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Variability in the fractionation of Cu, Ag, and Zn among cytosolic proteins in the bivalve *Macoma balthica*

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ABSTRACT: Gel filtration chromatographs of cytosols from the clam *Macoma balthica* analysed from both field and laboratory treated specimens showed that uptake of Cu, Ag, and Zn in the metallothionein-like protein (MLP) pool follows exposure both in nature and in the laboratory. Specimens collected from San Francisco Bay over 18 mo showed strong temporal variability in the fractionation of the metals among cytosolic proteins. A marked increase in Cu, Ag, and Zn in a very low molecular weight pool occurred when concentrations were highest in the MLP pool. The correlation between total cytosolic metal and MLP-metal also appeared to approach a hyperbolic character at the highest concentrations.

INTRODUCTION

It is well established that bivalve molluscs (among many other types of aquatic organisms) concentrate metals in their tissues, but the link between tissue metal concentrations and cytotoxic effects is not well understood. Although the largest fraction of the metal burden in many organisms may occur within membrane-limited vesicles (George et al. 1978, Lowe & Moore 1979), the small fraction extractable from the cell cytoplasm (the cytosol) plays an important role in sequestration, elimination and toxicology of many metals (Roesijadi 1981, Variengo et al. 1981a). Metals within the cytosol occur in association with 3 operationally defined protein fractions (as separated by gel filtration or HPLC): (1) high molecular weight proteins (> 30,000 daltons) which appear to include metalloenyzmes and which bind metals non-specifically (Frazier & George 1983); (2) a low molecular weight fraction of poorly understood composition; and (3) a metal-specific binding protein fraction of molecular weight 7 to 13,000 daltons, which displays many of the characteristics of mammalian metallothionein (e.g. low aromatic content, heat stability, synthesis induced by metal exposure, high affinity for binding trace metals).

The processes that determine the distribution (or partitioning) of metals among the operationally-defined protein fractions in the cytosol are not clearly understood, and inconsistencies are common among studies of cytosolic metal partitioning. For example, not all studies of partitioning in organisms from nature find metal associated with all 3 protein fractions (Roesijadi 1980, Variengo et al. 1981a, Kohler & Riisgard 1982). Similarly, the toxicological significance of shifts in partitioning among fractions is not clear. Metal concentrations associated with the metallothionein-like metal binding protein fraction (MLP) increase with metal exposure in laboratory studies. Some authors have proposed that metal accumulation in the MLP fraction approaches a hyperbolic character at high levels of exposure, and 'spillover' of metal from this pool into the high molecular weight fraction is indicative of the onset of metal toxicity (Brown & Parsons 1978, Winge et al. 1978, Roesijadi 1980). However, other work indicates increased metal partitioning to the very low molecular weight fraction coincides with the onset of metal induced stress (Sanders & Jenkins 1984) or conversely, the development of metal tolerance (Frazier & George 1983) perhaps even without saturation of the MLP fraction (Sanders & Jenkins 1984).

Two factors that could contribute to differences among studies of cytosolic metal partitioning have not been sufficiently discussed in the literature. One is the way laboratory exposures are conducted. For example, most studies employ short-term (relative to the life

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span of the organism) exposure to metal in solution. Animals in nature are exposed to metals in both solution and food, and the duration of an elevated exposure may vary from hours to a lifetime (Luoma 1983). If metal metabolism and thus cytosolic metal partitioning are affected by the length and the route (solution or food) of exposure, interlaboratory inconsistencies in results, and inconsistencies between laboratory and field comparions, should not be surprising. Secondly, few (if any) studies of metal fractionation in the cytosol of aquatic organisms have employed replicate samples. There is a paucity of information concerning the variability of intracellular metal partitioning either among individuals of the same population at any one time or within a population through time. Many conclusions appear to be based upon single samples despite the well-established variability that characterizes metal dynamics in aquatic organisms (Luoma & Cain 1979, Strong & Luoma 1981, Lobel et al. 1982). In fact, where more than 1 cytosol sample from the same species has been analyzed, a wide degree of variability has been observed (Ridlington et al. 1981).

The present study assesses the fractionation of Cu, Ag and Zn in the cytosol of the estuarine deposit feeding bivalve *Macoma balthica*. Uptake of Cu into the cytosol was studied in the laboratory in specimens exposed to Cu in solution and in Cu-enriched sediments. Changes also were followed in the intracellular fractionation of Cu, Ag and Zn over an 18 mo period in a population of *M. balthica* collected from South San Francisco Bay. This metal-tolerant population (Luoma et al. 1983) resides near an anthropogenic source of trace metal input (Thomson et al. 1984). Whole body concentrations of Cu and Ag fluctuate seasonally and from year to year (Luoma & Cain 1979, Strong & Luoma 1981). In the field study replicate samples were analyzed at each sampling interval to establish the extent of variability at any one time. Cadmium was not considered in this study because concentrations of Cd in *M. balthica* in South Bay are consistently low (< 1 mg g^{-1}) which seems typical of estuarine desposit-feeding organisms.

METHODS AND MATERIALS

Field sampling. Approximately 60 *Macoma balthica* were collected monthly from January 1981 through June 1982 in South San Francisco Bay near Palo Alto (Thomson et al. 1984). After a 2 d depuration period, 20 of these clams were separated into 5 to 7 samples, each containing individuals differing by 1 mm shell length or less. These specimens were removed from their shells, oven dried, reflux digested in concentrated nitric acid, evaporated and reconstituted in 3N HCl for whole body Cu, Ag, and Zn determination by flame atomic absorption spectrophotometry (AAS). The remaining specimens were separated into 4 size classes, dissected from their shells, wet weighed and analyzed for cytosolic metal partitioning.

Laboratory protocol. A laboratory experiment was designed to study the accumulation of Cu into different intracellular protein pools in *Macoma balthica* and to determine if prior exposure to relatively low concentrations of Cu affected accumulation. During the first phase of the experiment half the clams were exposed to 10 g of sediment in seawater with an initial solute Cu concentration of 75 $\mu g\ l^{-1}$ and half were held unexposed. This phase lasted 55 d. For the next 21 d all clams were held unexposed and fed suspended marine bacteria (Harvey & Luoma 1984). In the final phase of the experiment half the initially unexposed clams and all the previously exposed clams were held for 18 d with 10 g of sediment in seawater with an initial solute Cu concentration of 400 $\mu g\ l^{-1}$. In all phases sediment and seawater were changed every 3 d.

