitoring studies. The contribution of the work in this thesis in refining the use of MT, and its applicability to international biomonitoring protocols, is also discussed.

Before discussing the outcomes and applications of this research, the following provides a brief summary of the key findings presented in this thesis:

- Chapter 3: Effects of organism preparation in metallothionein and metal analysis in marine invertebrates for biomonitoring metal pollution
    - Transportation of organisms from field to laboratory on ice is recommended
    - Depuration is not necessary before analysis
    - Storing organisms at -20°C is acceptable for up to ten weeks

- Chapter 4: Seasonal effects to metallothionein responses to metal exposure in a naturalised population of *Ruditapes philippinarum* in a semi-enclosed estuarine environment
    - The relationship between MT and metal concentrations is disrupted during spring in the Manila clam (*Ruditapes philippinarum*) in the UK, probably due to gametogenesis
    - The relationship remains disrupted throughout summer, probably due to body condition of clams
    - Sampling of *R. philippinarum* for MT analysis should be restricted to winter, when at a resting reproductive state, as MT responds reliably to metal concentrations

- Chapter 5: Metallothionein responses in sympatric populations of *Ruditapes philippinarum, Ruditapes decussatus* and *Venerupis corrugata* using active sampling
    - MT in *R. philippinarum* and the European clam, or grooved carpet shell clam (*Ruditapes decussatus*) did not respond to differential exposure to metal contamination during spring and summer
    - MT concentrations in the pullet carpet shell clam (*Venerupis corrugata*) showed positive responses to metal exposure
    - *R. philippinarum* and *R. decussatus* MT response was probably disrupted by gametogenesis
    - *V. corrugata* may provide an alternative MT biomarker species during spring, due to an earlier gametogenic period

- Chapter 6: Metal accumulation and metallothionein response in *Fucus spiralis*
    - The use of MT in spiral wrack (*Fucus spiralis*) as a sensitive biomarker of metal contamination at low concentrations is limited, as MT does not appear to be induced
    - The toxicity of metals to *F. spiralis* may increase in summer months as they can inhibit photosynthetic processes and growth, and MT may respond
    - During periods of important physiological processes, such as photosynthesis and growth in summer, MT in *F. spiralis* may be able to relay the biological impact of metals at low environmental concentrations

7.2 Biomonitoring, biomarker use, and efficacy

Chemical monitoring has continued to be standard practise in regulatory legislation to monitor the risk of metals in the environment, comparing concentrations to environmental quality standards (EQSs) deemed to be acceptable (Hagger et al., 2006). The measurement of metals in biota (biomonitoring) has also been exploited to represent the availability of metals in the environment. This can be complimented by the use of biomarkers; a 'biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)' (Depledge, 1994). They offer extra information on the overall health of an organism, and potential effects and integrated toxicities of contaminants not offered by measuring contaminant concentrations alone (Hagger et al., 2008, Zhou et al., 2008). This is particularly relevant following the introduction of the Water Framework Directive (WFD) by the Commission (EC) of the European Union (EU; 2000/60/EC). This has shifted the emphasis from

monitoring chemical concentrations, to an incorporation of both chemical and ecological considerations, designed to protect and improve the health and functioning of aquatic ecosystems. The overall objective of the WFD is to achieve good ecological and chemical status for all European water bodies by 2015 (derogated to 2027 for underachieving water bodies). The WFD advocates the integration of various techniques, analyses, and disciplines for monitoring water quality, including the need to evaluate biological, chemical, physiochemical and hydromorphological components to determine water body status (Allan et al., 2006). A similar approach is being adopted for offshore waters under the Marine Strategy Framework Directive (MSFD) (EU; 2008/56/EC) (Lyons et al., 2010). As such, the incorporation of biomarkers within this framework, notably for risk assessment of specific contaminants, is promoted, representing a uniquely holistic approach to environmental management (Hagger et al., 2006).

The measurement of biological effects of contaminants is identified as a common topic of interest by international organisations such as: the International Council for the Exploration of the Sea (ICES); OSPAR Commission; Helsinki Commission (HELCOM); United Nations Environment Program Mediterranean Action Plan (UNEP/MAP); European Environment Agency; and, Arctic Monitoring and Assessment Program (Stagg, 1998, Hagger et al., 2006, Lyons et al., 2010). Biomarkers have also been employed by organisations such as Plymouth Marine Laboratory and the Centre for Environment Fisheries and Aquaculture Science (CEFAS) to study specific chemical exposure and general health (JNCC, 2004). However, they have had little use in a regulatory sense, as there has been no robust stand-alone tools (JNCC, 2004). Furthermore, the lack of a comprehensive quality assured method to robustly compare data is a key issue that has previously hampered the implementation of biomarkers into monitoring programmes (Stagg, 1998). Attempts have been made to develop quality assurance and control procedures for marine biological effects measures to increase repeatability and comparability across monitoring programmes. Such an example is the EU-funded Biological Effects Quality Assurance in Monitoring Programs (BEQUALM), which concluded in 2002, and defined quality standards for laboratories to adhere to in international marine monitoring programmes (BEQUALM, 2009). Although use of biomarkers is expanding with WFD and MSFD legislative practises, and programmes like BEQUALM, progress is slow, and concerns can still limit the enrolment of biomarkers in marine monitoring frameworks (Hagger et al., 2006).

It is pertinent to explore the traits of a biomarker that provide relevant information on ecological health, to select the biomarkers that provide practical and accurate information. Effective and reliable biomarkers must be sensitive to both pollutant availability and early biological effects (Van der Oost et al., 2003). Van der Oost et al. (2003), Hagger et al. (2006), and Le et al. (2016) describe a set of criteria for biomarkers (Table 16). They include: 1) availability of reliable (quality

assured) and low-cost measurements; 2) well-characterised dependence of the response on exposure dose and time; 3) sensitivity to pollutant exposure and effects; 4) known influence of confounding factors; 5) well-defined basal line in non-contaminated situations; and 6) established relationship between the response and effects on the organism. Essentially, this means that a predictable response to a contaminant is required of a reliable biomarker, which is not seen in a non-contaminated environment, and other influencers do not affect its concentration.

