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Application of trichloroacetic acid (TCA) to extraction of soft body for the determination of tissue Cd, Cu, Pb and Zn in the prosobranch *Hydrobia ulvae* (Pennant)

Adam Sokolowski [a,b,*], Pierre Richard [b], Denis Fichet [c], Gilles Radenac [c], Thierry Guyot [c]

[a] *Laboratory of Estuarine Ecology, Institute of Oceanography, University of Gdansk Al. M.Pilsudskiego 46, 81-378 Gdynia, Poland*
[b] *Centre de Recherche sur les Ecosystèmes Marins et Aquacoles de l'Houmeau (UMR10 CNRS-IFREMER), BP 5, 17137 l'Houmeau, France*
[c] *Laboratoire de Biologie et Environnement Marins (LBEM), EA 3168, Université de La Rochelle, Institut du Littoral, Avenue Michel Crépeau, 17042 La Rochelle Cedex, France*

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Abstract

The application of trichloroacetic acid (TCA) as a shell extractant for preparation of soft body parts with reference to tissue metal concentrations (Cd, Cu, Pb, Zn) in shellfish has been evaluated on the example of the mud snail *Hydrobia ulvae*, a small marine prosobranch densely present in rocky and soft-bottom habitats of the eastern Atlantic. A solution of 0.1 M TCA was tested on individuals treated according to two different protocols: (1) thawed after freezing ("non-dried") and (2) thawed and air-dried to a constant weight ("dried"). Two points were investigated in detail to improve the method: individual soft tissue dry weight and tissue metal concentration following a standard digestion method. In both instances, the results were compared with those from manually dissected snails. Conditions for total shell decalcification of 60 individuals (3–4 mm long) were 5.5 h in 20 ml of 0.1 M TCA.

No differences in individual soft tissue weight were observed between the treatments, indicating good efficiency of the TCA extraction with respect to weight of soft body parts. In contrast, tissue metal concentrations varied among treatments. The TCA extraction of the dried animals had a good recovery for Cd, most likely due to the lower solubility of Cd vital cellular components (proteins and mineral concretions) from the dried tissue. Satisfactory recoveries of the tissue concentrations of Cu and Pb were obtained for the non-dried individuals. This might be related to the specific distribution of metals in the organism (namely in the digestive glands and gonads) and their different chemical reactivity with TCA after the tissue was dried. Limited susceptibility of Zn-bearing protein bindings to complexing with TCA also accounts for significantly lower concentrations of Zn in the mud snail's soft tissue that was extracted. The 0.1 M TCA solution is therefore recommended for extraction of the shells of *Hydrobia ulvae* for tissue determination of Cd, Cu and Pb; however the treatment protocol does affect metal recovery and thus a consistent procedure should be followed.

The extracted metals from the soft tissues and shells of the mud snails (on the basis of both metal concentrations and contents) were ranked in order of increasing contribution of soft body parts to the total (shell + tissue): Pb < Cd < Zn < Cu.

Keywords: Trichloroacetic acid; Extraction; Soft tissue; *Hydrobia ulvae*; Metal determination

1. Introduction

In marine and estuarine molluscs metals taken up from the external environment (with food and/or from solution) preferentially accumulate in the soft parts of the body (including the foot, gills, mantle and internal organs) rather than the shell. The shell has rarely been selected as the target of chemical analysis (Koide et al., 1982) because it contains very low-metal concentrations as compared to the tissues, and the mechanisms of metal incorporation are still not fully understood (Thomas and Bendell-Young, 1998). Metal concentrations in soft tissue usually exceed those in the shell, offering a reasonable option for measuring and studying metal bioaccumulation processes in different shellfish species. Since the net accumulated metal concentration in soft

* Corresponding author. Tel.: +48-58-6601640; fax: +48-58-6202165.
E-mail address: oceas@univ.gda.pl (A. Sokolowski).

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tissue is a result of complex biological processes (and thus depends heavily on the accumulation strategy of the studied organism and the chemical speciation of the metal in the environment) analysis of metal concentrations contained in soft tissues offers a way in which to assess the potential for an organism to accumulate metals.