Clams for the study were collected from the field study site at low tide and acclimated for 5 d before the experiment was begun. Possible size (age) effects on experimental results were randomized by distributing an equal number of clams from different size classes among the experimental aquaria. The sample of clams pooled for analysis at each sampling interval included representatives from each size class.

Sediment used throughout the experiment was collected at one time from the oxidized surface layer of a mud flat where Cu concentrations in sediment were 40 to 50 % lower than at the field sampling site. The sediments were wet sieved through polyethylene mesh and particles less than 100 μm were stored wet at 4 °C to preserve microbial populations.

All experiments were conducted at pH 7.8 and 12 °C in seawater Water samples for Cu analysis were collected in acid-washed vials, acidified with 16N HNO_3 and analyzed by the standard additions method by flameless AAS. Replicate 1 ml aliquots of sediment suspension were pipetted directly from the aquaria into tared 20 ml glass vials for analysis. Soluble salts were washed from the sediment, then the samples were dried to constant weight at 90 °C and digested in a 2 1 mixture of 16N HNO_3 : 36N H_2SO_4. The digests were evaporated to dryness, reconstituted in 3N HCL and analyzed for Cu by flame AAS.

Cytosolic analysis. Pools of *Macoma balthica* were homogenized immediately after dissection at 12 °C in an extracting solution containing 0.5 M sucrose, 1 % 2-mercaptoethanol, and 10^{-3}M phenylmethyl-sulfonyl-fluoride (PMSF), a protease inhibitor (Variengo et al. 1981b, Sharma 1983). The homogenates were centrifuged at 37,000 × g for 45 min at 4 °C. The supernat-

ants were then heated under nitrogen for precisely 6 min in a 70 °C water bath to precipitate large molecular weight proteins. This mixture was centrifuged again at 37,000 × g for 45 min at 4 °C. The final supernatants were frozen at −20 °C. The pellets were oven-dried and digested in nitric acid to determine the insoluble Cu, Ag, and Zn portions of the total.

Aliquots of each supernatant were applied to a G-75 superfine gel filtration column and eluted at 4 °C with a 0.05M NH_4HCO_3 buffer solution at a flow rate of 0.25 ml min^{-1}. Five ml fractions were collected and measured for absorbance at 280 nm. Concentrations of Cu, Ag and Zn were determined on paired fractions immediately after elution. Cu and Ag were analyzed by flameless AAS and Zn by flame AAS. To allow comparison of different samples the 280 nm absorbance and trace metal concentrations were corrected for sample wet weight and homogenate volume. The integrated areas under the metal peaks on the chromatograph were used to quantify the 3 molecular weight pools. The molecular weights of the different fractions were identified using ovalbumin, chymotrypsin and cytochrome C as standard proteins.

The heat precipitation method of removing macromolecular organic material from the cytosol was compared to ultracentrifugation on a split sample collected in June 1982, to assess the relative differences in protein and trace metal content resulting from the 2 techniques.

RESULTS

Characteristics of the protein fractions

The elution profiles of Zn partitioning among cytosolic protein fractions for *Macoma balthica* shown in Fig. 1 characterize fractionation profiles observed for all 3 metals. Three distinct metal-containing protein peaks were evident (Fig. 1b). A high molecular weight protein peak (Peak I) occurred above 30,000 daltons; a middle molecular weight fraction (Peak II) at 12,400 daltons and a very low molecular weight fraction (Peak III) at less than 3000 daltons. Each of the metals (Cu, Ag, and Zn) was found associated with all 3 peaks at some times of the year, but only in Peaks I and II at other times (Fig. 1a).

Comparison of heat precipitation versus ultracentrifugation

Heating the supernatant caused a significant increase (48 %) in precipitation of the Peak I protein compared to that removed by ultracentrifugation. The small quantity of protein detectable at 280 nm in Peak II was not significantly different whether heat precipitation or ultracentrifugation was employed. The Cu and Ag content of the 3 fractions was affected by heat precipitation in a similar way to the protein content (Fig. 2a). A significant loss ($p < 0.01$) of each metal from the Peak I pool occurred upon heating (50 % more Cu and 34 % more Ag) but Peaks II and III had Cu and Ag concentrations similar to those observed in the ultracentrifuged samples. Variability among replicates was similar for the 2 procedures. The heat stability of

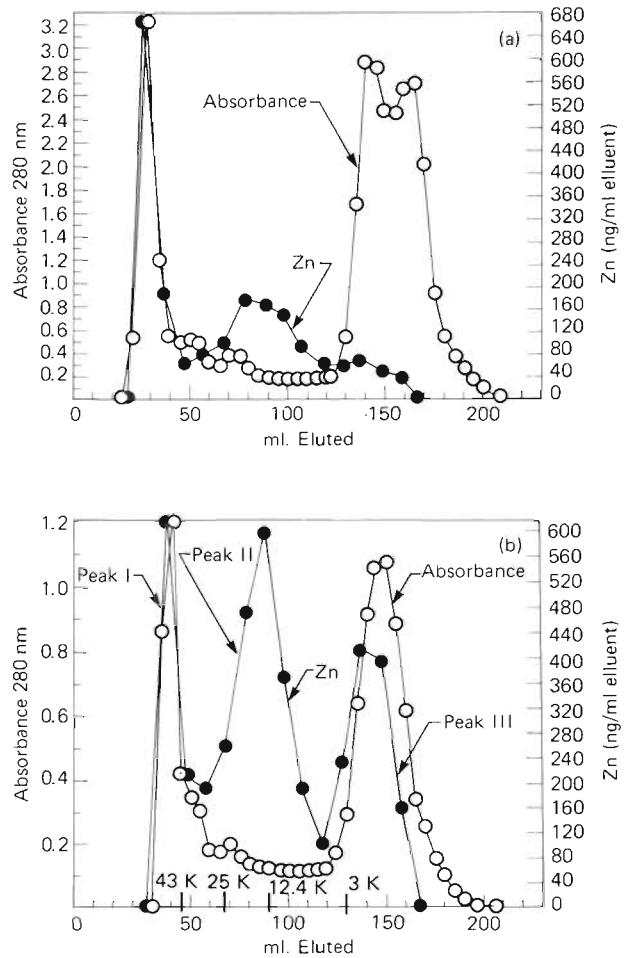

Fig. 1 *Macoma balthica*. Elution profiles. Absorbance (280 nm) and Zn (ng ml^{-1} elluent) elution profiles for samples from Palo Alto. Ovalbumin, chymotrypsin and cytochrome C were used for molecular weight calibration. Profile (a) is a sample with lower whole body concentrations of Zn than that of profile (b)