Table 16 Criteria for a reliable biomarker (adapted from Van der Oost et al. (2003), Hagger et al. (2006), Le et al. (2016))

Criterion	Description
1	Availability of reliable (quality assured) and low-cost measurements
2	Well-characterised dependence of the response on exposure dose and time
3	Sensitivity to pollutant exposure and effects
4	Known influence of confounding factors
5	Well-defined basal line in non-contaminated situations
6	Established relationship between the response and effects on the organism

7.3 Metallothionein reliability as a biomarker for metal pollution

To effectively biomonitor metal contamination, the potential of metallothionein (MT) as a biomarker in marine organisms became apparent in the late 1980s (Hennig, 1986, Engel and Roesijadi, 1987). MT makes a promising candidate due to its ubiquity among most living organisms, and its inducibility by non-lethal metal concentrations; it acts as an early warning signal for the impacts of metals to organisms (Viarengo et al., 1999). However, the problems with its use as a biomarker, due largely to its vulnerability to natural variation, have gained attention more recently. Effects on MT concentrations from tissue mass change (Raspor et al., 2004, Smaoui-Damak et al., 2009), gonad development (Geffard et al., 2001, Moschino et al., 2012, Scudiero et al., 2014), location (Smaoui-Damak et al., 2004), salinity (Hamer et al., 2008, Cravo et al., 2013), and temperature (Serafim et al., 2002, Bocchetti et al., 2008) have been highlighted; all issues which can reduce the consistency and reliability of biomonitoring activities. The reliability of MT is critiqued below, considering each criterion for a reliable biomarker separately (Table 16).

Criterion 1

Cheap and low-cost measurements certainly exist for MT analysis. Affordable and rapid assessment is often deemed imperative in large scale monitoring programmes, particularly in

countries where laboratory expertise or equipment may be lacking. Methodologies developed by Viarengo et al. (1997) offer a low-cost, rapid, and sensitive technique that has been inter-calibrated by a number of laboratories (UNEP/RAMOGE, 1999, Zorita et al., 2005). This has continued to be used as an effective means to measure MT in a variety of marine invertebrates (Moschino et al., 2012, Trinchella et al., 2013, Rodriguez-Iruretagoiena et al., 2016, Oaten et al., 2017). Many other methods for MT quantification are available, including electrochemical methods, chromatography, saturation-based methods, immunological methods, electrophoresis, and reverse transcript-polymerase chain reaction (RT-PCR) (Shariati and Shariati, 2011). Most methods for quantification differ in calculated concentrations of MT (Le et al., 2016). For example, the electrochemical method based on Brdicka's reaction might overestimate MT concentration as it lacks distinction between MT and SH-containing proteins, and requires careful pre-treatments (Raspor and Pavicic, 1996, Le et al., 2016). Quality control for various MT quantification is missing due to the absence of an appropriate reference material (Erk et al., 2002). Furthermore, the treatment of organisms before analysis is highly variable among studies assessing MT and metal concentrations (Oaten et al., 2015) (**Chapter 3**). Results may be ambiguous and unreliable, particularly if specimens are depurated before analysis (Oaten et al., 2015), and may compromise the reliability of MT. Therefore, although MT satisfies the availability of low-cost measurements of a reliable biomarker, an agreed international standard method for MT quantification is lacking.

Criteria 2 and 3

Some studies have shown MT to be a poor biomarker of metal contamination, and not represent bioaccumulated metal concentrations, or metal exposure within the environment. Examination of Table 17 shows that the majority of studies either find MT to be unreliable (independent, or insensitive to metal contamination), or to be moderately reliable (dependant, or sensitive to metal contamination to some extent). Therefore, in terms of dependence of response on exposure dose and time, criterion 2 of a reliable biomarker, MT is less agreeable. Ideally, the degree to which the magnitude of the biological response, MT production, relates to the dose of metal, will be known, enabling the severity of exposure to be quantified (Long et al., 2004). This relationship is inherently complex, and difficult to determine in the environment. This is partly due to the complex mixture of contaminants within the environment that interact (Ng and Wang, 2004), rendering the knowledge of a typical single response to a contaminant useless (Hagger et al., 2006). Furthermore, combinations of metals, rather than single metals, have been shown to cause MT induction (Serafim and Bebianno, 2010). Deciphering a dependant response of MT to a combination of metals, as observed in the environment, is very difficult. The dependence of MT response to metal exposure is also affected by natural and seasonal variation, causing concentrations to alter independently of metal exposure (Geffard et al., 2005).

MT does exhibit sensitivity to pollutant exposure, criterion 3 of a reliable biomarker, as it is regarded as an early warning signal for the impacts of metals to organisms (Viarengo et al., 1999). However, this concept is also affected by natural variation in MT concentration, causing MT to be unresponsive and insensitive to metal exposure due to overriding biotic or abiotic environmental factors (Table 17).

