



ELSEVIER

The Science of the Total Environment 225 (1999) 219–229

**the Science of the
Total Environment**
An International Journal for Scientific Research
into the Environment and its Relationship with Man

Metal levels in tissue granules of the freshwater bivalve *Hyridella depressa* (Unionida) for biomonitoring: the importance of cryopreparation

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Received 5 May 1998; accepted 2 October 1998

Abstract

The freshwater bivalve *Hyridella depressa* accumulates metals in extracellular granules distributed through its tissues and the elemental content of these granules was examined by X-ray microanalysis. The influence of two specimen-preparation techniques, chemical fixation and freeze-substitution, on the elemental data generated by granule microanalysis was compared to assess their application for environmental monitoring. Chemical fixation and wet sectioning resulted in lower proportions of Ca and Mn, and higher proportions of Fe, P, Cu, Zn and Pb compared with freeze-substitution and dry sectioning. Chemical fixation and wet sectioning also resulted in an increase in among-mussel variability. Wet sectioning of freeze-substituted tissue yielded similar results to chemical fixation and wet sectioning, suggesting that much of the dissolution and redistribution of elements associated with the chemical fixation procedure was due to aqueous processing. Granule microanalysis is a new approach to monitoring metal levels in the environment. Comparison of the data obtained by the two preparatory methods by analysis of variance and multi-dimensional scaling revealed the necessity of cryopreparation for maintaining elemental distribution and concentration. Use of the granules as biomonitors may be particularly useful when used with the mantle biopsy method for non-destructive sampling of mussel populations. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Metal accumulation; Freshwater mussel; EDS; Specimen preparation; Biological monitoring; X-Ray microanalysis

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1. Introduction

Metal-containing granules are commonly found in invertebrate tissues (Brown, 1982). In freshwater bivalves these granules are thought to have a number of functions including metal detoxification and storage of elements such as Ca and P (Taylor and Simkiss, 1989). In mussels, the presence of these metal-containing granules has led to the use of whole-body metal burdens for bio-monitoring (Jeffree et al., 1993; O'Connor, 1996). However, among-individual variability is high and large sample sizes are required for whole-body analyses. Normalization, by Ca content for example (Jeffree et al., 1993), may be necessary to provide a correction for the among-individual variability in amount of granular material in tissue. As an alternative approach, X-ray microanalysis of the granules shows promise for biomonitoring because the major site of metal accumulation in the mussels can be analysed directly and because it obviates the need for Ca normalization.

The validity of elemental data from X-ray microanalysis of biological specimens is ultimately dependent upon the techniques used for specimen preparation (Morgan, 1985). It could be argued that all preparation techniques induce artefacts, for we cannot observe and analyse specimens in a truly 'native' state. It is thus good practice to use different specimen-preparation techniques in parallel for the evaluation of preparation artefacts (Morgan, 1980). With aqueous chemical fixation, the primary concern is the dissolution of elements from the specimen during preparation, resulting in loss or redistribution of elements within the specimen (Morgan, 1980). Freeze-substitution is a major preparatory technique which has been developed to facilitate preservation of elements in biological specimens for microscopy and microanalysis (Marshall, 1980; Pålsgård et al., 1994). This method is the slow replacement of frozen specimen water by an organic solvent at very low temperature, thereby dehydrating the specimen and preserving structure and elemental localization with subsequent resin embedment. Freeze-substitution has found favour for preparation of 'difficult' specimens,

particularly structurally heterogeneous tissues such as plant material or coral (Marshall and Wright, 1991), and is a good alternative where cryosectioning is unavailable or impractical. Although debate continues over comparisons with other cryotechniques (Condron and Marshall, 1990; Zierold, 1992; Pålsgård et al., 1994), this approach is considered to maintain the content and location of elements during specimen preparation far better than chemical fixation.

