Bull. Environ. Contam. Toxicol. (1992) 48:179–184

Environmental Contamination and Toxicology

Method for the Estimation of Organic-Bound and Crystal-Bound Metal Concentrations in Bivalve Shells

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Bivalves have the ability to accumulate and concentrate heavy metals to levels several orders of magnitude above those found in their environment. One species, *Elliptio complanata*, has been used extensively as a biomonitor in freshwater systems (Hinch and Stephenson 1987; Imlay 1982). In most previous studies, only the tissues have been examined as to their metal content, distribution in those tissues and relationship to environmental concentrations.

Tissues have an inherently higher variability in their trace metal concentrations. Components of the shell, however, may have much less variability. For example, Bourgoin (1990) found that the nacreous layer of shell has a coefficient of variation for lead content of 22% while that for the tissues was 45%. Some factors contributing to the high variability of tissue-bound metal concentrations are season, mussel age, size, location and water column salinity can be standardized. However, intrinsic factors like sexual stage, growth rate and elevated uptake in specific organs tend to influence the trace metal concentrations. Shells, on the other hand, have a variety of advantages over tissues.

Previously, most trace metal levels in the shells were assumed to be solely due to passive adsorption onto the periostracum, the outer organic component of the shell and hence not a qualified measure of trace metal bioavailability (Keckes et al. 1968; Romeril 1971). However, these studies failed to examine bivalve shells in terms of their discreet structural components and therefore could not differentiate between trace metals passively adsorbed onto the shell surface from that which is incorporated within the various shell components.

The bivalve shell consists of two valves of crystalline calcium carbonate structured by an organic matrix. The entire shell is covered by the periostracum. All shell components are secreted by the various cells of the mantle epithelium. Excluding where the muscles attach the two valves together, the calcium carbonate and the organic matrix are deposited from a solution called the pallial fluid. The carbonate portion of the *Elliptio complanata* shell consists of an outer prismatic layer and an inner nacreous layer. The pallial fluid contains all the components involved in biomineralization as well as other constituents, including trace metals, assimilated from the water

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column. Therefore, any trace metal incorporated into the shell components during growth must have been assimilated by the organism itself. From this stage it is essential to choose the structural component which gives the best indication of bioavailability. In the case of *Elliptio complanata*, the component which has not been exposed to the water column either directly or by erosion, is the nacre. Since the incorporation of trace metals into the shell accompanies shell formation, and this process is slow compared to that of accumulation and release from the soft tissues, the relationship between trace metal concentrations in the nacre and the environment are less variable and more consistent than that for tissues. Shell nacre is by far easier to handle and store, no depuration is required since the nacre is not exposed to possible contamination from suspended particles. Finally, shell nacre appears to be a more sensitive indicator of trace metal concentrations in the environment due to the higher sensitivity over a greater period of time.

However, the nacre itself consists of an organic matrix and a crystal lattice of calcium carbonate. Thus, two sites of trace-metal binding potentially exist. The first, adsorption onto the organic matrix, ensues by passive adsorption processes from the pallial fluid, thereby functioning more as a sink for trace metal contamination. The second site of trace metal contamination is by isomorphic substitution. In such a case, the heavy metals substitute for the calcium ions and are actively incorporated into the crystal structure by annual layers. Since this process requires active assimilation and production of the metal-substituted carbonate in relation to the mussel's metabolic rate, the crystal provides an excellent index of trace metal bioavailability. However, it is not known at present where the metal ion goes within the crystals. It is important therefore, to determine which component of the shell nacre contains the trace metals. If it can be shown that the metals are crystal bound, where no post-depositional mobility is likely to occur to change the chronological deposition of trace metals, the shell nacre can be seriously considered as a reliable index for contamination. Therefore, by proving that trace metals are assimilated, then metabolically deposited into the nacre crystal as they lay down their annual layers with no mobility from the site of deposition, the nacre becomes the best and most reliable indicator of trace metal contamination with inherently lower variability.