Criterion 4

The knowledge of confounding factors, criterion 4 of a reliable biomarker, is key as it has influence over other criteria, particularly the dependence of response on exposure and sensitivity (criteria 2 and 3), and partly basal concentrations (criterion 5). Therefore arguably, vulnerability to the influence of non-metallic controls on MT concentration, caused by natural variation, is primarily responsible for reducing MT reliability. It is pertinent to explore which factors cause MT to be insensitive to, and independent of, metal exposure. In almost all cases where MT does not relate to metal exposure, gonad development or reproductive status is cited as a confounding factor (Table 17). This is in agreement with the outcomes from **chapters 4 and 5**. The effect seems to be due to either biological dilution due to increased tissue mass from gonadal growth (Bordin et al., 1997, Mouneyrac et al., 2000, Raspor et al., 2004, Raspor et al., 2005), hormonal induction of MT (Baudrimont et al., 1997, Mao et al., 2012), a link between reproductive status and metal metabolism, indicating a need of essential elements during sexual maturation (Geffard et al., 2001, Zorita et al., 2007a), or high concentrations of MT in gonadal tissue (Meistertzheim et al., 2009). The mechanisms of MT alteration occur in a range of species, and do not appear to be species specific. In *Mytilus galloprovincialis*, for example, both increases (Zorita et al., 2007a) and decreases (Raspor et al., 2004) in MT concentrations are reported during gametogenesis. This may be due to the amount of gonadal tissue that is included in dissected tissue for analysis (Mourgaud et al., 2002, Geffard et al., 2005). Nevertheless, a disruption of the positive relationship between MT and metal concentrations is consistently reported during gametogenesis and throughout spawning in a number of bivalve species. Therefore, the timing of sampling, and a good understanding of reproductive processes, is of crucial importance when designing a sampling strategy, to eliminate the key confounding factor of reproductive interference and increase MT reliability.

Criteria 5 and 6

Well-defined basal concentrations in non-contaminated environments, criterion 5 of a reliable biomarker, exist for MT in a variety of organisms. This is required for biomarkers in order to recognise a contaminant response, over a natural baseline concentration. Additionally, the signal-to-noise ratio should be clear, in which the signal, the change in MT in response to metal

exposure, is distinguishable from the noise, the natural variability in MT due to confounding factors (criterion 4), both biotic and abiotic (Geffard et al., 2005). An abundance of studies on MT in bivalves has identified MT concentrations in non-contaminated environments. Amiard et al. (2006), and the references cited therein, show that basal MT concentrations are broadly homogenous across a variety of species. It is notable that concentrations are higher in the digestive gland of organisms, compared to gills, and whole tissue. Due to this and because it is the main storage tissue for metals, the choice of MT in the digestive gland of bivalves as a biomarker is most common (Hamza-Chaffai et al., 2000, Geffard et al., 2005, Amiard et al., 2006, Serafim et al., 2011). However, some studies suggest MT in gills to be a more sensitive biomarker (Geffard et al., 2002, Cravo et al., 2013).

An established relationship between the response and effects on the organism, criterion 6 of a reliable biomarker, relates to the biological relevance of a biomarker. Biomarkers that are manifested at lower levels of biological organisations (i.e. molecular or cellular level), which are linked to population, community, or ecosystem consequences, are deemed more useful (Hagger et al., 2006). A clear example of this is the superimposition of male genitalia in female dog whelks (*Nucella lapillus*), known as imposex (measured as Vans Deferens Sequence Index), caused by tributyltin (TBT) (Bryan et al., 1987). This is an effect biomarker, which demonstrates the effect, or damage, caused by a contaminant (Galloway et al., 2008). It leads to sterility, and ecologically significant decreases in dog whelk populations (Bryan et al., 1986, Gibbs and Bryan, 1986). Another effect biomarker is lysosomal membrane stability (LMS), and is a measure of the weakening of cell membranes, caused by xenobiotics that may induce the diffusion of hydrolytic lysosomal enzymes into the cytosol (Petrovic et al., 2001). It has been developed as a biomarker for metal pollution (Castro et al., 2004, Domouhtsidou et al., 2004), and has direct linkages with scope for growth and is a good indicator for overall health of the organism (Moore, 2002). Although LMS is considered a very reliable biomarker (Domouhtsidou and Dimitriadis, 2001), it responds to a range of pollutants. Biomarkers of exposure, such as MT, are biomarkers that relay biological reactions due to a contaminant present in the environment (Galloway et al., 2008). Arguably, biomarkers of exposure are weakly linked to biological significance, and may be more susceptible to influence from general physiological processes or changes in protein metabolism (Amiard et al., 2006). However, it is important to realise that a lack of linkages to biological significance, generally attributed to biomarkers of exposure, do not invalidate their use. MT still demonstrates metals have entered organisms, have been distributed between tissues, and are eliciting a toxic effect (Shugart et al., 1992). Furthermore, although MT has shown evidence of protecting against general oxidative stress, it is poorly inducible by oxidants in mussels (Viarengo et al., 2000). Therefore, MT has appeal as a specific metal exposure biomarker.

Overall metallothionein reliability

Evidence suggests the robustness of MT as a biomarker is often compromised, and its use should perhaps be discouraged. This is primarily related to the dissatisfaction of criteria 4, knowledge of confounding factors, and effects of natural variation. The majority of studies cite gametogenesis or reproductive interference to cause MT to vary independently of metal exposure. However, some studies (including recent works) find MT to be a reliable biomarker for metal contamination (Table 17). Studies that report MT to be reliable, or report caveats with its use, find MT to be useable during resting periods in the reproductive cycle (Hamza-Chaffai et al., 2000, Mourgaud et al., 2002, Geret et al., 2003, Ivankovic et al., 2005, Oaten et al., 2017) (**Chapter 4**). Therefore, sampling should be undertaken during a resting reproductive period of the bioindicator organism, mainly during winter. This will improve the reliability of MT as a biomarker as it will conform better to criterion 4, and consequently criteria 2, 3, and 5. Provided this is adhered to, there is evidence that shows the use of MT is relevant and beneficial. Despite the mounting research that points towards the difficulties of using MT to inform on metal contamination it can still be used to good effect, and is a legitimate and worthwhile biomarker candidate, albeit at limited times of the year.

Table 17 Reliability of MT as a biomarker for metal pollution (NB: Reliability is described as 'not reliable' when MT is independent/insensitive to metal concentrations, 'moderately reliable' when MT is dependant on/sensitive to metal concentrations with caveats, or 'reliable' when MT is dependant on/sensitive to metal concentrations.