Hyridella depressa is an important component of the freshwater macroinvertebrate fauna of the Sydney region (McMichael and Hiscock, 1958). This mussel has been identified as a potential biomonitor of pollution due to its capacity to accumulate elements from the environment (Jeffree et al., 1993). *Hyridella depressa* sequesters elements in extracellular 'calcium phosphate' granules distributed throughout their body (Byrne and Vesik, 1996; Adams et al., 1997). As part of a wider study of the biomonitoring potential of *H. depressa* for the Hawkesbury–Nepean River system, X-ray microanalysis of these granules is being investigated (Byrne and Vesik, 1996; Adams et al., 1997). Previous microanalytical studies that document the elemental composition of the granules in several mussel species have used conventional chemical fixation or have isolated the granules by aqueous extraction (Davis et al., 1982; Jeffree and Simpson, 1984; Pynnönen et al., 1987; Silverman et al., 1987). Routine specimen preparation as used in these earlier studies would facilitate use of the granules as biomonitors because experience in conventional techniques is readily available in many laboratories. In contrast, experience in cryotechniques is less common. Indeed recent microanalytical studies of metals in the tissues of marine and freshwater mussels and other animals base their findings on metal distribution, bioavailability and detoxification on analysis of chemically-fixed tissues (Julliard et al., 1995; Giamberini and Pihan, 1996; Giamberini et al., 1996; Nigro and Leonzio, 1996; Soto and Marigómez, 1997). Consequently, we wished to determine whether conventional techniques could be applied to the granules of *H. depressa* for biomonitoring despite its expected shortcomings. To achieve this aim we undertook a parallel in-

vestigation using aqueous chemical fixation and freeze-substitution to compare the effects of these methods on the elemental data generated by X-ray microanalysis of the granules of *H. depressa* to assess the best approach for use of granules for biomonitoring.

2. Materials and methods

2.1. Initial preparation

Hyridella depressa were collected by divers from Lake Burragorang (34°S, 150°30'E) New South Wales, Australia. Mussels were kept cool in lake water during transport to the laboratory, and left overnight in large beakers of lake water. Male mussels were selected for dissection, and excess water was gently blotted from the mantle tissue. Using flattened forceps, the mantle was lifted away from the shell. Then, using copper-tipped pliers cooled in liquid nitrogen, a small section of mantle was frozen with the pliers, and pulled away from surrounding tissue and immersed in liquid nitrogen. Specimens for freeze-substitution were stored in liquid nitrogen until further processing. Cryosectioning of unfixed frozen tissue was attempted a number of times without success, hence the need for freeze-substitution. Pieces of tissue from the opposite mantle were excised and transferred to a vial of fixative (see below).

2.2. Freeze-substitution

Anhydrous 100% acetone was stored over a molecular sieve. Several grains of molecular sieve were placed in the bottom of a 5-ml plastic vial, and a square of Kimwipe tissue held in place over the grains with a ring of plastic cut from the bulb of a plastic pipette. This prevented specimens from falling between the molecular sieve grains and being damaged. Anhydrous acetone was added to the vials and the acetone cooled by standing the vials in a shallow bath of liquid nitrogen so that the acetone slowly froze. Frozen specimens were cut into pieces approximately 1 mm × 2 mm under liquid nitrogen, and transferred to the vials, into a shallow layer of liquid acetone overlying frozen acetone. Vials were

transferred to a low temperature freezer, and freeze-substitution was carried out over 3 days at 193 K, 16 h at 253 K and 4 h at 277 K. The vials were allowed to warm to room temperature, and the acetone replaced with fresh anhydrous acetone. Infiltration with Spurr's resin was graded over 4 h to 70% resin in acetone, left overnight, brought to 100% resin, left overnight, exchanged with fresh 100% resin, again left overnight before a final exchange with fresh resin and flat-embedding and polymerization at 333 K for at least 24 h.

Sections (0.5 μm) of freeze-substituted tissue were cut on a dry glass knife using an ultramicrotome, with the aid of an anti-static ionizator (Di-atome, Bern, Switzerland). Sections were placed onto Formvar film (lightly carbon coated) on a Ni slot grid using a hair probe. The grid was then placed face down on a film of Parlodion suspended on a plate with holes drilled in it, thus sandwiching the section. Sections were stored in a desiccator over silica gel until analysis (usually that afternoon). To examine the effects of wet sectioning on the resulting elemental profiles, two specimens were also sectioned conventionally onto a trough of distilled water and collected from the water surface directly onto Formvar coated Ni slot grids. For light microscopy, 0.5-μm dry sections were transferred to glass slides and stained with toluidine blue. Results from a small number of specimens which were freeze-substituted in 100% diethyl ether as an alternative to acetone (over 28 days), indicated little difference from acetone freeze-substitution (Vesk and Byrne, unpublished). As freeze-substitution with acetone was technically simpler and much faster than diethyl ether, this was adopted as our standard method (Morgan, 1980).