The soft tissues of marine shellfish, in contrast to the shell, have inherently highly variable metal concentrations due to factors such as season, size, sex and weight (Bryan, 1984; Rainbow, 1996; among others). Furthermore, metal concentrations in the soft tissues may be substantially higher than the levels in the environment and tend to be indicative of different metal forms, providing a direct and time-integrated measure of the bioavailability of the metals in aquatic ecosystems (Phillips, 1980). This observation is the basis of biomonitoring programs that use shellfish species to assess the biological availability of metals in the environment.

In most ecotoxicological and monitoring studies using shellfish organisms, the chemical detection of contaminant metals is performed on whole soft tissue homogenates (de Kock and Kramer, 1994) and many standard laboratory protocols for the preparation and measurement of metal concentrations in tissue samples exist. These techniques include: manual dissection of fresh (or freshly thawed) or previously lyophilised individuals, using non-metallic instruments, followed by homogenisation and acid-digestion of shucked tissue. Removing the shell manually is feasible when the organism is large enough to be easily handled and sufficient tissue remains for analyses on the dissected individual tissue samples. For smaller animals, several individuals are dissected, then pooled (before homogenisation and digestion) and thus the manipulations become increasingly time-consuming (not only must more individuals be dissected, but the smaller animals require more skilled handling by laboratory staff). These practicalities may, in part, account for the relatively scarcity of studies on metal concentrations in the tissue of small marine and coastal shellfish organisms and the available data generally refers to a total body burden.

The objective of this study was to develop a chemical method for decalcifying small estuarine shellfish species with trichloroacetic acid (TCA) and to evaluate its usefulness for the analysis of soft tissue metals (Cd, Cu, Pb and Zn). Based on metal concentrations (and contents) in the soft tissues and shells (indirectly calculated from remaining TCA solutions), metal partitioning into soft tissues versus the external skeleton has also been calculated. The common prosobranch mud snail, *Hydrobia ulvae*, was chosen as the test organism; this organism occurs widely on the Atlantic coast of Europe, in particular on muddy substrates, where its density often exceeds 10 000 ind. m^{-2} and 10 gof dry weight m^{-2} (Sauriau, 1987).

2. Materials and methods

Surface sediments (1–2 mm deep) containing the mud snail, *Hydrobia ulvae*, were sampled from intertidal mudflats located in Aiguillon Bay, on the Atlantic coast of France (46°15′ N, 1°10′ E) in March 2001. The uppermost layer of mud was gently scraped, then stored cool in plastic boxes and transported immediately to the laboratory for further processing. The animals were separated from the sediment by sieving through a 1mm mesh. To avoid size effects on the results, only animals with shell lengths between 3 and 4 mm were used. The snails were then depurated for 24 h in GF/F filtered natural seawater in a temperature-controlled room under conditions similar to the natural environmental situation ($T = 14.9$ °C, $S = 25$), prior to being frozen at 20 °C.

Two different criteria were evaluated for testing the suitability of TCA as an extractant: (i) individual soft tissue dry weight and (ii) tissue concentrations of Cd, Cu, Pb and Zn per unit of dry weight following a standard digestion method. The values that were obtained after manual dissection of all soft parts were used as a reference for both criteria.

Two sets of three replicate pools (60 individuals per pool) were thawed as needed for the experiments. One set was dried at 55 °C to a constant weight and weighed to obtain the individual total dry weight (shell + soft tissue). The soft tissue of the snails from the second set was removed manually from the shells by using a disposable plastic knife under a dissecting microscope. Attention was paid to dissect full digestive glands and gonads, which are known to serve as storage sites for metals in some mollusc species (Bryan, 1984) and are located in a posterior-most part of a shell. Dissected tissue was dried at 55 °C and weighed to determine the average individual body dry weight. Next, the whole individuals (first set) and an aliquot of soft tissues (from the second set) were digested in a mixture of concentrated nitric/perchloric acid (14N/22N Suprapur Merck®) at 80 °C for 24 h in covered beakers. Samples were then uncovered and dried to evaporation in an acid digestion fumehood. The dry residues were re-dissolved into 0.3 N nitric acid and stored at 4 °C until the samples were analysed.