Peak II, coupled with its high metal content, low absorbance at 280 nm and molecular weight near 12,000 daltons is characteristic of a metallothionein-like, metal specific binding protein. The loss of protein and its associated metal in Peak I suggests the operational definition of Peak I will differ among studies employing heat precipitation or ultracentrifugation.

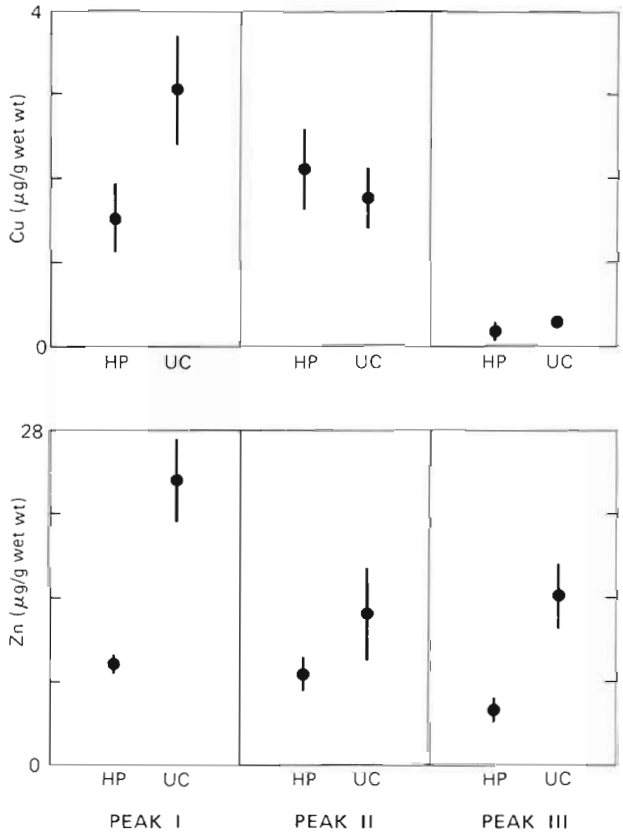

Fig. 2. *Macoma balthica*. Comparison of Cu (a) and Zn (b) concentrations in Peaks I, II, and III after sample preparation with either heat precipitation (HP) or ultracentrifugation (UC). Heat precipitation and ultracentrifugation values are the means and SDs of 4 and 3 replicate samples of pooled specimens, respectively

In contrast to the results with Cu and Ag, significantly more Zn was removed from Peak III (67 %) by heating than by ultracentrifugation (Fig. 2b). However Zn in Peak II was not significantly affected ($p > 0.10$).

Laboratory uptake of Cu

Copper in seawater in the laboratory experiments quickly became associated with particulates upon mixing of the 2 phases, although no attempt was made to determine if the Cu completely reached phase equilibrium during the 3 d between renewals of Cu-laden water and sediment. For the first 55 d of the experiment mean Cu concentrations in the Cu-treated system were 13 µg l^{-1} and 92.8 µg g^{-1} (dry weight) for the water and sediment, respectively Concentrations in the control system were 7 µg l^{-1} and 54 µg g^{-1} in water and sediment. During the last 18 d of the experiment concentrations of Cu were raised to 70 µg l^{-1} in water and 246 µg g^{-1} (dry weight) in sediment.

No significant accumulation of Cu in *Macoma balthica* was observed in any of the protein fractions of the Cu-exposed clams during the first (low) exposure and the second, non-exposure phase of the experiment. Upon exposure to higher levels of Cu, however, both clams that had been previously exposed to low levels of Cu, and those that had not, displayed Cu accumulation within cytosolic fractions. In Peak I, Cu concentrations approximately doubled during the initial 3 d of exposure. The rate of accumulation decreased during the next 11 d, but then displayed another period of rapid accumulation. Within Peak II an immediate and continuous accumulation of Cu occurred. Cu accumulation in Peak III lagged behind that of Peaks I and II (Fig. 3). No accumulation occurred until the 6th day of exposure, then uptake increased 2 to 3 fold within the next 8 d.

In contrast to the clear accumulation of Cu in cytosolic Peaks I and II, net changes in whole body concentrations of Cu were not detectable within the variability among samples. On the last day the only