2.3. Chemical fixation

Specimens were prepared as described previously (Adams et al., 1997). Briefly, specimens were fixed in 2.5% glutaraldehyde in 0.02 M Hepes buffer pH 7.2, post-fixed in 2% OsO₄ in 0.01 M Hepes pH 7.2, dehydrated through an ethanol series, replaced with 100% acetone, infiltrated and embedded in Spurr's resin. Sections, 0.2 μm thick, were sectioned onto a water bath using an

ultramicrotome and picked up on Ni hole grids with carbon coated Parlodion films.

2.4. X-ray microanalysis

Specimens were analysed in a Philips CM12 120 kV scanning transmission electron microscope (STEM) with a Be thin window, energy dispersive X-ray detector and Edax 9900 analyser using a Be low-background specimen holder. Individual spherical granules were analysed with a raster $0.4 \mu\text{m} \times 0.4 \mu\text{m}$ for 100 live seconds, using a beam current of approximately 0.3 nA. Spectra were processed with the Edax Super Quant programme, by subtracting a standard manually fitted background and using net peak counts of Mg, Al, P, S, Ca, Ba, Mn, Fe, Cu, Zn and Pb. Data were transferred to a spreadsheet (Microsoft Excel) and converted to proportions for statistical analyses by dividing the counts from each peak by the sum of all peak counts. This was necessary as beam current varied among analyses and section thickness varied among the preparation methods.

2.5. Data analysis

A total of 11 *H. depressa* were used for this study. From each mussel, 18 granules were chosen randomly for X-ray microanalysis. Prior to analysis of variance (ANOVA), variance heterogeneity was tested with the *F*-ratio of the variances (Sokal and Rohlf, 1995). Where heterogeneity was significant, data were transformed using a combination of arcsine and either square root or natural logarithms. Two-factor unreplicated ANOVA (analogous to paired *t*-tests) was used to compare element proportions between specimens prepared by freeze-substitution and chemical fixation. Plots were made of means and standard errors using pooled estimates of standard errors from the ANOVA and back transforming the data where appropriate. Non-metric multi-dimensional scaling of fourth root transformed proportional data and Bray–Curtis similarities was used to display relationships among the samples combining information from the 11 elements listed above (Clarke, 1993). Determina-

tion of percentage contribution by the individual elements to multivariate dissimilarity was calculated with SIMPER (Clarke, 1993). SIMPER and multi-dimensional scaling were carried out with the multivariate statistical analysis software package PRIMER (Plymouth Marine Laboratory, UK).

3. Results

Examination of light microscopic sections of the mantle of *H. depressa* revealed the presence of aggregations of granules (Fig. 1A). Unstained dry cut sections viewed with STEM showed that these granules are spherical ($0.5\text{--}1 \mu\text{m}$ diameter) and electron dense (Fig. 1B). A representative X-ray spectrum from one of these granules shows characteristic Ca, P and Fe peaks with smaller Ba and Mn peaks (Fig. 2a). Occasionally, aggregations of small, diffuse and less regular granules were observed (Fig. 1B). An X-ray spectrum from one of these granules shows a dominant S peak, with substantial Ca, Fe and Zn peaks (Fig. 2b). Copper was often present in high proportions in these S-type granules.

Bar graphs of mean elemental proportions generated from 18 granules from 11 mussels show that proportions of P, Fe, Cu, Zn and Pb were lower with freeze-substitution compared with chemical fixation (Fig. 3). In contrast, proportions of Ca and Mn were higher in freeze-substituted tissue than in fixed tissue (Fig. 3). Unreplicated two-way ANOVA established significant differences between freeze-substitution and chemical fixation for these elements (Table 1).

Paired bar graphs of means obtained for Cu and Zn with both methods for each of the 11 mussels show high among-mussel variability with chemical fixation (Fig. 4). The *F*-tests showed that the among-mussel variance was significantly greater for chemically fixed tissue than for freeze-substituted tissue, for the elements Al ($F = 8.92$), S ($F = 90.9$), Cu ($F = 444.5$), Zn ($F = 106.7$), Pb ($F = 12.79$); the critical $F_{0.05(10,10)} = 3.72$. Trends in proportions of these elements among mussels were not consistent between preparation techniques (Fig. 4). Arcsine transformation of proportional data reduced the observed