Species	Setting	Tissue	Reliability	Confounding factor	Reference
Cerastoderma edule	Field	Whole tissue	Not reliable	None given	Galloway et al. (2004b)
Cerastoderma edule	Field	Whole tissue	Not reliable	High tolerance and adaptation to metal exposure	Aly et al. (2014)
Crassostrea gigas	Field	Gills	Reliable	-	Geffard et al. (2002)
Crassostrea gigas	Field	Digestive gland	Not reliable	Mass changes due to reproductive cycle and food availability	Geffard et al. (2001)
Dreissena polymorpha	Laboratory	Whole tissue	Reliable (only for Cd)	-	Lecoeur et al. (2004)
Mytilus edulis	Field	Digestive gland, gills	Reliable (digestive gland) Not reliable (gills)	-	Geffard et al. (2005)
Mytilus galloprovincialis	Field	Digestive gland	Reliable (natural variability mentioned)	-	Serafim et al. (2011)
Mytilus galloprovincialis	Field	Digestive gland	Reliable	-	Pytharopoulou et al. (2006)
Mytilus galloprovincialis	Field	Digestive gland	Moderately reliable	Mass changes due to reproductive cycle and food availability	Ivankovic et al. (2005)
Mytilus galloprovincialis	Field	Digestive gland, whole tissue	Reliable	-	Mourgaud et al. (2002)
Mytilus galloprovincialis	Field	Digestive gland	Moderately reliable	-	Marigomez et al. (2013)
Mytilus galloprovincialis	Field	Digestive gland	Not reliable	Mass change and sexual maturation	Raspor et al. (2004)
Mytilus galloprovincialis	Field	Digestive gland	Not reliable	Gonadal development, food abundance, mass changes	Raspor et al. (2005)
Mytilus galloprovincialis	Field	Digestive gland, reproductive gland	Not reliable	Reproductive status	Scudiero et al. (2014)
Mytilus galloprovincialis	Field	Digestive gland, mantle	Not reliable	Physiological function (e.g. gonad development) and high metabolism	Trinchella et al. (2013)
Mytilus galloprovincialis	Laboratory	Gills	Not reliable	Salinity	Hamer et al. (2008)
Mytilus galloprovincialis	Laboratory	Digestive gland, gills, mantle	Moderately reliable	Tissue and cell specific response to different metals	Zorita et al. (2007b)
Ruditapes decussatus	Field	Digestive gland, gills	Reliable	-	Geret et al. (2003)
Ruditapes decussatus	Field	Digestive gland, gills	Not reliable (digestive gland) Moderately reliable (gills)	Salinity, temperature, condition index, gonad maturation	Cravo et al. (2013)
Ruditapes decussatus	Field	Digestive gland	Reliable	-	Hamza-Chaffai et al. (2000)
Ruditapes decussatus	Field	Gills	Not reliable	Body mass and site	Smaoui-Damak et al. (2004)
Ruditapes decussatus	Field	Digestive gland, gills, remaining tissue	Reliable (only for Cd)	-	Bebianno and Serafim (2003)
Ruditapes decussatus	Laboratory	Digestive gland, gills, remaining tissue	Moderately reliable	Only reliable when exposed to metal mixture	Serafim and Bebianno (2010)
Ruditapes decussatus	Laboratory	Digestive gland, gills, remaining tissue	Reliable	-	Serafim and Bebianno (2007a)
Ruditapes philippinarum	Field	Digestive gland	Not reliable	Gonadal development	Moschino et al. (2012)
Ruditapes philippinarum	Field	Digestive gland	Not reliable	Reproductive cycle	Bocchetti et al. (2008)
Ruditapes philippinarum	Laboratory	Digestive gland	Reliable	-	Won et al. (2012)
Ruditapes philippinarum	Laboratory	Digestive gland, gills	Moderately reliable	Adaptation to chronic metal exposure and parasitic infection	Paul-Pont et al. (2010b)
Scrobicularia plana	Field	Whole tissue	Reliable	-	Boldina-Cosqueric et al. (2010)

7.4 The current use of metallothionein in biomonitoring programmes

MT is currently employed by a number of international biomonitoring programmes. The OSPAR Commission, which was created from the Oslo and Paris Conventions, and ICES set up the Joint Assessment and Monitoring Programme (JAMP) to protect and monitor the marine environment in the northeast Atlantic. This came following the need to clarify an integrated approach to biological effects and chemical monitoring under the MSFD requirements (Davies and Vethaak, 2012). A report by the OSPAR Commission compiles JAMP guidelines and advice from ICES, and outlines a suite of biomarkers for integrated chemical and biological effects monitoring (OSPAR Commission, 2013). The report includes MT in digestive gland of *Mytilus edulis* and *M. galloprovincialis* as a biomarker for metal pollution. This replaces the use of MT in fish, specifically cod (*Gadus morhua*), dab (*Limanda limanda*), and flounder (*Platichthys flesus*), which was recommended in previous JAMP guidelines, where MT in invertebrates was reported to be useful but further study was required before inclusion (OSPAR Commission, 2007). UNEP/MAP created another assessment programme, called MED POL following the Barcelona Convention, which monitors pollution in the Mediterranean. It helped develop and calibrate analytical methodologies for measuring MT in marine invertebrates, namely the spectrophotometric method devised by Viarengo et al. (1997) (UNEP/RAMOGE, 1999). MT is currently employed as a biomarker for metal pollution as part of the framework of UNEP/MAP MED POL Phase IV (2006-2013) using caged molluscs, primarily mussels (UNEP/MAP, 2015). It is also included in Natural England's suite of assays to assess the condition of the marine environment (Galloway et al., 2008). These uses of MT to inform environmental management actions are founded upon previous academic literature advocating its use. Emerging literature, and the findings of this thesis, have highlighted the difficulties in using MT and the specific controls required for it to become reliable. In light of this, the legitimacy of the current use of MT in monitoring programmes is discussed below.