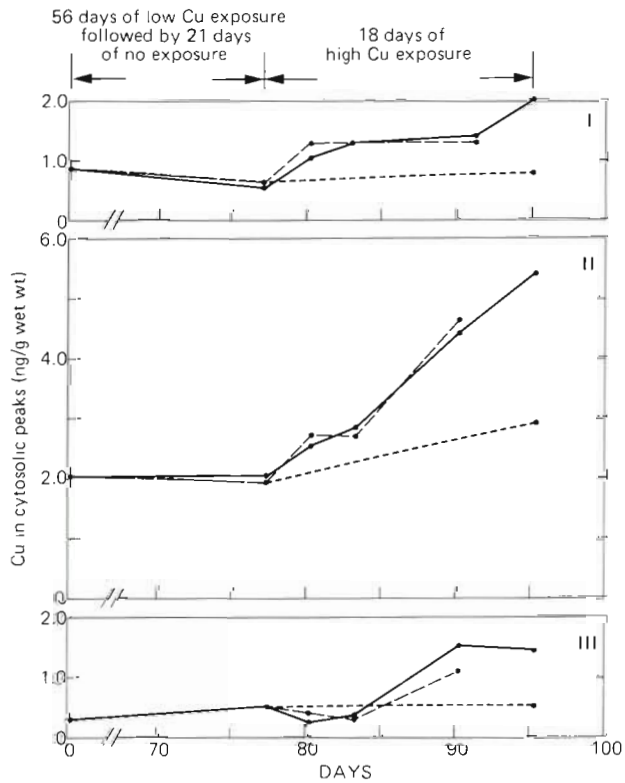

Fig. 3. *Macoma balthica*. Copper concentrations in Peaks I, II, and III. Copper exposure for 18 d period starting on Day 77 was 70 µg Cu l^{-1} in water and 246 µg Cu g^{-1} (dry weight) in sediment. Two groups of clams were exposed: (—) clams previously exposed to low Cu conditions (see text) and (--) clams that had been held under control conditions for the first 77 days. (----): clams held under control conditions throughout course of expriment. Coefficients of variation (cv) for the initial sample (n = 2) are 3.5, 3.1 and 18.9 % for Peaks I, II and III, respectively

statistically significant increase occurred in whole body concentrations, but this increase was only 50 % (Fig. 4).

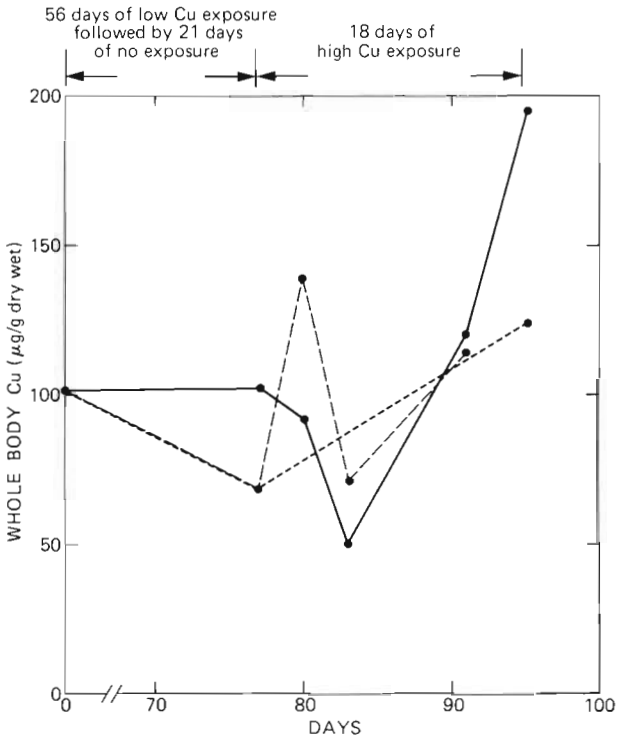

Fig. 4. *Macoma balthica*. Whole body Cu concentrations. Initial value is the mean (± 1 SE) of replicate (n = 2) samples. All other values are for a single sample consisting of pooled individuals. Exposure conditions and symbols are the same as in Fig. 3

Concentrations of Zn in the cytosolic fractions showed no consistent pattern of change during the period of Cu exposure.

Variability in Cu fractionation in nature

Variability among replicates

Concentrations of Cu, Ag, and Zn in specific peaks within the cytosol of *Macoma balthica* varied among replicate samples by as little as 2 to 5 % at some times and by as much as 50 to 70 % at others (Table 1; also see error bars in Fig. 8). The percent variability was greatest in Peak III because metal concentrations at some times of the year were near analytical detection limits in this fraction. The coefficient of variation averaged 20 to 26 % for Peaks I and II (where metals were readily detectable) and was quite similar to the variation observed in whole body metal concentrations from similar sample sizes (25 to 40 individuals) (Lobel et al. 1982, Luoma et al. unpubl.). At each sampling date enough differences occurred among replicate chromatographic profiles to suggest caution should be exercised in interpretations based only upon a single profile (Fig. 5).

Table 1. *Macoma balthica*. Mean and range of the coefficient of variation in metal concentrations associated with cytosolic fractions. n = 18. Mean and range for each sampling time (i. e. each n) was calculated from 3 to 5 replicate pools of specimens collected at that time

	Coefficient of variation (% of mean)					
	Cu		Ag		Zn	
	Mean	Range	Mean	Range	Mean	Range
Peak I	23	5–49	15	2–68	21	6–71
Peak II	20	4–46	26	15–59	21	6–53
Peak III	47	3–112	84	38–150	41	4–63

Temporal variability

The percent of total Cu, Ag, and Zn extracted in the cytosol from *Macoma balthica* was dependent on sampling times. In general, the highest whole body metal concentrations coincided with both a higher concentration and a higher proportion of cytosolic metal (Fig. 6). However, the relation between cytosolic metal and whole body metal concentrations was quite variable at lower concentrations. Soluble Cu varied between 4 and 14 % of total Cu in soft tissues; soluble Ag varied between less than 0.3 and 3.0 %; and soluble Zn varied between 25 and 70 %.

The temporal pattern of fluctuation in whole body concentrations of Ag, Cu and Zn in *Macoma balthica* at Palo Alto has been demonstrated by Luoma & Cain (1979), Strong & Luoma (1981) and Thomson et al. (1984). In nearly all years, maximum soft tissue Cu, Ag, and Zn concentrations occur in the early winter (the rainy season, Dec to Mar), then concentrations decline through the spring to a summer or fall minimum (Fig. 7a). Concentrations of Cu in cytosols followed a generally similar regime in 1981–1982 (Fig. 7b). Concentrations were highest in the cytosols when sampling began in January 1981, declined nearly 5-fold through the summer and fall, then increased in December through April 1982. The differences between 1981 and 1982 in maximum Cu concentrations are typical of year-to-year differences observed during our 10 yr of study on this mudflat (Luoma et al. in press). Both whole body and total cytosolic concentrations of Zn and Ag were similar to Cu except that the summer decline in Zn concentrations was somewhat more rapid than that of Cu and Ag (data not shown).

