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Characterization of the cytosolic distribution of priority pollutant metals and metalloids in the digestive gland cytosol of marine mussels: Seasonal and spatial variability

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HIGHLIGHTS

- Seasonal and spatial differences in cytosolic metal profiles were revealed by SE-HPLC-ICP-MS
- Spatial differences of Cu, Cd and Zn in MMW range are probably associated with MTs.
- Pb elutes mostly with HMW ligands but also markedly in LMW range at polluted locations.
- Arsenic predominantly elutes bound to LMW cytosolic ligands.
- Cytosolic metal profiles could be suitable as potential indicator of metal exposure.

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ABSTRACT

Cytosolic profiles of several priority pollutant metals (Cu, Cd, Zn, Pb) and metalloid As were analyzed in the digestive gland of the mussel (*Mytilus galloprovincialis*) sampled at locations with different environmental pollution levels along the Croatian coast in the spring and summer season. Size-exclusion chromatography (SEC) connected to inductively coupled plasma mass spectrometry (ICP-MS) was used to determine selected elements bound to cytosolic biomolecules separated based on their molecular size. Copper, cadmium and zinc eluted mostly associated with high molecular weight (HMW) and medium molecular weight (MMW) biomolecules, but with a more prominent elution in the MMW peak at polluted locations which were probably associated with the 20 kDa metallothionein (MT). Elution of all three metals within this peak was also strongly correlated with cytosolic Cd as strong inducer of MT. Lead mostly eluted in HMW biomolecule range, but in elevated cytosolic Pb concentrations, significant amount eluted in low molecular weight (LMW) biomolecules. Arsenic, on the other hand eluted almost completely in LMW range, but we could not distinguish specific molecular weight biomolecules which would be predominant in detoxification mechanism. Seasonal variability in element abundance within specific peaks was present, although not in the same extent, for all elements and locations, especially for As. The results confirm the suitability of the distribution of selected metals/metalloids among different cytosolic ligands as potential indicator for metal exposure. Obtained findings can also serve as guidelines for further separation and characterization of specific cytosolic metal-binding biomolecules.

1. Introduction

Coastal environments are subjected to several forms of anthropogenic disturbances. In particular trace metal pollution is still of major concern. Although concentrations of priority metals in the seawater are regularly monitored worldwide, great effort is being made towards the application of biomarkers that indicate an early response in selected target organisms that finally provide evidence of the exposure to the chemical pollutants and may indicate a toxic effect. Especially effects based on the response at molecular and cellular levels represent the earliest warning signals of an environmental disturbance.

Metal toxicity arises predominantly from the binding of metals to essential biomolecules such as enzymes and transporter proteins or the role of certain metals in the formation of radicals (Mason and Jenkins, 1995). Therefore, the excess of metal ions needs to be removed from the vicinity of important biological molecules. The major types of internal sequestration are achieved by accumulation of metals in

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granules and lysosomes or by binding of metals to cytosolic proteins like metallothioneins (MTs) (Vijver et al., 2004). In order to get a better insight not only into detoxification mechanisms but also in the mechanisms of metal toxicity, it is important to determine which biomolecules bind metals specifically in normal metabolism and whether the same biomolecules are involved in binding processes under exposure conditions.

Size-exclusion liquid chromatography (SEC) has been frequently used as the basis for the protein separation. The performance under mild, physiological chromatographic conditions keeps proteins as intact as possible which is important when analyzing natural, metal-binding proteins (De la Calle Guntiñas et al., 2002). Often, the subsequent step in characterizing metal-binding proteins is the analysis of metals in the separated fractions. In recent time the mass spectrometry as a metal detection system for different chromatographic separation techniques is being increasingly used due to the possible low detection limits and (hetero)element-specific detection (De la Calle Guntiñas et al., 2002; Pröfrock and Prange, 2012). Since cytosol is a complex biological matrix, SEC hyphenated to inductively coupled plasma mass spectrometer (ICP-MS) is a valuable tool for the screening of cytosolic metal distribution in the biomolecules of different molecular sizes. Using this methodology, cytosolic molecules involved in the normal metal metabolism, detoxification mechanisms or metal toxicity could potentially be identified — a step towards defining biomarkers. In addition, quantitative and/or qualitative differences in the distribution of metals among different cytosolic ligands at different levels of environmental metal pollution could also be identified and used as a potential indicator of metal exposure.

Either the offline or online combination of SE-HPLC and ICP-MS for determination of the cytosolic metal distribution in various aquatic invertebrates and vertebrates has been used by various authors (Mason and Storms, 1993; Ferrarello et al., 2002; Li et al., 2005; van Campenhout et al., 2008; Krasnići et al., 2013). In our knowledge cytosolic distribution of metals was not investigated with reference to seasonality. Yet, it is known that the level of total tissue and cytosolic metals shows a seasonal variability dependent on the abiotic and biotic factors even when the environmental concentrations of metals remain unchanged (Regoli and Orlando, 1994; Raspor et al., 2004; Ivanković et al., 2005). Similarly, it is found that biomarkers are also affected by seasonality (Ivanković et al., 2005; Bocchetti and Regoli, 2006). Therefore, in order to use the cytosolic metal distribution as indicator of the metal exposure, it is necessary to identify cytosolic metal-binding ligands that best reflect the environmental metal pollution level by not only applying this hyphenated approach of separation of biomolecules and detection of associated metals, but also to investigate the possible seasonal variability in cytosolic metal distribution.