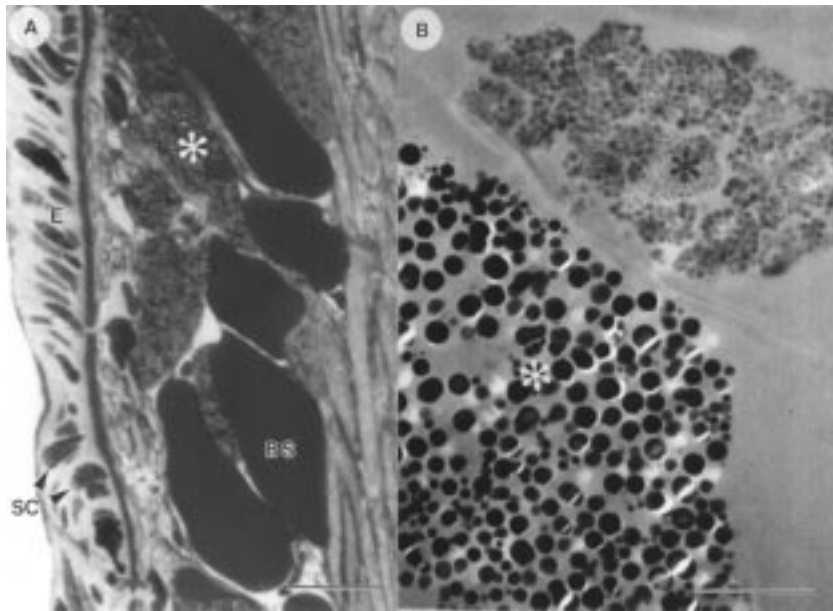


Fig. 1. Micrographs of *Hyridella depressa* mantle, showing extracellular granules. (A) Light micrograph of a toluidine blue stained section showing mantle epithelium (E) with secretory cells (SC), aggregations of granules (asterisk) and blood spaces (BS). Bar = 40 μm . (B) Scanning transmission electron micrograph of unstained, dry cut section showing Ca–P granules (white asterisk) and uncommon diffuse S-type granules (black asterisk). Bar = 5 μm .

F values, but did not remove the variance heterogeneity entirely. Subsequently, transformation to square roots (Al), fourth roots (Cu, Pb) or natural logarithms (Zn) were used before carrying out ANOVA. Even logarithmic transformation of arcsine values for Zn was not successful in eliminating variance heterogeneity, however, the ANOVA was carried out, keeping in mind the likely effect of variance heterogeneity (increased Type 1 error) and using a critical significance level of $P = 0.01$, at which the result was significant (Table 1). ANOVA of the data obtained for S was not carried out as variance heterogeneity was not substantially reduced by data transformations.

Ordination of the elemental data from 11 mussels using multi-dimensional scaling resulted in a relatively tight clustering of the data from freeze-substituted tissue in the lower left of the plot (Fig. 5). These were distinctly separated from the elemental data obtained from chemically-fixed tissue. The data from chemically-fixed tissue were also more widely spread over the ordination.

Within-group, multivariate (Bray–Curtis) similarity was higher for freeze-substitution (96.2%) than for chemical fixation (93.9%). In other words, among-mussel variability for multiple elements was higher with chemical fixation than freeze-substitution. Most dissimilarity between the preparation techniques as represented in the ordination (Fig. 5) was due to differences in proportions of Cu (23%), Mn (13%), Zn (12%), Ca (12%) and Fe (10%).

Wet sectioning of the freeze-substituted tissue yielded results similar to those obtained for chemically-fixed tissue, with lower Ca and Mn and increased among-mussel variability for Cu and Pb (Fig. 6). Cu and Pb were substantially higher in one wet-sectioned specimen, compared with the corresponding dry-sectioned specimen (Fig. 6). The analysed section was observed to contain, in addition to the Ca–P granules, an aggregation of S-type granules (Fig. 1B, Fig. 2b) that contained substantial proportions of S, Fe, Cu, Zn and traces of Pb.