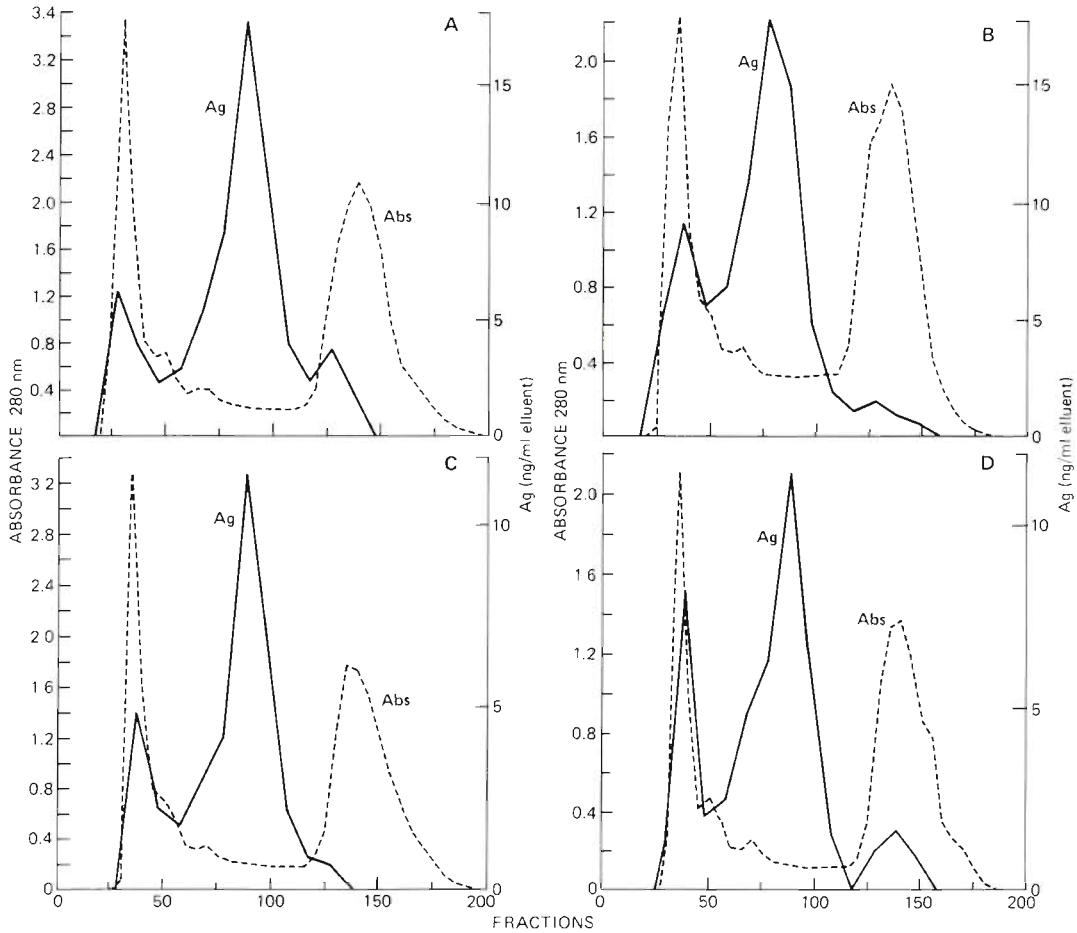

Fig. 5. *Macoma balthica*. A comparison of chromatographic profiles for Ag and protein concentration from 4 size classes of clams collected from Palo Alto on March 31, 1981. Each sample comprised of tissues from 7 to 15 individuals. Average shell lengths of size classes: (A) 2.57 cm, (B) 3.30 cm, (C) 2.40 cm, (D) 2.81 cm

Mean concentrations of Cu in Peak I showed no discernible pattern of change through the 1981–1982 study period (Fig. 8). Concentrations of Cu in Peak II followed a temporal trend similar to cytosolic Cu. Concentrations in Peak III were high in winter of 1981 when cytosol and total Cu concentrations were at their highest, but Peak III contained little Cu during the remainder of the study. Again, Ag followed the same trends as Cu, so the data are not shown.

Zn concentrations in all 3 protein fractions generally followed a pattern of fluctuation similar to cytosolic and total Zn (Fig. 8c); but, as with Cu and Ag, the greatest similarity to cytosol and total Zn was with the Peak II fraction.

Differences in temporal trends among protein fractions resulted not only in differences in the percent metal associated with different fractions at different times, but also in substantial temporal differences in the gel filtration profiles of each metal (e.g Fig. 1, Fig. 9).

Concentrations of Cu, Ag, and Zn in Peak II correlated with concentrations in the cytosol (Fig. 10). At low metal concentrations in the cytosol the relation was strongly linear. However, the linearity broke down above 7 $\mu g\ g^{-1}$ cytosolic Cu, 0.4 $\mu g\ g^{-1}$ cytosolic Ag and 40 $\mu g\ g^{-1}$ cytosolic Zn, when the relation appeared to approach a hyperbolic character. In contrast, metal concentrations in Peak III showed little relation to cytosolic metal at low concentrations, but increased at high cytosolic metal concentrations (Fig. 10). At approximately the point where Peak II metal concentrations began to demonstrate a saturable relation with cytosolic metal, concentrations in Peak III began to increase. No increase in Cu or Ag in the high molecular weight Peak I was observed at the point of apparent saturation in the Peak II relation.