Methodologies for measuring metallothionein

Multiple methodologies for measuring MT in organisms are suggested and have been validated within international monitoring programmes. OSPAR Commission notes three main methods for MT quantification: electrochemical differential pulse polography (DPP) method, metal substitution, and spectrophotometric sulphydryl method (OSPAR Commission, 2013). As mentioned, the spectrophotometric method devised by Viarengo et al. (1997) was inter-calibrated by a number of laboratories, and was included as a means to measure MT in MED POL (UNEP/RAMOGE, 1999, Zorita et al., 2005). However, a standardised method has not been agreed across monitoring programmes despite acknowledgement that different methods yield different

concentrations. Furthermore, the methods for sampling and the treatment of organisms before analysis have not been tested, but are advised by Davies and Vethaak (2012) for inclusion in OSPAR Commission guidelines. The treatment of organisms before analysis varies considerably among studies assessing MT and metal concentrations (Oaten et al., 2015), as discussed in section 7.3. OSPAR Commission (2013) advises samples to be dissected within 24 hours and snap frozen in liquid nitrogen, under 'Technical Annex for integrated chemical and biological monitoring of mussel (*Mytilus* sp.)'. 'Technical Annex on sampling and analysis for integrated chemical and biological effects monitoring in fish and shellfish' also denotes organisms to be transported in an insulated container at 4°C in a damp atmosphere. However, the effects of these pre-treatments to MT are not cited. Oaten et al. (2015) (**Chapter 3**) addresses the effects of the pre-treatment of organisms on MT concentrations and recommends: transportation on ice in isothermic container; dissection of organisms as soon as possible after sampling, without depuration; and storage at -20°C for a period of up to 10 weeks is acceptable. This information would increase cost-effectiveness, as storage in liquid nitrogen is not necessary for measuring MT concentrations, and help standardise methodological approaches in international biomonitoring programmes.

Confounding factors and the timing of organism sampling

The reproductive interference with MT response to metal exposure is the most common issue in MT biomarker studies (Table 17), and this is acknowledged in monitoring programmes. Within 'Chapter 10: Metallothionein (MT) in blue mussels (*Mytilus edulis, Mytilus galloprovincialis*)' of OSPAR Commission's guidelines (OSPAR Commission, 2013), confounding factors are described, and it is stated that large changes in MT during the spawning period in mussels occur, with lower concentrations of the protein, citing Raspor et al. (2004), Geffard et al. (2005), and Zorita et al. (2007a). However, the latter two studies show highest MT concentrations in spring, with positive correlations between MT and gonad index (stage of maturity). This shows indiscretion in the interpretation of background literature to support the use of MT in OSPAR Commission's JAMP.

Despite slight misinterpretation of the literature, OSPAR Commission's 'Technical Annex on sampling and analysis for integrated chemical and biological effects monitoring in fish and shellfish' advises sampling to be undertaken outside of the spawning period of *M. galloprovincialis* and *M. edulis* when undertaking MT analysis. However, Mourgaud et al. (2002) and Ivankovic et al. (2005) specifically recommend MT use in *Mytilus* sp. outside of the reproductive cycle entirely (i.e. in the resting reproductive period of winter or early spring). Oaten et al. (2017) (**Chapter 4**) shows further evidence that gametogenesis, which occurs in spring for most species, can disrupt the relationship between MT and metal concentrations in *R. philippinarum*. The relationship remains disrupted throughout the spawning period in summer,

perhaps partly due to a reduction in condition index (**Chapter 4**). This renders MT an unreliable biomarker during any reproductive period, from early gametogenesis to post-spawning. Therefore, international biomonitoring programmes should consider adopting a precautionary approach and advise a protocol to restrict the use of MT to periods when organisms are in a resting reproductive state.

Monitoring programmes aiming to gain information on the severity of metal contamination from MT concentrations that are within the reproductive period of a species should be interpreted with caution, or disregarded. A project to evaluate the feasibility and value of establishing a rapid, cost-effective methodology of biological-effects based sampling, and analysis of Special Areas of Conservation (SAC) designated features in England, was undertaken in partnership with Natural England (Galloway et al., 2008). A series of field surveys were carried out to assess two SACs in southwest England, employing a suite of biomarkers, including MT. This sits alongside other monitoring programmes in the UK, such as OSPAR/National Marine Monitoring Programme (NMMP) work, to inform condition assessment of European Marine Sites and provide advice to government. MT analysis in *M. edulis* digestive gland and gills from the Plymouth Sound and Estuaries SAC, and the Fal and Helford SAC, was undertaken. It was concluded that although MT induction in gills is occurring in metal contaminated, poorly flushed, areas upstream in the SACs, MT induction due to metal contamination in digestive gland is masked. However, samples were taken in spring, as well as autumn and winter. Therefore, reproductive interference may be compromising MT reliability in this study. This is an example where MT has been employed unadvisedly, and evidence gathered from its use could be refined and improved by sampling exclusively in resting reproductive periods.