Table 1

Summary of results of unreplicated two-factor ANOVA of element proportions in mantle tissue granules from *Hyridella depressa* ($n = 11$ mussels), testing for the effect of specimen preparation (freeze-substitution or chemical fixation)

Element	Transformation	Mean square	<i>F</i>	<i>P</i>
Ca	–	0.033	39.72	< 0.001
P	–	7.8×10^{-3}	53.32	< 0.001
Ba	–	6.9×10^{-7}	0.23	n.s.
Mn	–	3.3×10^{-7}	18.52	< 0.005
Fe	–	7.8×10^{-3}	5.62	< 0.05
Mg	–	1.9×10^{-7}	1.24	n.s.
Al	Arcsine, $\sqrt{\quad}$	1.8×10^{-3}	2.27	n.s.
Cu	Arcsine, $\sqrt[4]{\quad}$	0.11	51.32	< 0.001
Zn	Arcsine, \log_e	1.7	15.82	< 0.005
Pb	Arcsine, $\sqrt{\quad}$	7.8×10^{-3}	39.53	< 0.001

Note. $\sqrt{\quad}$ indicates square root transformation, $\sqrt[4]{\quad}$ indicates fourth root transformation, \log_e indicates transformation to natural logarithms, n.s. indicates not significant at $P = 0.05$.

4. Discussion

Comparison of the X-ray microanalytical data

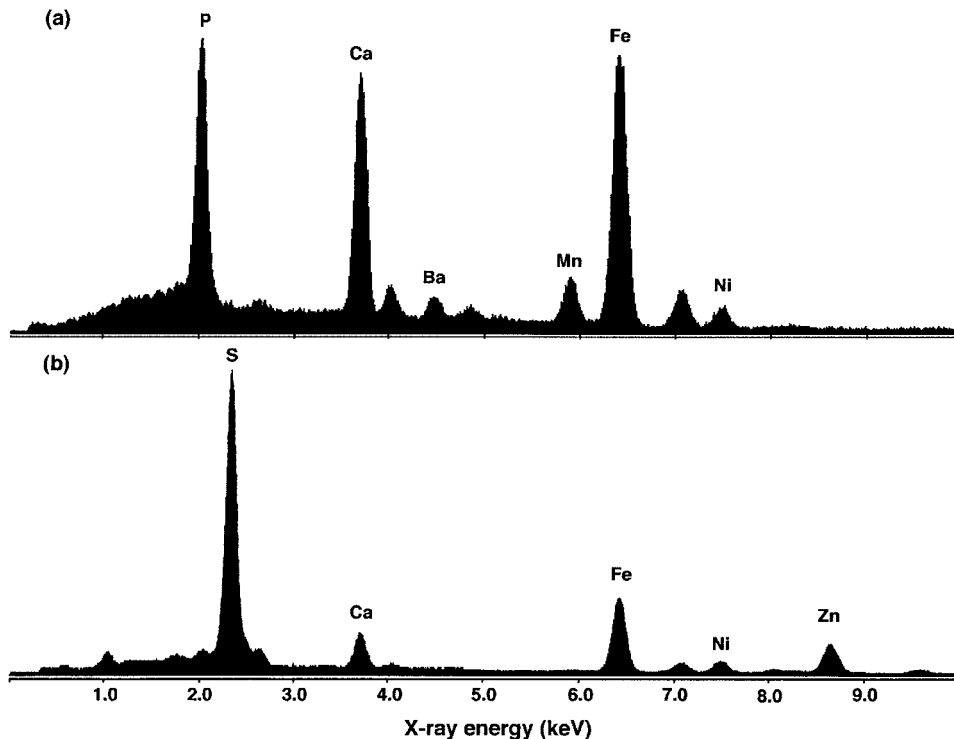


Fig. 2. Representative spectra from energy dispersive X-ray microanalyses of dry cut, freeze-substituted *Hyridella depressa* mantle tissue (Ni peaks are from specimen support grid): (a) common Ca–P type granule; and (b) uncommon S-type granule, note substantial Zn peak.

obtained for the granules in chemically-fixed and freeze-substituted tissue revealed that the proportions of Ca and Mn were lower in granules from chemically-fixed tissue. In contrast, the proportions of P, Fe, Cu, Zn and Pb were higher with chemical fixation. The loss of Ca and use of proportional data based on total counts meant that at least part of the observed increase in some elements in fixed tissue was due to loss of Ca. However, examination of the raw counts showed that while overall the total counts were lower with chemical fixation, raw counts of Cu, Zn and Pb were higher, verifying the observed increase in these metals.