DISCUSSION

The 5 to 15 % extractable Cu found in *Macoma balthica* is typical of the proportion of cytosolic Cu

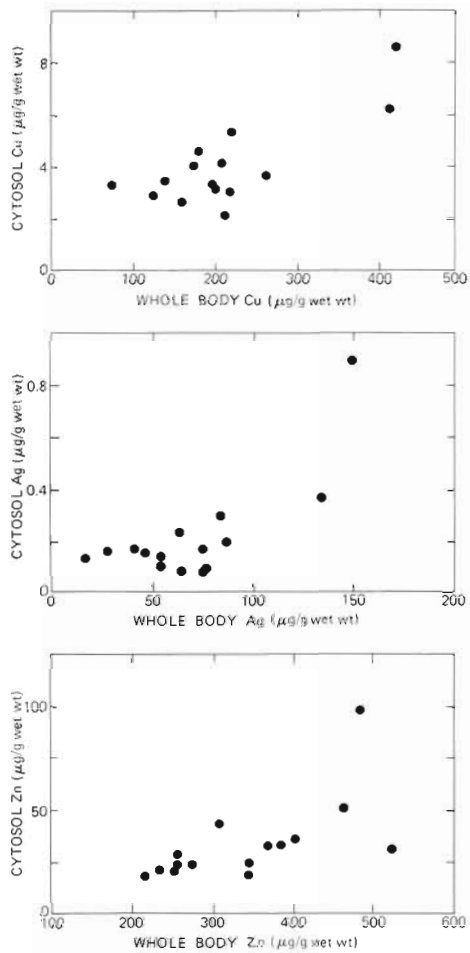

Fig. 6. *Macoma balthica*. Variation of cytosolic Cu, Ag and Zn as a function of whole body concentrations. Whole body values are the means of 17 to 23 individuals and cytosolic values are the means of 3 to 5 pools totaling approximately 40 individuals

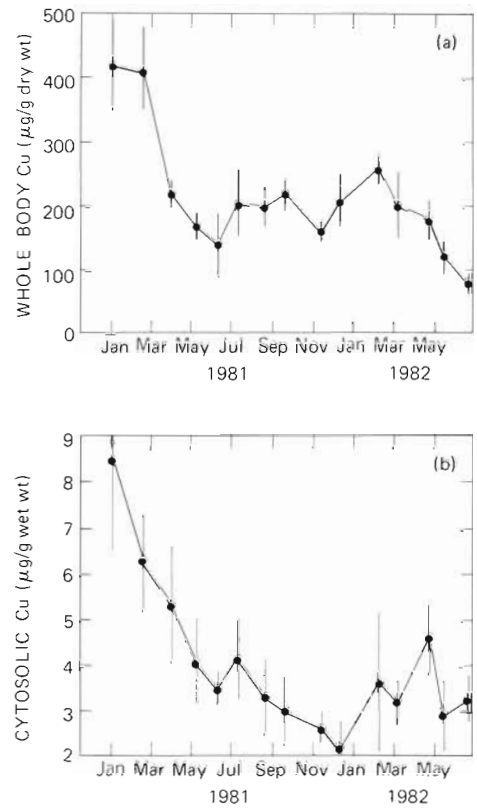

Fig. 7. *Macoma balthica*. Temporal fluctuation of whole body (a) and cytosolic (b) concentrations of Cu. Bars represent ± 1 SD for 17 to 23 individuals in (a) and approximately 40 individuals pooled into 3 to 5 samples in (b)

found in crabs, mussels and oysters (Coombs 1974, Variengo et al. 1981b, Sharma 1983). Although proportionately small, the cytosol comprises an important bioactive metal pool in aquatic invertebrates (Roesijadi 1980, Variengo et al. 1981) and a pool that responds rapidly to metal (i.e. Cu or Ag) exposure. Whole body or whole organ concentrations may include large pools of metals that are minimally involved in metal metabolism (George et al. 1978, Lowe & Moore 1979). High concentrations of non-extractable Cu appeared to mask biologically important uptake in *M. balthica* when whole body metals were analysed in our laboratory study. Uptake was much more evident when the cytosolic pool of metals was studied directly. Thus cytosol metal concentrations may be more sensitive than whole body concentrations when studying metal uptake in organisms with pre-established high concentrations of metals in their tissues (such as metal-tolerant populations).

Although excellent replication of results was observed in the laboratory exposure study, a relatively large degree of variation in metal and protein concentrations was evident among replicate samples of *Macoma balthica* collected from nature. This suggests that the same statistical cautions commonly exercised in interpreting whole body metal concentrations in organisms must be used in interpreting chromatographic profiles or in describing other aspects of cytosolic metal fractionation.

Temporal variability or differences in exposure histories could explain some of the inconsistencies among studies of cytosolic metal fractionation. For example some authors have reported the occurrence of Cu associated with a very low molecular weight fraction (equivalent to our Peak III) in the cytosol of crabs (Rainbow & Scott 1979), mussels (Variengo et al. 1982), oysters (Sharma 1983) and crab larvae (Sanders & Jenkins 1984). Other studies have not found low molecular weight Cu (Roesijadi 1980). Laboratory studies with *Macoma balthica* indicate detectable levels of Cu appear in the very low molecular weight fraction of the cytosol during elevated exposures, but only after a lag of several days. This fraction was only present in

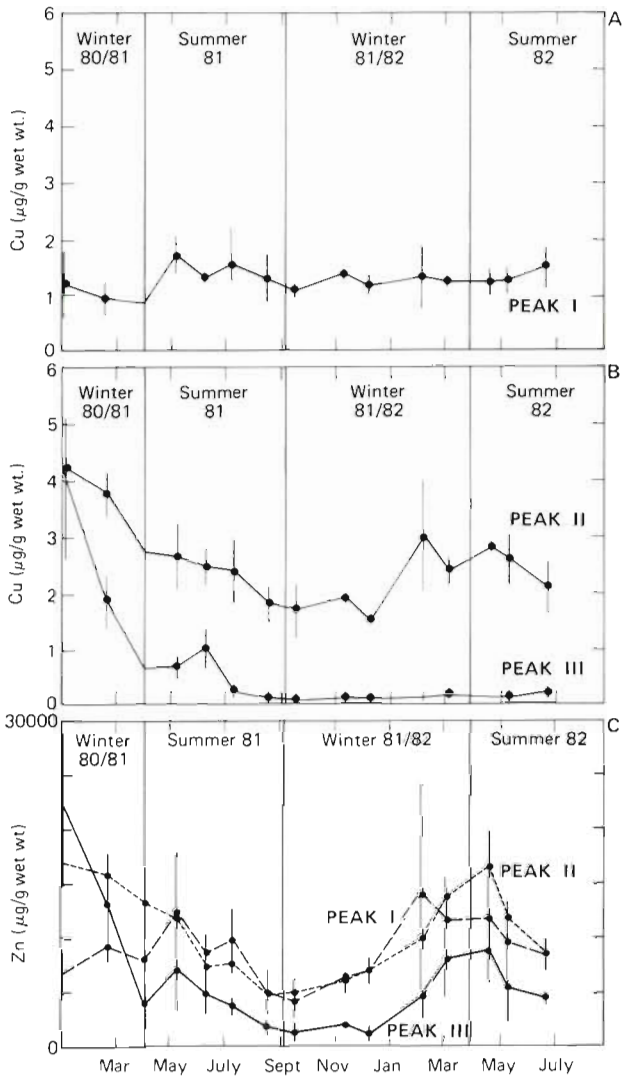

Fig. 8. *Macoma balthica*. Temporal changes in concentrations of Cu and Zn in Peaks I, II and III in the cytosol of clams from Palo Alto. Each value is the mean and SD from approximately 40 individuals pooled into 3 to 5 samples. Trends for Ag were similar to those for Cu