Use of multiple species

Bivalves are primarily chosen in MT and biomonitoring studies due to their ease of sampling, their sedentary nature and consequent representation of a study site, and similarities with Class I MT (Amiard et al., 2006). The wealth of literature on the use of MT in various bivalve species is growing, but biomonitoring programmes still rely on mussel species. Clam species such as *R. philippinarum,* the global distribution of which is extending beyond its native boundaries, and *R. decussatus* are examples of species which has been positively examined in the literature for MT biomarker use (Bebianno et al., 2000, Hamza-Chaffai et al., 2000, Bebianno and Serafim, 2003, Serafim and Bebianno, 2010, Zhao et al., 2010, Wang et al., 2011, Figueira et al., 2012, Won et al., 2012), but not employed in monitoring programmes. It is important to consider that diverse phyla, with different feeding strategies and habitats, will allow the holistic ecological relevance of contaminant exposures to be more readily determined (Galloway et al., 2004a, Galloway et al.,

2004b). Therefore, alternative bivalve species should be assessed and promising candidates should be added to biomonitoring programmes with standardised methodological protocols, to provide a holistic approach to environmental management (Hagger et al., 2006). It will also extend the geographical locations where protocols in biomonitoring programmes can be applied. Another advantage lies with the utilisation of sympatric species, with differently timed reproductive strategies, that could be sought as alternative bioindicator species to sample when other species may be unreliable at certain times of year (**Chapter 5**).

MT in lesser-studied organisms should also be pursued as potential bioindicator species. For example, an early indication for the use of MT in *F. spiralis* as a biomarker has been shown during summer (**Chapter 6**). Metal toxicity during peak photosynthetic rates during summer is suspected to illicit a MT response. MT in seaweeds may be advantageous compared to bivalves, as processes associated with reproduction may be less influential. Subsequently, a diverse range of organisms in biomonitoring programmes may improve utilisation across seasons, as they will have varied susceptibility to confounding factors. However, further research is required to evaluate the validity of using MT in seaweeds, particularly in heavily contaminated environments, before it is employed in biomonitoring programmes.

7.5 The future use of metallothionein as a biomarker of metal pollution

Marine environments face mounting pressures and increasing metal pollution due to globalisation, and a shift in the anthropogenic use of metals. Effective biomonitoring procedures are needed in order to conserve marine environments for their economic value to society, ecological health, and recreational use. Moreover, key pieces of legislation such as the WFD and MSFD are reflecting the importance and use of biomarkers.

A suite of biomarkers, at different levels of biological organisation, is being promoted in pollution monitoring studies as it allows for a thorough evaluation of the contaminant, and the risk it poses (Schlenk, 1999, Brown et al., 2004, Galloway et al., 2004b, Hagger et al., 2006, Galloway et al., 2008, Serafim et al., 2011). This adopts a weight-of-evidence approach, whereby a single biomarker is not relied upon to measure the effects of a contaminant. Rather, the consideration of multiple biomarkers contributes information to ascertain the overall condition of marine environments, and minimises the importance of natural variation to individual biomarkers (Hagger et al., 2006).

If used with care and awareness, MT can offer a valuable contribution to a weight-of-evidence approach in biomonitoring programmes. It is the primary biomarker that responds specifically to metal exposure at non-lethal concentrations, providing an early warning system for metal

pollution (Viarengo et al., 1999). Although the use of MT as a biomarker of metal pollution is beneficial, its limitations must be recognised and incorporated into experimental and survey design. Implementation of standardised protocols for the treatment of organisms and MT quantification within international biomonitoring programmes is highly important if the use of MT is to be continued. Additionally, sampling bivalve species within a resting reproductive period is vital in order to reduce the natural variability of MT associated with gametogenesis and spawning, and avoid consequent unreliable results. Different reproductive strategies of sympatric species may allow for the use of alternative bioindicators to continue sampling across seasons, if required. Continued research of MT in alternative bioindicator species (to mussels) will also promote a more holistic and ecologically relevant inclusion of MT in international biomonitoring programmes. This would also increase the geographical extent where the uses of standard monitoring protocols are available.

Overall, the findings of this thesis, and the resulting recommendations, refine the use and increase our knowledge of the reliability of MT as a biomarker for metal pollution. Provided these are adopted as protocol, MT is a worthwhile biomarker of metal pollution as part of international biomonitoring programmes, and can contribute to the assessment of metal pollution in marine environments.

Appendices

Appendix A

Table 18 One-way ANOVA LSD post-hoc test showing differences between each pre-treatment for metal concentrations (µg/g) (P = 0.05). NB: DG is digestive gland, WT is whole tissue.

LSD

Dependent Variable	TREATMENT	TREATMENT	Mean Difference	Std. Error	Sig.
NiDG	ICE	SEA	.63375	.59227	.291
		DEP	.50150	.48359	.306
		DIS	-.74017	.48359	.134
	SEA	ICE	-.63375	.59227	.291
		DEP	-.13225	.59227	.825
		DIS	-1.37392*	.59227	.026
	DEP	ICE	-.50150	.48359	.306
		SEA	.13225	.59227	.825
		DIS	-1.24167*	.48359	.014
	DIS	ICE	.74017	.48359	.134
		SEA	1.37392*	.59227	.026
		DEP	1.24167*	.48359	.014
NiWT	ICE	SEA	.60833*	.16863	.001
		DEP	.56667*	.13768	.000
		DIS	-.22667	.13768	.108
	SEA	ICE	-.60833*	.16863	.001
		DEP	-.04167	.16863	.806
		DIS	-.83500*	.16863	.000
	DEP	ICE	-.56667*	.13768	.000
		SEA	.04167	.16863	.806

Appendix A

LSD

Dependent Variable	TREATMENT	TREATMENT	Mean Difference	Std. Error	Sig.
CuDG	DIS	DIS	-.79333*	.13768	.000
		ICE	.22667	.13768	.108
		SEA	.83500*	.16863	.000
		DEP	.79333*	.13768	.000
	ICE	SEA	2.56750*	.98939	.013
		DEP	5.77750*	.80783	.000
		DIS	-.80083	.80783	.328
	SEA	ICE	-2.56750*	.98939	.013
		DEP	3.21000*	.98939	.002
		DIS	-3.36833*	.98939	.002
	DEP	ICE	-5.77750*	.80783	.000
		SEA	-3.21000*	.98939	.002
		DIS	-6.57833*	.80783	.000
	DIS	ICE	.80083	.80783	.328
		SEA	3.36833*	.98939	.002
		DEP	6.57833*	.80783	.000
CuWT	ICE	SEA	1.42750*	.64873	.034
		DEP	2.90500*	.52969	.000
		DIS	.39333	.52969	.462
	SEA	ICE	-1.42750*	.64873	.034
		DEP	1.47750*	.64873	.028
		DIS	-1.03417	.64873	.119
	DEP	ICE	-2.90500*	.52969	.000

