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

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HIGHLIGHTS

- Pre-treatments to organisms before metallothionein (MT) analysis were examined.
- Depuration, transportation, storage temperatures, and tissue types were tested.
- Storage temperature of samples does not alter MT concentrations determined.
- Depuration of organisms is not recommended before metallothionein analysis.
- Whole tissue and digestive gland MT concentrations can be mathematically related.

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ABSTRACT

Metallothionein (MT) is established as a potentially useful biomarker for monitoring aquatic pollution. This paper addresses widespread inconsistencies in storage conditions, tissue type selection and pre-treatment of samples before MT and metal analysis in biomarker studies. This variation hampers comparability and so the widespread implementation of this monitoring approach. Actively sampled *Mytilus edulis* in Southampton Water, UK were exposed to different storage temperatures, a variety of tissue types were analysed, and various pre-treatments of transportation on ice, transportation in seawater, depuration, and rapid dissection in the field were examined. Storage temperatures of −20 °C were found to be adequate for periods of at least ten weeks, as MT was not reduced by protein degradation compared with samples kept at −80 °C. Whole tissue and digestive gland concentrations of MT and metals were significantly positively correlated and directly relatable. MT in the digestive gland appeared to be more responsive to metals than in whole tissue, where it may be diluted, masking MT responses. However, longer study periods may suffer the effects of mass changes to the digestive gland, which alters MT concentration, and it may therefore be advisable to measure whole tissue. Depuration and transportation in seawater reduced both MT and metal concentrations in the digestive gland, and few correlations between MT and metals were identified for these treatments. It is therefore recommended that: i) samples are transported to the laboratory on ice and dissected as soon as possible thereafter, ii) depuration should not be used when examining MT response to metal exposure until further research clarifying its utility is reported, iii) either whole tissue or the digestive gland can be used to measure MT, though whole tissue may be preferable on long-term studies, and iv) organisms can be stored at −20 °C before analysis for up to ten weeks. These practices can be applied to future biomonitoring studies and will improve the comparability and repeatability of using MT as a biomarker.

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). 33 priority substances are listed under an

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amendment to the WFD (Environmental Quality Standards Directive 2008/105/EC), including Cadmium (Cd), Mercury (Hg), tributyl tin (TBT), Nickel (Ni), and Lead (Pb). Other relevant legislation addressing water quality and pollution includes the Bathing Waters (76/160/EEC), Fish (78/659/EEC) and Shellfish Waters (79/923/EEC) Directives, as well as those based on substances or sources of pollution such as 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 measurement of xenobiotics in biological tissue (biomonitoring) provides a synoptic measure of pollution exposure to elements of direct ecotoxicological relevance, and allows spatial and temporal derivations to be examined more readily than traditional techniques (Rainbow, 1995). 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 accompanied 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). 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 (MEDPOL) (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 (Aly et al., 2014; Forbes et al., 2006).

Furthermore, 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 1). Close examination of Table 1 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 may be contributing to the ambiguity of MT results. Davies and Vethaak (2012) report, 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-analysis procedures on MT are 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 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 (2007) 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 (see table); however it will not be addressed in this study as it is an expensive and has exclusive availability, and therefore is not feasible to implement as a 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.

2. Methodology

2.1. Active sampling and pre-treatment

Seven hundred, *Mytilus edulis*, each 45 to 50 mm long, from Poole Harbour, United Kingdom, were actively sampled from the National Oceanography Centre (NOC), Southampton 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 3 h. A group of organisms was also left to depurate in filtered, UV treated seawater for 24 h, as this is the time period used by previous studies (Table 1), 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 °C or −80 °C for ten weeks before analysis.

2.2. MT analysis

A modified spectrophotometric method, as described by Viarengo et al. (1997) with few modifications by Aly et al. (2014), was used to