For the TCA extraction method, two treatments were tested: (1) 0.1 M TCA (made from TCA Crystalline Certified ACS grade) extraction of "non-dried" animals (used just after thawing) and (2) 0.1 M TCA extraction of "dried" animals (thawed and dried to a constant weight). Three replicate pools of 60 individuals each of the non-dried and dried snails were decalcified in three different volumes of 0.1 M TCA (5, 10 and 20 ml) at 20 °C. The efficiency of the extraction was checked every 15 min under a dissecting microscope and was regarded as complete when the calcareous parts of shells were no

longer visible; an extraction period of 5.5 h in 20 ml of 0.1 M TCA was sufficient to achieve total shell decalcification for both non-dried and dried snails.

After extraction was complete, the tissues were strained onto acid-washed polypropylene net of a 63 μm pore diameter and the remaining solution of 0.1 M TCA was collected into a glass beaker and then evaporated to dryness at 60 °C. The tissues were rinsed with Milli-Q water, allowed to dry at 55 °C to a constant weight and then weighed. Aliquots of tissue and the residues of the evaporated TCA extraction solutions were digested using concentrated acids, following the same procedure as described above.

The concentrations of Cd, Cu, Pb and Zn were measured in the solutions using a graphite furnace AAS (Varian SpectraAA 250 instrument in background correction mode and with a NiPd modifier added). Appropriate standard reference material (muscle of dogfish DOLT-2 from National Research Council of Canada NRC, Institute for National Measurements Standards NMS) was systematically analysed according to the same procedure as for samples to control the quality of the results. The agreement between the analytical results for the reference material and their certified values was satisfactory: the recovery (total certified concentration versus total metal concentration obtained in this study) ranged from 72.6% to 113.2% and the average precision was <7.2%. To check for contamination, process control blanks of TCA and the digestion mixture of concentrated nitric/perchloric acid were also analysed using the same procedure every 10 samples.

Metal content, or the total amount of metal per individual, was computed for each treatment to compare metal body burdens. For manual dissections, calculations of the metal content were based on direct metal concentration measurements in the whole animals (direct method). For the TCA extractions, metal concentrations determined in soft tissue and in the remaining TCA solution were summed (indirect method). Finally, in order to determine metal partitioning between the soft parts and the shell in *Hydrobia ulvae*, the contribution of the soft tissue and the shell was calculated in terms of metal concentrations and contents along with the ratio of metal concentration and content in the shell to the respective values in the soft tissues (fractioning ratio, FR).

3. Results

3.1. Dissecting methods

Individual total dry weight (shell + soft tissue) of the snails did not differ among the various treatments (*t*-test, $p < 0.005$). Although mean soft tissue dry weight of the dried decalcified snails was slightly lower then those dissected manually and the non-dried decalcified snails, no significant differences were found (Table 1). The contributions of tissue dry weight to the total were around 12% among the three treatments (Table 1).

The concentrations of metals in the soft tissue and TCA solutions varied markedly among the elements and the treatment methods (Table 2). The overall trend is an inverse relationship between metal concentrations in the soft tissue as compared to the metal concentrations in the TCA solutions.

The tissue of the non-dried decalcified animals contained significantly less Cd than tissue from animals that were manually dissected or the dried snails that underwent TCA decalcification (*t*-test, $p < 0.005$). Consequently, the TCA solution remaining after extraction of the non-dried snails had a higher level of Cd than the solution remaining after extraction of the dried snails (Table 2).

The soft tissue concentrations of Cu and Pb in animals that were dissected manually and in the non-dried animals extracted with TCA were similar (*t*-test, $p < 0.005$) and significantly different from the tissues of the dried snails extracted with TCA. The concentration of Cu in the latter was lower and that of Pb higher than in the two remaining analytical groups. As a result, a lower concentration of Cu and a higher concentration of Pb were measured in the TCA solutions after extraction of the non-dried snails as compared to the respective TCA solutions after extraction of dried animals.