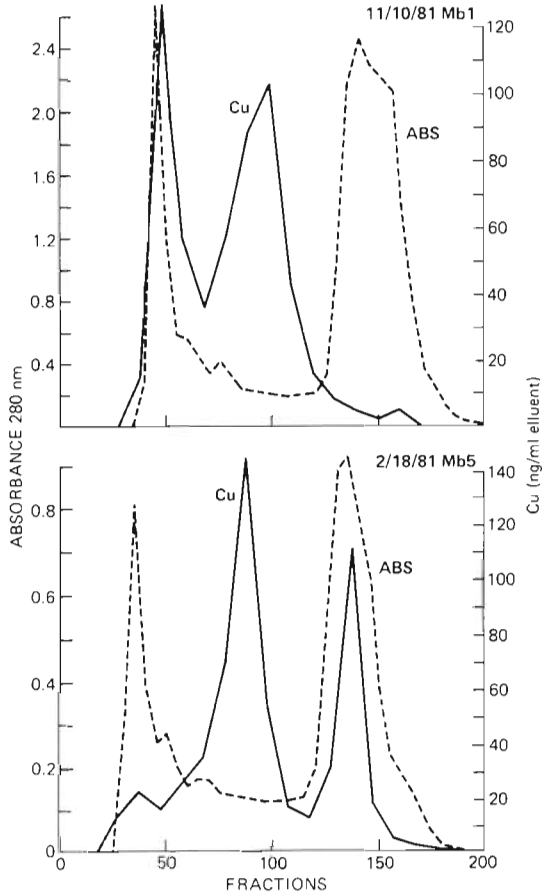

Fig. 9. *Macoma balthica*. Absorbance, 280 nm, and Cu, ng g^{-1} wet weight, elution profiles for clams from Palo Alto. Profile (a) is from a sample with lower whole body Cu concentrations than profile (b)

nature during the periods of highest metal exposure. Thus the dynamics of the very low molecular weight fractions of Cu are complex and may vary not only among species, but within a species with the seasonal, year-to-year, and spatial differences in metal exposure that are common in nature.

Recent studies have raised several other major questions concerning metal fractionation in the cytosol of aquatic invertebrates. The first concerns the induction of thionein-like proteins by metal exposure. Induction of Cu-thionein has been observed in many organisms (e.g. Variengo et al. 1981b). Frazier & George (1983) concluded that Cu exposure did not induce Cu-thionein production in oysters. Their conclusions were mainly based upon the low concentrations of Cu-thionein in animals from an estuary where sediments were heavily contaminated with Cu (although no evidence was presented demonstrating that the Cu was biologically available). The population of *Macoma balthica* studied in our work also is from a metal-enriched environment, and is exceptionally tolerant to Cu (Luoma et al. 1983) and Ag (D. J. Cain, unpubl.). In these organisms Cu concentrations in the metallothionein-like fraction (Peak II) increased during a period of Cu exposure in the laboratory, and increased with increasing concentrations of cytosolic Cu in the field. Both observations imply induction of Cu-thionein-like protein and suggest that, at least below some maximum level, Cu in Peak II reflects biologically available Cu in the environment (Olafson et al. 1979, Sanders et al. 1983). Although reports of a Ag-binding protein in aquatic organisms are not common, our field results also clearly show the presence of Ag in the metallothionein-like fraction of the cytosol of *M. balthica*, implying induction of the synthesis of this protein in this species.

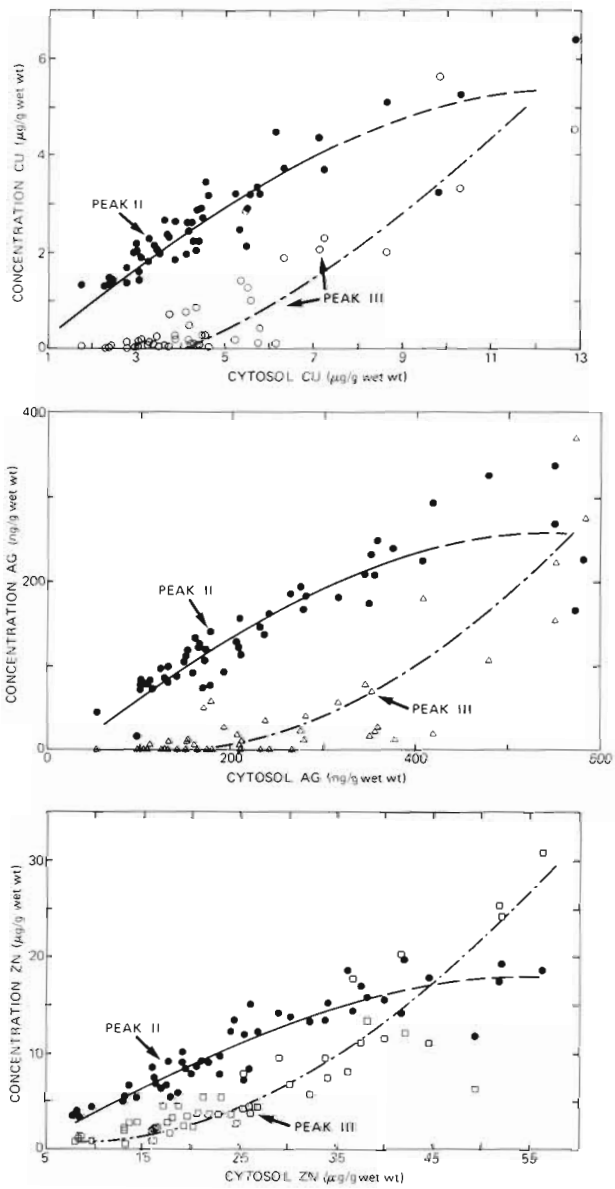

Fig. 10. *Macoma balthica*. Concentrations of Cu, Ag, and Zn in Peaks II and III as a function of cytosolic metal concentration