Ca (among other elements such as K, Na and Mg) is known to be susceptible to extraction and redistribution with conventional aqueous specimen preparation (Morgan, 1980). Morgan et al. (1975) found that Ca was lost from chemically fixed sperm cells and that most of the loss occurred in glutaraldehyde. Morgan (1980) summa-

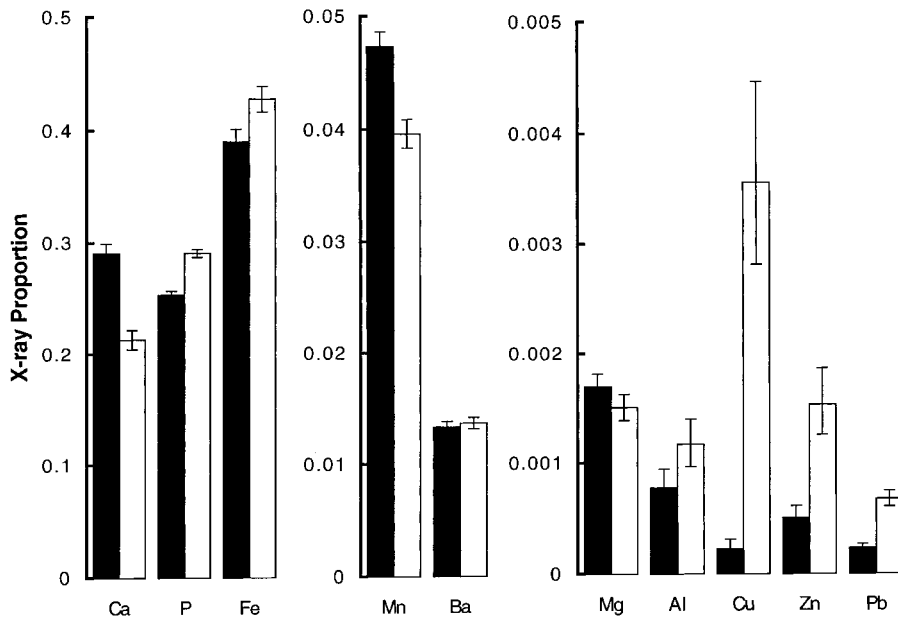


Fig. 3. Mean (\pm S.E.M., $n = 11$ mussels) proportions of characteristic X-ray counts for Ca, P, Fe, Mn, Ba, Mg, Al, Cu, Zn and Pb (calculated as peak counts divided by the sum of all peak counts, see text) in Ca-P type granules from *Hyridella depressa* mantle tissue prepared by chemical fixation (open bars) or freeze-substitution (solid bars).

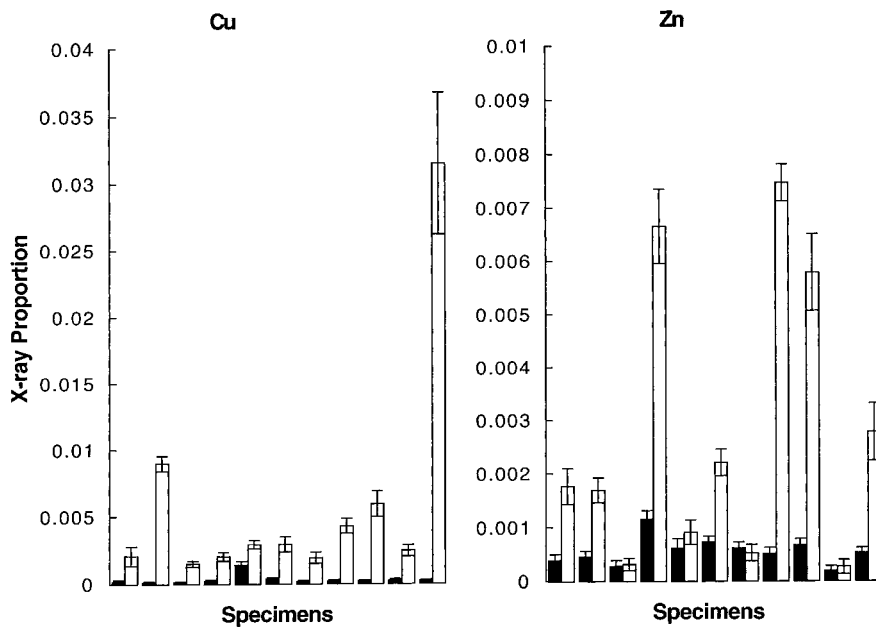


Fig. 4. Mean (\pm S.E.M., $n = 18$ granules) Cu and Zn proportions of characteristic X-ray counts (of sum of all peak counts, see text) in Ca-P type granules from 11 paired specimens of *Hyridella depressa* mantle tissue prepared by chemical fixation (open bars) or freeze substitution (solid bars). Note the high among-mussel variability with chemical fixation and lack of consistent trends among the preparation techniques.

