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Lake Maggiore population of *Unio pictorum mancus* (Mollusca, Bivalvia)

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ABSTRACT

*The study focused on using a fresh-water mussel (*Unio pictorum mancus*) as a bioindicator of various pollutants, and particularly metals. The elements considered were: Al, As, Ca, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, V and Zn. This research was carried out at a site where various other studies have been conducted on important characteristics of the same population of* Unio. *This site is a small bay called "Sabbie d'Oro" located on the south-east coast of Lake Maggiore. Our study involved quantifying the capacity of this mollusc to concentrate large amounts of metal in its body without evident consequences by using detoxification mechanisms. We analysed not only element concentrations, but also their variability (expressed as CV%) in the soft tissues and in the shell, for two main reasons: i) the sampling design should include a preliminary analysis to determine how many specimens is necessary collect to ensure a specified level of precision; ii) the sample variability value may be combined with the value of analytical precision (BCR) to obtain an estimate of the "experimental" precision. In soft tissue, Ca accumulation tended to increase with size, and Al accumulation decreased with size. In shell, Ca and Ni were stored more than in soft tissue, but not proportionally to size. V, Cr and Mo tended to accumulate in the shell progressively over the years, whereas Co and Al are "diluted" during growth, or are absorbed mainly during the juvenile stage. Partition between shell and soft tissue resulted roughly the same in the juvenile and adult stages for the following elements: Fe, Al, Co, As, Pb. The relationships among the various elements were schematized in a hierarchical tree plot.*

Key words: freshwater mussel, metals, shell size, bioindicators

1. INTRODUCTION

Pollutants in freshwater can originate from a variety of sources, including municipalities, agriculture, construction, industry and atmospheric depositions. Once in the water column, trace element inputs are removed *via* sediment deposition, complexation by dissolved organic carbon (DOC) (Swift 1989) or uptake and accumulation in aquatic organisms (Adriano 2001). Aquatic organisms can be grouped into two categories based on their uptake of pollutants: excluders (low uptake) and accumulators (extreme uptake). Accumulators, such as bivalve molluscs, possess a detoxification system based on sequestration and tend to show elevated concentrations of metals even in less contaminated environments (Mason & Jenkins 1995). Mussels accumulate many metals from the water they filter, without discriminating between essential and non-essential elements. In fact, they are exceptionally resistant to toxicity as a result of a variety of detoxification mechanisms, such as the production of metal-binding thyoneins or calcium phosphate granules (Ravera *et al*. 2003a). For this and many other reasons (e.g., abundance in many terrestrial and aquatic ecosystems, easy availability for collection, sedentary way of life and ability of several species to survive in both clean and polluted waters), unionidae have long been regarded as useful bioindicators (Boyden 1977; Lau *et al*. 1998; Gundacker 2000; Ravera *et al*. 2007a). Accumulation follows different pathways in the shell and in the soft tissues of the mussel producing different chemical compositions, with different turnover times (much shorter in the soft tissues). As a consequence, the chemical composition of the soft tissues may be considered an indicator of the most recent environmental conditions, whereas the shell composition represents the average conditions from the birth of the mussel up to the moment the sample was collected (Ravera *et al*. 2007b). Because the ring number corresponds approximately to the age of the mussel (Grefsurd *et al*. 2006), shell analysis can be used to obtain valuable information on water characteristics (pollutants, trophic level and physical conditions such as temperature and pH) (Ravera *et al*. 2007b) for a well-defined past period, which is impossible or very difficult to achieve by water sampling (in terms of time and money). In a previous study, Metcalfe-Smith *et al*. (1996) highlighted the fact that seasonal cycles, the physical environment and the biological state of the organisms strongly influence data variability when this monitoring method is used. This research deals with a species of mussel (*Unio pictorum mancus*) which is particularly suitable as an indicator of trace metal abundance thanks to its capacity to accumulate trace pollutants from the surrounding environment, its colonization of aquatic environments ranging from oligotrophic to

hypertrophic, and its availability in all seasons. The specimens were collected from a site on the oligo-mesotrophic Lake Maggiore. Soon after collection, the mussels were divided into various size-classes. Zn, Fe, Mn, Cu, Al, V, Cr, Co, Ni, As, Mo, Cd and Pb, which are essential or non-essential for mussels in relation to their concentration (Nielsen 2000; Ravera *et al*. 2003a), were measured in the soft tissues and shells along with Ca, which plays an important role in detoxifying soft parts (Markich *et al*. 2001) and is very abundant in the shell. Following a hypothesis described in previous works (Beone & Ravera 2003; Ravera *et al*. 2003a, 2003b, 2005, 2007a, 2007b), the objective of this study was to determine the advantages of using *Unio pictorum* as biomonitor for heavy metal pollution in freshwater ecosystems.

2. MATERIALS AND METHODS

2.1. Sampling site

The mussel were all collected from one only station, located on the south-eastern side of the deep oligo-mesotrophic Lake Maggiore (Tab. 1), in a location called Sabbie d'Oro, which is a gently sloping bay with silty-muddy sediments and very active water renewal attributable to wave action (latitude N 45°50'19", longitude E 8°37'17"; Fig. 1).