A higher percentage of total Zn was extracted in the cytosol of *Macoma balthica* than or Cu or Ag. Zn is not consistently observed in the metallotheionein fraction of molluscs in nature (Variengo et al. 1982), but was always present in Peak II in *M. balthica*. Studies have shown that metallothioneins induced by trace metals other than Zn (e.g. Cd, Hg, Cu and Ag) may contain Zn in high concentrations (Bremner & Davies 1975, Winge et al. 1975, Leber & Miya 1976). This may explain the abundance of Zn in Peak II in *M. balthica*, especially at the low whole tissue concentrations of Zn (for a tellenid clam) found at Palo Alto.

Another important area of inconsistency in recent studies is the relation between metal exposure and metal accumulation in the high molecular weight protein fraction of the cytosol (Peak I). The concentration of Cu associated with high molecular weight protein increases with increasing cytosolic Cu in the liver of ducks (Brown & Chatel 1978) and the liver of trout (Roch et al. 1982). In *Macoma balthica* no change in Cu and Ag concentrations associated with high molecular protein was observed through a large gradient in cytosolic Cu concentrations in nature. In contrast Cu was accumulated in the high molecular weight pool when *M. balthica* was exposed in the laboratory. It is not clear whether the laboratory results represented a transient response to a high Cu exposure, a kinetic effect in which total Cu uptake occurred more rapidly than Cu binding in Peak II and the remaining Cu appeared in Peak III, or a short-term interaction between the high molecular weight and very low molecular weight fractions of Cu. In any case it is again obvious that Cu fractionation into high molecular weight protein in response to Cu exposure is a complex phenomenon.

A third unresolved issue is whether metal binding to thionein-like protein is a saturable process, and whether spill-over from the thionein protein pool is specifically indicative of metal stress. A number of laboratory studies have indicated that toxicity ensues when metal uptake exceeds the binding capacity of metallothionein (Piotroski & Bolanowska 1970, Brown & Parsons 1978, Roesijadi 1980). None of these studies collected extensive data in the region of metallothionein saturation. Sanders et al. (1983) showed a decrease in the growth of crab larvae that coincided with the half saturation constant of the hyperbolic relation between Cu-thionein and cytosolic Cu. However their hyperbolic relation depended heavily upon a single data point (and none of the data included estimates of variance). In more extensive work (Sanders & Jenkins 1984) they demonstrated a linear relation between Cu-thionein and cytosolic Cu over a wider data range. The apparent saturation had either represented simply variance in the data or a temporary plateau in accumulation of Cu into metallothionein (Jenkins & Sanders 1984). Toxicity in the latter study coincided with the onset of Cu accumulation in a very low molecular weight fraction of the cytosol. In nature, Harrison & Lam (1983) observed apparent saturation in the relation of Cu-thionein to cytosolic Cu in blue gill from the cooling pond of a power plant. As the linearity of the Cu-thionein relation broke down, Cu concentrations in high molecular weight protein increased. A breakdown in the linearity of the accumulation of Cu, Ag and Zn into thionein-like protein (Peak II) was also observed in our study with *Macoma balthica* in nature. Metal accumulation in the very low molecular weight

fraction (Peak III) accompanied apparent saturation of the relation between metal in Peak II cytosolic metal in *M. balthica,* but no 'spillover' of metal into high molecular protein was evident. Both our study and that of Harrison & Lam (1983) rely upon a small quantity of data at the high concentrations where partitioning in the cytosol changed. Whether additional data at higher concentrations of metals would show a continuation of the hyperbolic nature of the relation or a return to linearity in the relation is unknown. Of course, obtaining data at higher concentrations may be difficult if changes in cytosolic partitioning are indeed indicative of stress, because, in nature, stressed animals are not likely to survive.

Macoma balthica at Palo Alto show many symptoms of a metal-stressed population. As reported here, changes in cytosolic metal partitioning characteristic of the onset of metal stress reported by Sanders & Jenkins (1984) occurred in 1981. This population is as much as 50-fold more tolerant to Cu than several other populations of *M. balthica* in San Francisco Bay (Luoma et al. 1983). Electrophoretic studies indicate differences in isozyme distributions of the protein leucine amino peptidase between clams at Palo Alto and clams from several other environments in the Bay (Humphreys et al. unpubl.). If the tolerance to Cu is genetically-based, it suggests that at least a portion of the genetic pool of *M. balthica* in San Francisco Bay cannot survive the metal enriched conditions at Palo Alto (Luoma 1977). The population of *M. balthica* at Palo Alto also completely disappeared from our sampling area several times in the last 8 yr, each time following the appearance of very high concentrations of whole tissue Cu and Ag. In both of the later instances, however, it is not clear that the changes in *M. balthica* populations were linked to metal exposure because other changes in ecological conditions were also involved (Nichols & Thompson 1985).

Despite the indications of stress, we have not proven conclusively that trace metals are adversely affecting *Macoma balthica* at Palo Alto. Conclusive proof would depend upon *in situ* measurement of (1) physiological stress in specimens from nature; (2) a coincident shift in cytosolic metal fractionation of a type that has been linked conclusively to metal stress in the laboratory; and, (3) ideally, a measured change in the abundance of *M. balthica* relative to other members of the invertebrate community. Given the gaps in our knowledge of how to detect physiological stress in populations in nature, how to physiologically interpret changes in cytosolic metal fractionation, and the relation between cause and effect when benthic communities change, it is not surprising that conclusively documenting the effects of trace metals upon populations of organisms in nature is difficult (Bascom 1981).

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