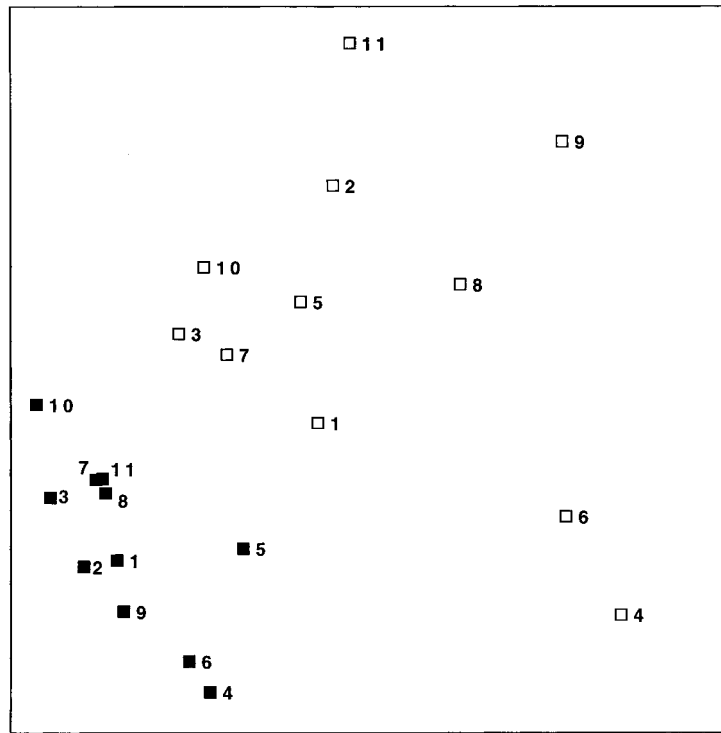


Fig. 5. Ordination of paired specimens (pairing indicated by numerals) prepared by chemical fixation (open symbols) or freeze-substitution (solid symbols), using non-metric multi-dimensional scaling, which combines information from 11 elements analysed in Ca–P type granules to produce a map of specimen similarities, stress = 0.08.

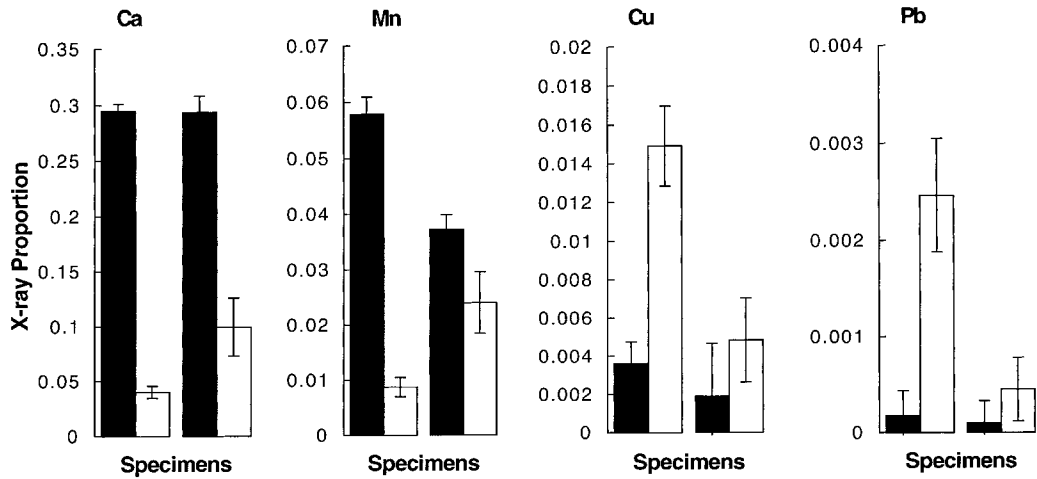


Fig. 6. Mean (\pm S.E.M., $n = 11, 18, 13, 14$ granules) Ca, Mn, Cu and Pb proportions of characteristic X-ray counts (of sum of all peak counts, see text) in Ca–P type granules from two paired specimens of freeze-substituted *Hyridella depressa* mantle tissue that was wet sectioned (open bars) or dry sectioned (solid bars).

rized data from a number of studies showing that major elemental losses occurred during the initial fixation step. In contrast to the observed increase in Cu and Zn observed here, George et al. (1978) found that conventional preparation methods led to a *loss* of these metals and substantial redistribution of Zn from granules in oyster tissue.