Tab. 1. Morphometric parameters of Lake Maggiore (Ambrosetti *et al*. 1992).

Altitude (m a.s.l.)	194
Watershed area (km^2)	6599
Lake area (km^2)	212.5
Mean depth (m)	177
Maximum depth (m)	370

2.2. Mussel sampling and preparation

We selected Painter's mussel, *Unio pictorum* subspecies *mancus* (Bivalve, Unionidae), and tested its usefulness as an indicator of trace elements in the environment (Beone & Ravera 2003; Ravera *et al*. 2003a, b, 2005, 2007a, b). In July 2002, we randomly collected 37 adult stage mussels by hand from the littoral zone of Lake Maggiore, and preserved them in plastic bags in an ice-box for transportation to the laboratory. Even if a single sampling period, combined with one only sampling station, cannot be considered representative of the ecological situation in the littoral zone of the entire lake, this choice represents an attempt to remove any influence of trophic level, population density, seasonal variation of biomass and bioaccumulation (Ravera *et al*. 2007a, b), because our objective was to focus differences between various sizes and age groups in a single population of one sub-species.

The mussels were collected in summer because in this period the growth is at its maximum after the growth interruption that occurs in winter (Negus 1966). We assumed that the number of the growth rings in the shell corresponds to the individual age expressed in years.

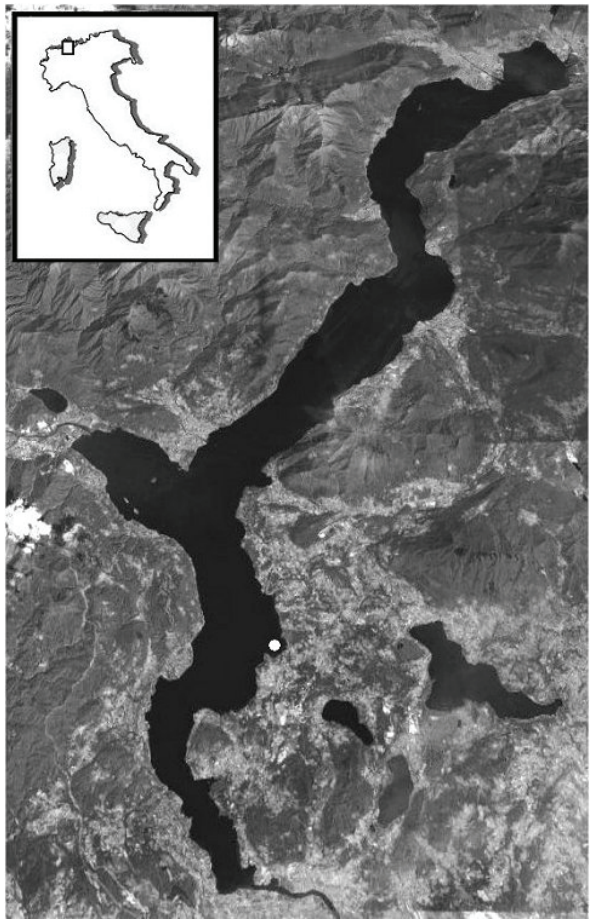

Fig. 1. Sampling station on Lake Maggiore (white spot).

Age was determined by counting the annual rings in the shell, although this becomes more difficult for adults older than 3 years, like the specimens in our study, and can result in a margin of error of approximately 1-2 years because the more recent rings are concentrated in a small space on the shell (Ravera & Sprocati 1997). Determining age using the annual rings in the ligament raises similar objections. A more reliable method involves examining thin sections (Kilada *et al*. 2007), but it can only be applied to a small number of specimens, because a specialized technician is required. Once in the lab, shell length, width and thickness were measured using a calliper, while ring number was obtained from the most frequent of the estimates realized independently by three scientists. An additional class of specimens (9), corresponding to the juvenile stage (characterized by the absence of rings and the presence of a protoconch), was collected in September 2003, despite the difficulties resulting from their small size and burrowing way of life (Patzner & Müller 2001). The month of September was chosen because it was suitable for collecting samples at the juvenile stage. *Unio* has a

Tab. 2. Means of metal concentrations in shell and mussel of the 43 samples under investigation and in BCR (Community Bureau of Reference) mussel tissue CRM 278. Values found in certified sample are means of 11 replicates (different amounts analysed repeatedly) ± SD for Zn, Fe, Mn, Ca, Cu and Al, and of 19 replicates (different amounts analysed repeatedly) ± SD for V, Cr, Co, Ni, As, Mo, Cd and Pb. Values in brackets are reported by BCR and are indicative only.