Zinc was the only element whose soft tissue concentration differed significantly between the manually dissected individuals and the TCA extracted individuals (*t*-

Table 1
Comparison of total individual dry weights (shell + soft tissue) and soft tissue individual dry weights of the mud snail *Hydrobia ulvae* treated by different shell removal techniques

Shell removal technique	Number of individuals per pool	Number of pools	Individual total dry weight (mg)	Individual soft tissue dry weight (mg)	Contribution of tissue dry weight to the total (%)
Manual dissection	60	3	4.02 ± 0.53	0.48 ± 0.07	11.9
0.1 M TCA, dried snails	60	3	3.98 ± 0.48	0.47 ± 0.01	11.8
0.1 M TCA, non-dried snails	60	3	4.12 ± 0.41	0.50 ± 0.07	12.1

Results are presented as mean ± standard deviation. Values not significantly different from each other (*t*-test, $p < 0.05$) are grouped by the dashed line.

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Table 2
Comparison of metal concentration in soft tissue ($\mu g\,g^{-1}$ dry weight), the remaining TCA solutions ($\mu g\,dm^{-3}$) and metal content ($ng\,ind^{-1}$) in *Hydrobia ulvae* dissected manually and extracted with 0.1 M TCA

Metal		Shell removal technique		
		0.1 M TCA, dried snails	Manual dissection	0.1 M TCA, non-dried snails
Cd	Soft tissue concentration	0.110 ± 0.018	0.119 ± 0.014	0.021 ± 0.003
	Concentration in TCA	0.658 ± 0.018	–	2.357 ± 0.047
	Content	0.213 ± 0.008[a]	0.221 ± 0.006[b]	0.204 ± 0.013[a]
Cu	Soft tissue concentration	103.8 ± 10.2	135.2 ± 18.9	137.9 ± 21.2
	Concentration in TCA	97.2 ± 13.0	–	52.8 ± 7.6
	Content	125.8 ± 8.2	74.9 ± 27.3	127.8 ± 7.3
Pb	Soft tissue concentration	41.1 ± 0.8	10.5 ± 2.2	11.7 ± 3.0
	Concentration in TCA	61.4 ± 22.6	–	73.1 ± 11.2
	Content	62.7 ± 12.0	63.0 ± 0.0	60.2 ± 3.8
Zn	Soft tissue concentration	24.4 ± 4.6	61.3 ± 9.2	8.2 ± 1.4
	Concentration in TCA	337.1 ± 0.0	–	829.0 ± 108.9
	Content	72.6 ± 2.4	75.8 ± 1.7	71.2 ± 14.8

Results are presented as mean ± standard deviation ($n = 3$). Values not significantly different from each other (t-test, $p < 0.05$) are grouped by a common dashed line.
[a] The sum of metal concentration in soft tissue and in the TCA solution was re-counted for one individual (indirect method).
[b] Organisms were analysed as a whole (direct method).

test, $p < 0.005$). The highest concentration was measured in the manually dissected samples and the lowest Zn concentration after the TCA decalcification of the non-dried snails. Lower concentrations of Zn were measured in the TCA solutions from the dried snails (Table 2) than from the non-dried snails' TCA solutions.