LSD

Dependent Variable	TREATMENT	TREATMENT	Mean Difference	Std. Error	Sig.
ZnDG	ICE	SEA	-1.47750*	.64873	.028
		DIS	-2.51167*	.52969	.000
		ICE	-.39333	.52969	.462
	DIS	SEA	1.03417	.64873	.119
		DEP	2.51167*	.52969	.000
	ICE	SEA	9.31750	6.93912	.187
		DEP	4.02917	5.66576	.481
		DIS	-2.23583	5.66576	.695
	SEA	ICE	-9.31750	6.93912	.187
		DEP	-5.28833	6.93912	.451
		DIS	-11.55333	6.93912	.104
	DEP	ICE	-4.02917	5.66576	.481
		SEA	5.28833	6.93912	.451
		DIS	-6.26500	5.66576	.276
	DIS	ICE	2.23583	5.66576	.695
		SEA	11.55333	6.93912	.104
		DEP	6.26500	5.66576	.276
AsDG	ICE	SEA	-.49667	.76015	.517
		DEP	2.11917*	.62066	.002
		DIS	-1.06583	.62066	.094
	SEA	ICE	.49667	.76015	.517
		DEP	2.61583*	.76015	.001
		DIS	-.56917	.76015	.459

LSD

Dependent Variable	TREATMENT	TREATMENT	Mean Difference	Std. Error	Sig.
CdDG	DEP	ICE	-2.11917*	.62066	.002
		SEA	-2.61583*	.76015	.001
		DIS	-3.18500*	.62066	.000
	DIS	ICE	1.06583	.62066	.094
		SEA	.56917	.76015	.459
		DEP	3.18500*	.62066	.000
	ICE	SEA	.09817	.07910	.222
		DEP	-.03050	.06458	.639
		DIS	-.00733	.06458	.910
	SEA	ICE	-.09817	.07910	.222
		DEP	-.12867	.07910	.112
		DIS	-.10550	.07910	.190
	DEP	ICE	.03050	.06458	.639
		SEA	.12867	.07910	.112
		DIS	.02317	.06458	.722
	DIS	ICE	.00733	.06458	.910
		SEA	.10550	.07910	.190
		DEP	-.02317	.06458	.722

Table 19 Kruskall-Wallis Test and pairwise comparisons showing differences between each pre-treatment for metal concentrations (P = 0.05). NB: DG is digestive gland, WT is whole tissue.

Dependent Variable	TREATMENT	TREATMENT	Test Statistic	Std. Error	Sig.
CrDG	ICE	SEA	20.417*	6.134	.005
		DEP	18.667*	5.008	.001
		DIS	-3.500	5.008	1.000
	SEA	ICE	20.147*	6.134	.005
		DEP	-1.750	6.134	1.000
		DIS	-23.917*	6.134	.001
	DEP	ICE	18.667*	5.008	.001
		SEA	-1.750	6.134	1.000
		DIS	-22.167*	5.008	.000
	DIS	ICE	-3.500	5.008	1.000
		SEA	-23.917*	6.134	.001
		DEP	-22.167*	5.008	.000
CrWT	ICE	SEA	19.208*	6.133	.010
		DEP	19.708*	5.007	.000
		DIS	-2.917	5.007	1.000
	SEA	ICE	19.208*	6.133	.010
		DEP	.500	6.133	1.000
		DIS	-22.125*	6.133	.002
	DEP	ICE	19.708*	5.007	.000
		SEA	.500	6.133	1.000

Dependent Variable	TREATMENT	TREATMENT	Test Statistic	Std. Error	Sig.
		DIS	-22.625*	5.007	.000
		ICE	-2.917	5.007	1.000
	DIS	SEA	-22.125*	6.133	.002
		DEP	-22.625*	5.007	.000
		SEA	12.667	6.134	.234
	ICE	DEP	21.417*	5.008	.000
		DIS	-5.000	5.008	1.000
		ICE	12.667	6.134	.234
	SEA	DEP	8.750	6.134	.922
		DIS	-17.667	6.134	.024
FeDG		ICE	21.417*	5.008	.000
	DEP	SEA	8.750	6.134	.922
		DIS	-26.417*	5.008	.000
		ICE	-5.000	5.008	1.000
	DIS	SEA	-17.667	6.134	.024
		DEP	-26.417*	5.008	.000
		SEA	13.167	6.134	.191
	ICE	DEP	21.167*	5.008	.000
		DIS	-5.000	5.008	1.000
		ICE	13.167	6.134	.191
FeWT	SEA	DEP	8.000	6.134	1.000
		DIS	-18.167*	6.134	.018
	DEP	ICE	21.167*	5.008	.000

Dependent Variable	TREATMENT	TREATMENT	Test Statistic	Std. Error	Sig.
ZnWT	ICE	SEA	8.000	6.134	1.000
		DIS	-26.167*	5.008	.000
	DIS	ICE	-5.000	5.008	1.000
		SEA	-18.167*	6.134	.018
		DEP	-26.167*	5.008	.000
	ICE	SEA	9.417	6.134	.748
		DEP	11.917	5.008	.104
		DIS	-1.750	5.008	1.000
	SEA	ICE	9.417	6.134	.748
		DEP	2.500	6.134	1.000
		DIS	-11.167	6.134	.412
	DEP	ICE	11.917	5.008	.104
		SEA	2.500	6.134	1.000
		DIS	-13.667*	5.008	.038
	DIS	ICE	-1.750	5.008	1.000
		SEA	-11.167	6.134	.412
		DEP	-13.667*	5.008	.038
AsWT	ICE	SEA	6.500	6.134	1.000
		DEP	9.333	5.008	.374
		DIS	-10.250	5.008	.244
	SEA	ICE	6.500	6.134	1.000
		DEP	2.833	6.134	1.000
		DIS	-16.750*	6.134	.038