The object of this study was to provide a technique to separate and analyze the adsorbed heavy metals in the nacreous layer of the shells of *Elliptio complanata* from that of the isomorphically substituted metals, thereby determining the relative distribution of the total metal content between the two nacre components. In doing so, it will test the hypothesis that the shell is a legitimate and better index of bioavailability. By understanding the distribution of trace metals through the tissues and into the shell nacre components, it allows for a better understanding of the uptake processes that occur and ultimately the significance of the observed concentrations.

MATERIALS AND METHODS

Elliptio complanata mussels were collected from Bird Lake, Ontario (79°03', 45°03') a soft water lake, and transported live to the laboratory. Fifty mussels (7-9 cm) with little or no erosion were selected for examination.

The mussels were shucked and the shells were subsequently washed on the

inside with concentrated H_2O_2 followed by a rinse of ultrapure water. The mussels were then placed in a drying oven at 60° C for 24 hr. The periostracum and outer prismatic layers were mechanically ground off using an industrial grinding stone. The final product was determined visually as the clean nacreous layer.

The isolated nacre layers were ground first using an electrical grinder and then an agate mortar and pestle. The ground nacre was then sieved through a 120 um Nytex screen to obtain uniform crystal clusters and allow for easier digestion. The <120 um nacre was then pooled and shaken to allow for even distribution of the combined shell nacres.

Ten subsamples of 200 mg each of the ground nacre were analyzed for the total cadmium and lead concentration. These samples were ashed at 400° C for 18 hr to destroy the organic matrix. No loss of metal ions occurs under these conditions (Bourgoin 1987). After the samples were cooled, they were reweighed to obtain an ashed weight and loss on ignition value. The ashed sample underwent digestion with a small volume of sodium acetate buffer (0.2 M, pH 9.2) and subsequent additions of concentrated hydrochloric acid (trace metal grade) until all the calcium carbonate was dissolved. These dissolved samples were then filtered through a Millipore cellulose filter of 0.45 um using the sodium acetate buffer as a rinse. Sample filtrates were then adjusted to pH 1.5 and analyzed on a Metrohm anodic stripping voltammeter (ASV model 626, Brinkmann) for cadmium and lead concentrations using standard additions.

To isolate the metal adsorbed to the organic matrix from that which was isomorphically substituted, a set of ten subsamples of 1.50 mg each were used. These subsamples were vigorously shaken with 20 mL of 0.80 M EDTA (ethylene diamine tetraacetic acid) for a period of five minutes. The EDTA wash was used to extract any adsorbed metals from the organic matrix without breaking down any of the crystal structure. Prolonged exposure to EDTA will eventually degrade the calcium carbonate crystal. However, five minutes proved to be adequate for metal extraction as subsequent washings contained little or no metal. The samples were then filtered through pre-weighed glass microfibre filters (Whatman GF/A). The filtrates, containing the adsorbed metals, were adjusted to pH 1.5 and analyzed using ASV.

The remaining crystals on the glass fibre filters were dried and then ashed at 400° C for 18 hr to break down the organic matrix fragments remaining after EDTA washing, which yielded comparable LOI to that of total metal analysis; therefore, no chemical dissolution occurred. After cooling in a dessicator, the filters were weighed to determine the ashed weight of the samples of isomorphically substituted metals. These ashed samples underwent the same HCl digestion, filtration, pH adjustment and analysis as for the total samples.

Contamination arising from the sodium acetate buffer, EDTA and glass microfibre filters was determined and subtracted from the corresponding sample concentrations. The first two samples from each set were used as tests for subsequent analysis parameters.

As a check for any loss of the metal concentrations to the glassware and filtering apparatus, a radiotracer experiment using 1.45×10^{-3} uCi of ^{109}Cd

(Sigma) was added to 1.00 g samples and the entire experimental protocol repeated. All glassware and apparatus were rinsed with trace concentrated HCl and the rinsings analyzed by NaI gamma detection.