3.2. Metal partitioning

Metal contents calculated by direct (manual dissection) or indirect means (TCA extractions) did not, in general, differ among the different sample preparations (Table 2). The exception was Cu, the content of which was significantly higher after the two TCA extractions (t-test, $p < 0.05$). These results indicated that during the two-step chemical extraction used here, no losses or contamination of Cd, Pb and Zn occurred. The differences in the content of Cu presumably resulted from the contamination of the TCA solutions used for the extraction: a hypothesis that was confirmed by the analyses of the blanks (not shown here). However, it is worthwhile to note that the contamination of the extractant did not affect the concentrations of Cu measured in the soft tissue. The high-standard deviation in the total Cu content of snails (34.5%, Table 2) dissected manually may be due to a high-natural variability in Cu concentration of the snails or size differences. The lower Cu content in the manually dissected samples suggests that, the Cu contamination present in the TCA also contaminated the tissues during the shell extraction procedure. Thus less Cu would be expected in tissue samples that were dissected manually. When Cu is calculated as a concentration (per gram of dry tissue) the average values are not significantly different. This is not conclusive evidence that the contaminated extractant solution affected the Cu concentration as measured in the soft tissues.

Metal partitioning in the soft tissues (versus the shell) varied markedly between the different metals analysed (Table 3). The metal content in the soft body parts relative to the total was highest for Cu (86.7%), intermediate for Zn and Cd (38.8% and 25.8%, respectively) and lowest for Pb (8.0%); the FR values were 0.2, 1.6, 2.9 and 11.5, respectively. A similar order (in terms of percentages) was apparent on the basis of metal concentrations, with the relative contributions of soft tissue (and the FR values) being 72.3% (0.4), 14.3% (6.0) and 13.8% (6.2) for Cu, Cd and Pb, respectively.

4. Discussion

4.1. Comparison of extraction methods

Marine and estuarine shellfish species have long been known to be an essential component of aquatic ecosystems in terms of energy flow due to their high abundance and pervasive distribution (Rainbow, 1995). These organisms generally represent first-order consumers, feeding on primary producers, and being themselves consumed by higher trophic levels, they are a potentially important link for metal transfer between different food web compartments and trophic levels. Most ecotoxicological studies are biased towards investigations of macroinvertebrates because of their commercial value and ease of laboratory manipulation. Studies are scarce of metal accumulation in the soft tissues of smaller shellfish (including species of bivalves, gastropods, barnacles and brachiopods); one reason for this is because smaller organisms are more difficult to manipulate during manual dissection. Therefore a chemical extraction method that quickly and efficiently separates tissue from a calcareous skeleton could be of widespread use for studies of small mollusc species.

In this study 0.1 M TCA was used to dissolve the shell from a mud snail *Hydrobia ulvae* prior to digesting the soft tissues for determination of Cd, Cu, Pb and Zn. TCA solutions have not been evaluated for analyses of tissue metal concentrations, but they have been employed successfully to decalcify bones of mammals for the detection of nucleic acids (Shibata et al., 2000; Yamamoto-Fukuda et al., 2000), and to decalcify the shells

Table 3
Metal contents expressed in ng (per compartment) in soft tissue and shell, their contributions to the whole body content (%) and the ratio of metal content and concentration in the shell to those in the tissue (FR) in the mud snail *Hydrobia ulvae*

Compartment	Cd		Cu		Pb		Zn	
	(ng)	%	(ng)	%	(ng)	%	(ng)	%
Metal content								
Soft tissue	0.057	25.8	64.9	86.7	5.04	8.0	29.4	38.8
Shell[a]	0.164	74.2	10.0	13.3	57.96	92.0	46.4	61.2
FR								
Content	2.9		0.2		11.5		1.6	
Concentration	6.0		0.4		6.2		–	

Metal contents refer to the values obtained after manual dissection, metal concentrations correspond to the results of the 0.1 M TCA extractions that gave a satisfactory recovery (in the case of Zn FR was not calculated).
[a] Calculated as a difference between total metal content and metal content in soft tissue.

of some bivalves in order to estimate their organic content (Goulletquer and Wolowicz, 1989).

Data from the present study showed no differences between the efficiency of tissue chemically extracted (relative to individual soft tissue dry weight) and tissue dissected manually from the shell. The TCA extractions of both dried and non-dried snails confirmed the utility of TCA solutions for decalcification of marine shellfish as reported in an earlier study (Goulletquer and Wolowicz, 1989). In contrast, there were considerable differences between metal concentrations in the soft tissue. Since the recoveries per dry weight of soft tissues extracted with TCA did not differ among treatments, the reasons for the discrepancy in tissue metal concentrations are attributed to differences between the: affinities of TCA for the suite of metals studied, and/or the relative solubilities of the stored forms of the respective metals within the organism.