This study was focused on the characterization of cytosolic profiles of several priority pollutant metals (Cu, Cd, Zn, Pb) and As in mussel digestive gland (*Mytilus galloprovincialis*) from coastal locations of different environmental pollution levels with reference to seasonal variability in two most distinct seasons (late winter/early spring vs. advanced summer) concerning reproductive cycle of mussels and abiotic factors.

Mussels are widely accepted as useful biological indicators of marine pollution on both the organism as well as the cellular level (Goldberg and Bertine, 2000; Narbonne et al., 2005; Zorita et al., 2007), while digestive gland represents the central detoxification organ of the organism. Although, Cu and Zn are essential trace metals required for a wide range of metabolic processes of proteins, carbohydrates and lipids, for cell signaling, cell growth and respiration (Flemming and Trevors, 1989; Murakami and Hirano, 2008), they can also be toxic if present in a high enough concentration. Cadmium, on the other hand, is generally considered as a toxic trace element and identified as a priority hazardous substance in many EU countries (Water Framework Directive, 2000) although some recent researches have shown that Cd can be of benefit for some marine diatoms (Lane and Morel, 2000). Lead is not involved in any biological mechanism necessary for life (Gnassia-Barelli and Romeo, 1993) and because of a deleterious effect on the aquatic environment it is included in the list of the priority toxic pollutants within the European Water Framework Directive (Water Framework Directive, 2000). Arsenic is a widespread metalloid in the marine environment existing in many chemical forms. Tissues of marine invertebrates and fish contain high concentrations of As, mainly in different organic less toxic forms (Neff, 1997), compared to a lower concentration of inorganic As species, which are considered of being more toxic (Del Razo et al., 2001; Thomas, 2007).

Among sources of coastal pollution with the investigated metals and metalloid in particular antifouling paints, used to prevent growth of the fouling organisms on the ships' hulls and submerged structures, play an important role. Since the ban of tributyltin, antifouling paints are mainly based on Cu(I) and zinc oxide boosted by other mostly organic co-biocides, while they also contain other metals as additives and non-biocidal pigments (Turner, 2010) that represent an important threat on the marine invertebrates (Bellas, 2006). Around boatyards, besides Cu and Zn, elevated concentrations of numerous other metals such as Cd, and Pb were found (Tapinos et al., 2006; Turner, 2010), while constituents of prohibited paints (e.g. As, Sn and Hg) (Almeida et al., 2007) could also be released to the surrounding areas during the ship maintenance. Therefore, in this study we focused on the investigation of the cytosolic metal distribution in particular on organisms living sedentary in highly metal polluted aquatic environments such as marinas and harbors. In addition, the harbor selected for this research is located in a stratified estuary with frequent salinity and temperature changes, which could pose an additional stress for the organisms living in that place. Therefore, the results obtained by this study will also provide information on the cytosolic metal distribution in organisms, which in addition to the stress caused by metal pollution, are also chronically exposed to the natural stress caused by salinity and temperature variations.

2. Materials and methods

2.1. Sampling

Mussels *M. galloprovincialis* were collected in two sampling periods (March and August, 2010) at three locations in the central part of the eastern coast of the Adriatic Sea. Since marinas and harbors are generally considered as sites of the metal exposure, the marina (M) located in the south west end of Kaštela Bay and the harbor (H) of the town of Šibenik were chosen as polluted locations. One location (village Zablaće in the vicinity of Šibenik) under less anthropogenic influence was selected as the reference location (R). Mussels ranging from 3.8 to 8.0 cm in length size were collected from the coastal rocks or concrete embankment structures between 0.5 m and 1 m below the sea surface. Digestive gland was dissected and frozen in liquid nitrogen immediately upon the sampling. Digestive glands of 6–8 individuals were pooled to form representative composite sample. Since it was not always possible to collect a sufficient number of similar size specimens, the samples were formed in such a way that they contain digestive glands of both larger and smaller mussels. Three composite samples per location were analyzed.

2.2. Analysis of total dissolved metals in seawater

The concentrations of dissolved Cu, Cd, Zn and Pb were determined in the filtered (0.45 μm) seawater using differential pulse anodic stripping voltammetry as described by Omanović et al. (2006) and Cukrov et al. (2008).

2.3. Isolation of cytosolic fraction

The digestive gland tissues were cut into small pieces, diluted 6 times with a cold homogenization buffer (20 mM Tris–HCl/Base,