RESULTS AND DISCUSSION

The isolated nacre was determined by loss on ignition to consist of 4.25% organic matrix by weight, consistent with previous studies in which molluscan shells were found to contain approximately 0.3 to 4.0% organic matrix by weight depending upon the species (Bourgoin 1987).

The cadmium extracted from this organic matrix was 13.97% of the total cadmium within the nacre, while the calcium carbonate crystals contained 48.33% of the total (Table 1). The organic matrix contained 2.95% of the total lead, while the calcium carbonate crystals contained 62.32% of the total. Radiotracer experiments confirmed that the remaining 37.71% and 34.73% of the total cadmium and lead content, respectively, was lost to adsorption onto the glass microfibre filters.

The Cd and Pb concentrations were calculated on a per weight basis for each component of the shell nacre (i.e., 4.25% organic matrix, 95.75% crystal - Table 1). The concentration of Cd in the organic matrix was 369.14 ng/g and 56.77 ng/g in the carbonate crystal; a ratio of 6.5 in favor of the organic matrix. Similarly, the concentration of Pb in the organic matrix was 1529.53 ng/g, while that in the carbonate crystal was 1435.02 ng/g; thus the distribution coefficient was approximately 1. The higher partitioning of Cd to the organic matrix presumably is a result of Cd having a higher adsorption coefficient for the organic molecules than lead. This phenomenon is seen with other types of organic matter as well (Campbell and Evans 1986). Differences in distribution coefficients, however, may be explained partially by the fact that lead undergoes isomorphic substitution for calcium more readily than cadmium due to its atomic size being more similar to calcium ($Cd^{2+} = 1.14\text{A}^{\circ}$, $Pb^{2+} = 1.20\text{A}^{\circ}$, $Ca^{2+} = 1.18\text{A}^{\circ}$). There is also a trend for ions somewhat larger than calcium to concentrate in the mussel shell (Imlay 1982).

For Cd the ratio of organic matrix to calcium carbonate crystals is the driving force behind metal partitioning. Generally, the ratio of organic matrix to calcium carbonate crystal is quite low (0.04), therefore substantiating the hypothesis that the carbonate crystals contain the majority of the metals. However, if the organic content should increase, as is the case for some bivalves, then a substantial portion of the metal content could be incorporated into the organic matrix, making these species less suitable for environmental monitoring.

Further evidence for the mechanism controlling metal concentrations in shells can be drawn from the ratios of tissue concentration to shell concentration. The average Cd concentration in tissue was 19 μg/g, much higher than the 6.8 μg/g found for Pb. Whether this was due to a higher cadmium concentration in the lake, or to the mussel having a greater ability to accumulate and assimilate cadmium is under investigation. Regardless, as can be seen from the concentration factors, the shell actively assimilates only 0.01 of the total cadmium from the tissues into the nacre. However, the assimilation of lead between the tissue and shell was found to be 0.32. This is con-

sistent with the suggestion that lead can more easily substitute for calcium within the carbonate crystals than can cadmium.

Table 1. Metal distribution in shell nacre and soft tissues (N=8).

Assay	Percent of Total	Shell Compartment Concentration (ng/g ± 1sd)	Tissue Concentration (μg/g ± 1sd)
Cd			
Total	100		19.4 ± 7.7
Adsorbed	14.0	369 ± 106	
Crystal	48.3	57 ± 13	
Loss	37.7		
Pb			
Total	100		6.9 ± 3.5
Adsorbed	3.0	1530 ± 411	
Crystal	62.3	1435 ± 455	
Loss	34.7		

In conclusion, bivalve shell nacre has potential to be a good monitor for trace metal contamination from the environment. Since the majority of the metals are crystal-lattice bound and these crystals are deposited on an annual basis, the shell nacre should provide a reliable chronological index of metal exposure with no significant post-depositional mobility occurring. Species with a high organic matrix component of their shell, however, should be avoided until the role of this matrix in post-depositional mobility is better understood.

Acknowledgements. This work was supported financially by research grants to RDE from the Natural Sciences and Engineering Research Council of Canada.

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Received July 12, 1991; accepted October 10, 1991.