4.1.1. Cadmium

The concentration of Cd in the soft tissue of *Hydrobia ulvae* snails that were manually dissected was similar to the concentration after TCA extraction of the dried animals, and significantly higher than that obtained after TCA extraction of the non-dried animals. Relatively higher concentrations of Cd were recorded in the remaining TCA solution of the non-dried animals. The difference in the efficiency between the dried and non-dried snails might be linked to the selective solubility of the stored Cd compounds in the organism. Cadmium, as a non-essential element, has been shown to be preferentially detoxified by complexation with metallothioneins in the digestive glands and kidneys of marine and estuarine gastropods (Noël-Lambot et al., 1980). Once inside a cell, Cd is bound to low-molecular-weight sulphur-rich proteins with low solubilities and these compounds remain associated with the internal organs (Rainbow, 1996). Accumulated Cd may also be detoxified into precipitated mineral concretions in the apical vacuoles of kidney cells (Bryan, 1984). According to Bernhard and George (1986), Cd in marine invertebrates can also be present in particulate subcellular structures (lysosomes) containing up to 50% of the total metal content of an individual. In the present study, the destruction of cell membranes caused by freezing, could have released sequestered Cd-bearing compounds into the intracellular space. As a result, these compounds may have been present in the solution and been subject to selective extraction by the 0.1 M TCA: the process being more enhanced in the non-dried tissues.

4.1.2. Copper and lead

Cu and Pb had similar concentrations in the TCA extracted tissue of the non-dried snails and those dissected manually. This indicates that 0.1 M TCA can be used to decalcify mud snails for determination of tissue Cu and Pb in animals that have not been air-dried beforehand. The level of Cu in the soft tissue of the dried and TCA extracted snails was significantly lower than in the snails dissected manually and the non-dried TCA extracted snails; the opposite was true for Pb.

The generally good recovery of tissue Cu concentrations in mud snails extracted with TCA (for both treatment methods) can be attributed to the chemical reactivity of Cu and its compartmentalization within the organism. Copper is an essential element and some of the accumulated metal pool leave available (soluble) for enzymatic requirements of an organism (White and Rainbow, 1985). Soluble Cu in the subcellular space ranges between 10% and 40% in most aquatic invertebrates (Bordin et al., 1992); the balance is stored in different chemically bound (and thus detoxified) forms such as in organic complexes with metallothioneins or precipitated in calcareous granules. The metabolically available fractions of Cu (remaining in an easy accessible form or in intercellular body liquids to fulfil physiological needs) were presumably extracted with 0.1 M TCA from tissues of the non-dried *Hydrobia ulvae* resulting in a satisfactory extraction efficiency by this technique. Furthermore, denaturation of some Cu-stored protein structures could also contribute to the measured extracted metal concentrations (particularly in the non-dried tissue), as TCA solutions were found to precipitate peptides through reaction with hydrolytically unstable carboxyl groups (Ivanov et al., 2000).

Lead, like Cd, may cause potentially toxic effects in marine invertebrates. Therefore, accumulated Pb is primarily stored bound to high-molecular-weight ligands and unavailable for significant back release into the metabolically available fraction. Unlike Cd, however, the Pb concentration in soft tissues after TCA extraction of the dried individuals was substantially higher than those obtained after both manual dissection and TCA extraction of non-dried animals. The high-specific affinity of Pb for sulphur groups may provide an explanation for our observations. In many aquatic invertebrates, accumulation of Pb is due to the ability of the metal to form mercaptides (primarily in digestive glands; Abdallah and Moustafa, 2002) and the –SH group of amino acid side chains. These compounds are relatively easily dissolved by weak hydrochloric and trichloroacetic acids (Crenshaw, 1972). However, if mercaptides were dissolved in the extraction, it should have occurred during TCA decalcification of the snails treated by both methods tested here, i.e. dried and dried, and not solely in the latter. It is therefore hypothesized that drying the tissue prior to extraction must have altered the chemical resistance of the Pb-bearing compounds in the tissues, resulting in their increased solubility in TCA. The physico-chemical mechanism responsible for differential recovery of soft tissue Pb concentrations after the different TCA treatments re-

mains unclear; further investigations with special focus on the chemical nature of Pb store compounds in soft tissues of marine shellfish species and the reactivity of these bindings with ionic TCA are required.