Similar to the results obtained for chemical fixation, freeze-substituted tissue that was wet sectioned yielded lower Ca and Mn proportions than dry sectioned, freeze-substituted tissue. Previous studies have reported that wet sectioning caused elemental loss from freeze-substituted specimens [Na, Cl (Harvey et al., 1976); Na, Cl, K, Ca, Zn (Chandler, 1980)], and also from chemically fixed tissues [P, K (Morgan et al., 1975)]. Morgan (1980) found that extracellular calcitic spherules in earthworms were dissolved by wet sectioning and also by section staining. Similar to this, we noted that the granules of *H. depressa* in freeze-substituted tissue that was wet sectioned appeared less electron dense than those that were dry sectioned, and were also annulated as reported previously for fixed tissue from this species (Adams et al., 1997). Metal-containing granules in invertebrates typically have an annulated appearance in ultrathin sections of chemically-fixed tissues (Brown, 1982). Our results suggest that the annulations may, at least in part, be an artefact caused by a leaching of elements from the organic matrix associated with the light annulations.

Compared with the relatively low among-mussel variability of the elemental profiles obtained for the dry sectioned freeze-substituted tissue, both chemical fixation and wet sectioning of freeze-substituted tissue were characterized by high among-mussel variability. This was particularly the case for elements such as Cu, Zn and Pb. It was not possible to determine whether the losses and redistribution of elements in the chemically-fixed tissue occurred at the stage of sectioning or prior to this step. We predict that chemically-fixed tissue that was dry sectioned would be less prone to redistribution or loss of elements and have less among-mussel variability than wet sectioned specimens.

The infrequent presence of diffuse S-type granules which contain varying but often substantial

proportions of Cu, Zn and Pb (Adams et al., 1997), suggests an explanation for the increased among-mussel variability in proportions of these metals with aqueous preparation. These S-type granules are similar in metal composition and appearance to granules thought to contain metallothionein (George and Pirie, 1979; High et al., 1997) and may serve as a source of Cu, Zn and Pb for dissolution and redeposition into the Ca-P granules during aqueous processing. An aggregation of S-type granules were observed in one of the wet-cut sections of freeze-substituted tissue, and the Ca-P granules in this section were found to have high proportions of Cu and Pb. The loss of Ca and Mn and increase of Cu and Pb in Ca-P granules with aqueous processing agrees with the Linear Free Energy Relationship as applied to freshwater mussel tissue (Brown et al., 1996) where the former metals replace the latter due to greater stability when complexed by P-containing ions. Investigations of the response of animal cells and tissues to the presence of metals in the environment often use X-ray microanalysis (Julliard et al., 1995; Giamberini and Pihan, 1996; Giamberini et al., 1996; Nigro and Leonzio, 1996; Soto and Marigómez, 1997) and our results emphasize the necessity of using cryopreservation and anhydrous preparation. Clearly metal distributions determined for chemically-fixed tissues may reflect the artefactual loss and redistribution of elements. Although chemical fixation may be justified in cases where the metals are tightly bound, it is unlikely that the experimenter will know this in advance, and it is certainly unwise to assume it. Nor are precipitation reactions satisfactory alternatives, they are still susceptible to ion loss and redistribution (Morgan, 1985). Consequently, wet chemical preparation of tissues in X-ray microanalytical studies should be restricted to providing images with improved ultrastructure to accompany cryoprepared specimens.

X-ray microanalysis of granules in mussel tissue is a new approach for monitoring metal levels in the freshwater environment. This approach should be particularly useful in catchments where conservation concerns preclude intensive sampling of threatened populations by a 'mussel watch' type sampling programme which involves

whole body analyses of a large number of specimens (O'Connor, 1996). *Hyridella depressa* recovers well from biopsies of mantle tissue (Byrne, unpublished) and X-ray microanalysis of granules in biopsies shows promise as a strategy for sampling low density mussel populations (Berg et al., 1995).

Acknowledgements

This study was supported by the NSW Government through its Environmental Research Trust. We thank S. Adams for data from fixed tissue, Dr C. Nockolds and M.J. Anderson for advice and discussion, and I. Wright, G. Capararo and G. Hunter for assistance with sampling. Thanks to Sydney Water Corporation for providing boat access to field sites. We gratefully acknowledge the Electron Microscope Unit, University of Sydney for assistance and use of facilities.

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