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Dependent Variable	TREATMENT	TREATMENT	Test Statistic	Std. Error	Sig.
AgDG	DEP	ICE	9.333	5.008	.374
		SEA	2.833	6.134	1.000
		DIS	-19.583*	5.008	.001
	DIS	ICE	-10.250	5.008	.244
		SEA	-16.750*	6.134	.038
		DEP	-19.583*	5.008	.001
	ICE	SEA	18.500*	6.131	.015
		DEP	-6.250	5.006	1.000
		DIS	-1.250	5.006	1.000
	SEA	ICE	18.500*	6.131	.015
		DEP	-24.750*	6.131	.000
		DIS	-19.750*	6.131	.008
	DEP	ICE	-6.250	5.006	1.000
		SEA	-24.750*	6.131	.000
		DIS	5.000	5.006	1.000
	DIS	ICE	-1.250	5.006	1.000
		SEA	-19.750*	6.131	.008
		DEP	5.000	5.006	1.000
AgWT	ICE	SEA	18.250*	6.031	.015
		DEP	-3.417	4.925	1.000
		DIS	2.167	4.925	1.000
	SEA	ICE	18.250*	6.031	.015
		DEP	-21.667*	6.031	.002

Dependent Variable	TREATMENT	TREATMENT	Test Statistic	Std. Error	Sig.
		DIS	-16.083*	6.031	.046
	DEP	ICE	-3.417	4.925	1.000
		SEA	-21.667*	6.031	.002
		DIS	5.583	4.925	1.000
	DIS	ICE	2.167	4.925	1.000
		SEA	-16.083*	6.031	.046
		DEP	5.583	4.925	1.000
SnDG	ICE	SEA	17.333*	6.134	.028
		DEP	18.000*	5.008	.002
		DIS	8.333	5.008	.577
	SEA	ICE	17.333*	6.134	.028
		DEP	0.667	6.134	1.000
		DIS	-9.000	6.134	.854
	DEP	ICE	18.000*	5.008	.002
		SEA	0.667	6.134	1.000
		DIS	-9.667	5.008	.322
	DIS	ICE	8.333	5.008	.577
		SEA	-9.000	6.134	.854
		DEP	-9.667	5.008	.322
SnWT	ICE	SEA	18.375*	6.117	.016
		DEP	16.000*	4.995	.008
		DIS	7.625	4.995	.761

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Dependent Variable	TREATMENT	TREATMENT	Test Statistic	Std. Error	Sig.
PbDG	SEA	ICE	18.375*	6.117	.016
		DEP	-2.375	6.117	1.000
		DIS	-10.750	6.117	.473
	DEP	ICE	16.000*	4.995	.008
		SEA	-2.375	6.117	1.000
		DIS	-8.375	4.995	.562
	DIS	ICE	7.625	4.995	.761
		SEA	-10.750	6.117	.473
		DEP	-8.375	4.995	.562
	ICE	SEA	17.583*	6.134	.025
		DEP	17.833*	5.008	.002
		DIS	-1.833	5.008	1.000
	SEA	ICE	17.583*	6.134	.025
		DEP	.250	6.134	1.000
		DIS	-19.417*	6.134	.009
	DEP	ICE	17.833*	5.008	.002
		SEA	.250	6.134	1.000
		DIS	-19.667*	5.008	.002
		ICE	-1.833	5.008	1.000

Dependent Variable	TREATMENT	TREATMENT	Test Statistic	Std. Error	Sig.
PbWT	DIS	SEA	-19.417*	6.134	.009
		DEP	-19.667*	5.008	.002
	ICE	SEA	20.042*	6.133	.007
		DEP	19.125*	5.008	.001
		DIS	-.417	5.008	1.000
	SEA	ICE	20.042*	6.133	.007
		DEP	-.917	6.133	1.000
		DIS	-20.458*	6.133	.005
	DEP	ICE	19.125*	5.008	.001
		SEA	-.917	6.133	1.000
		DIS	-19.542*	5.008	.001
	DIS	ICE	-.417	5.008	1.000
		SEA	-20.458*	6.133	.005
		DEP	-19.542*	5.008	.001

Appendix A

Table 20 Paired samples T-Test showing differences between metal concentrations in digestive gland (DG) and whole tissue (WT) (P = 0.05).

		t	df	Sig. (2-tailed)
Pair 1	NiDG - NiWT	46.531	41	.000
Pair 2	CuDG - CuWT	24.524	41	.000
Pair 3	ZnDG - ZnWT	.066	41	.947
Pair 4	AsDG - AsWT	17.962	41	.000
Pair 5	CdDG - CdWT	1.288	41	.205

Table 21 Wilcoxon Signed Rank Test showing differences between metal concentrations in digestive gland (DG), and whole tissue (WT) (P = 0.05).

		Z	Sig. (2-tailed)
Pair 1	CrDG - CrWT	-5.645	.000
Pair 2	FeDG - FeWT	-5.645	.000
Pair 3	ZnDG - ZnWT	-1.132	.258
Pair 4	AsDG - AsWT	-5.633	.000
Pair 5	AgDG - AgWT	-4.896	.000
Pair 6	CdDG - CdWT	-2.132	.033
Pair 7	SnDG - SnWT	-5.133	.000
Pair 8	PbDG - PbWT	-5.470	.000

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