4.1.3. Zinc

Zinc was the only metal whose concentration in the soft tissue of manually dissected *Hydrobia ulvae* was significantly higher than with the TCA treated snails (both the dried and dried). The solution of 0.1 M TCA did not thus appear to be a suitable shell extractant of marine and estuarine gastropod shellfish for tissue Zn determination. This might be related to the specific subcellular distribution of Zn in the soft tissue of aquatic invertebrates and the selective reactivity of TCA on proteins. Many marine molluscs are regarded to actively (partial) regulate the level of Zn within a physiologically required range (Rainbow, 1996). A relatively large portion of Zn in the internal organs (primarily the digestive glands and kidney) exists in a cytoplasmatic fraction, bound with enzymes and in carbonate-based granules that act as temporary reusable and easily transformable metabolic reserves for these animals (Simkiss, 1980; Mason and Nott, 1981). These fractions represent soluble forms of Zn with easy physiological access from sensitive vital cellular components, available to play required roles in metabolism of an organism. The proportion of cytosol Zn has been determined in a number of marine molluscs: *Littorina littorea* (46%; Langston and Zhou, 1986); *Ostrea edulis* (40%; Coombs, 1974); *Patella vulgata* (33–45%; Coombs, 1974) and *Mercenaria mercenaria* (27%; Carmichael et al., 1980). In the Baltic clam *Macoma balthica*, Johansson et al. (1986) determined a distribution of up to 70% Zn in the cytosol, and Sharma (1983) has measured a percentage as high as 70% in *Ostrea lutaria*. Similar partitioning of soluble fractions of Zn (mainly in a protein-bound form) can also be expected in the mud snail *Hydrobia ulvae* and thus a large part of the metal in the soft tissue of this gastropod might have been dissolved by TCA. This likely accounted for lower Zn concentrations in the TCA treated tissue. The previously mentioned effect of TCA on proteins has also been described and used to separate soluble and insoluble (membrane) proteins (Stancic, 1997).

4.2. Tissue versus shell partitioning

A question that often arises in studies that use shellfish species as biomonitors of metal levels in the marine environment is whether or not the shell should be analysed. For most marine invertebrates, metals preferentially accumulate in the soft parts of the organism (Szefer and Szefer, 1990; Thomas and Bendell-Young, 1998). There are some exceptions where higher metal concentrations have been found in bivalve shells. For example, Babukutty and Chacko (1992) observed elevated levels of Pb and Mn in the shells of the estuarine bivalve *Villotoria cyprinoids* var. *cochinensis*.

Shell partitioning has also been observed in the present study, with FR values > 1 for the concentrations and the contents of Cd, Pb and Zn, and FR < 1 recorded for Cu (Table 3). On the basis of the FR value (for both metal concentration and content) the metals can be ranked into the following order of increasing contribution to the soft tissues: Pb < Cd < Zn < Cu. The shell appeared to be the main repository for Cd, Pb and Zn in the mud snail *Hydrobia ulvae* suggesting that shell analyses could provide a good resource for long-term monitoring of metal pollution in nearshore aquatic environments. The use of shell material has also been suggested by Koide et al. (1982), who emphasised some of the benefits of using bivalve shells in place of tissues: less variability due to factors such as season, age, sex, negligible depuration rate and ease of laboratory handling and storage. However, tissue-bound metal concentrations would be a more useful indicator of metal assimilation in an organism during short-term experiments where the organism is exposed to elevated metal concentrations.

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