Element	Found ($\mu g\ g^{-1}$)	Certified ($\mu g\ g^{-1}$)	Shell ($\mu g\ g^{-1}$)	Mussel ($\mu g\ g^{-1}$)
Ca	981 ± 50	1000	368000	53900
Cu	9.5 ± 0.4	9.6 ± 0.2	5.1	34
Fe	128 ± 6	133 ± 4	57	1600
Mn	6.9 ± 0.6	7.3 ± 0.2	210	4880
Zn	73 ± 2	76 ± 2	n.d.	631
Al	39 ± 7		40	237
V	0.54 ± 0.07		0.04	0.60
Cr	0.94 ± 0.09	0.80 ± 0.08	0.87	4.27
Co	0.34 ± 0.03	(0.34)	0.49	0.80
Ni	1.1 ± 0.1	(1.0)	10.7	6.1
As	5.7 ± 0.2	5.9 ± 0.2	0.22	8.2
Mo	0.37 ± 0.05		0.02	0.66
Cd	0.32 ± 0.03	0.35 ± 0.07	0.04	2.09
Pb	1.89 ± 0.18	1.91 ± 0.04	5.92	0.30

short breeding season, from April to mid-August, and the glochidia (larval stage) are released from a marsupium as soon as they are mature. Glochidia are generally species-specific and attach to fish for two to five weeks (depending on temperature), forming a cyst on the external surface of the fish. After completing their development in this parasitic stage, they break free from the host, and drop to the bottom of the lake to begin an independent life (Bauer & Wächtler 2001).

We obtained 9 shells with no rings and 4, 10, 14 and 7 shells characterized by 3, 4, 5 and 6 rings respectively. Individuals with 7 rings were two only and were consequently excluded. Shells with 3 or more rings were measured and afterwards divided into three length classes with the same width: 51-58 mm, 58.5-65.5 mm and 66-73 mm, each one containing 9 individuals at least. To avoid any loss of trace elements in the samples, the mussels were not permitted to discharge undigested materials in clean water, and the soft tissues were separated from the shells. The soft tissues of each specimen were weighed and frozen at -20 °C until analysis. Before analysis, the soft tissues of the mussels were freeze-dried, kept at 40 °C for 24 hours and then pulverised using a Planetary Micro Mill with agate bowl and balls. The shells were broken up before pulverisation. The powders were passed through a 0.2 mm sieve and mineralised in a CEM MDS-2000 a Microwave Digestion System (Mattews, NC, USA) using HNO_3 (65% w/v) and H_2O_2 (30% w/v) at 180 °C.

2.3. Sample analysis and instrumentation, precision and trueness

The nitric acid (65% w/v) was purified using a subboiling system (Milestone mod. SubPURE) and hydrogen peroxide (30% w/v) was Suprapur reagent (Merck, Darmstadt, FRG). High purity water was produced using a Milli-QTM deionizing system (Millipore, Bedford, MA, USA). The solution obtained from acid digestion was filtered before analysis. Zn, Fe, Mn, Ca, Cu and Al were measured *via* Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES Perkin Elmer mod. Optima 2100 DV), while V, Cr, Co, Ni, As, Mo, Cd and Pb (which are commonly present at trace level concentrations) were measured *via* Inductively Coupled Plasma-Mass Spectrometry (ICP-MS Agilent 7500 ce). Calibration for each ICP was obtained using external standards. Standard solutions of each element investigated were prepared by diluting a 100 mg L^{-1} multielement solution obtained from CPI International with the same amount of acid used to dissolve the samples. Glassware was cleaned by soaking with the contact overnight in a 10% (w/v) nitric acid solution and then rinsed with deionized water. Analytical precision and trueness were checked by using Certified Reference Material of *Mytilus edulis* (mussel tissue BCR 278) and conducting repeated analyses of different amounts of it (Tab. 2). The recovery rate for the certified elements always ranged between 80 and 120%. Quality control gave good precision (SD <10%) in almost all samples.

2.4. Statistics

We adopted the coefficient of variation (CV%) to test the significance of the variability of elements concentrated in the shell and soft tissues. This approach allowed to compare datasets with different units or widely different means. Paired comparisons of variability between age or size groups were realized by Wicoxon signed rank test, by examining the ranking of elements by each age or size group. Level of statistical significance was set at 0.05.

Statistical analysis of the results was performed using SAS System (S.A.S. Institute 1996). A hierarchi-

Tab. 3. Coefficient of variation (% CV) calculated from mean and standard deviation of 9 measurements at least, for each element quantified in BCR (Community Bureau of Reference) mussel tissue CRM 278 and mussel shell and tissue samples. *Unio pictorum mancus* samples were divided into size classes (juvenile stage, 51-58 mm, 58.5-65.5 mm and 66-73 mm). * = values not detectable.

	CRM 278	juvenile stage		51-58 mm		58.5-65.5 mm		66-73 mm	
	mussel	mussel	shell	mussel	shell	mussel	shell	mussel	shell
Ca	5.17	15.6	4.67	30.2	0.84	43.5	1.37	26.3	1.32
Cu	4.65	15.3	11.1	39.6	26.6	15.1	25.9	38.6	29.6
Fe	5.04	18.4	19.2	28.4	21.2	26.6	39.5	20.3	44.8
Mn	9.26	20.6	16.4	26.3	19.4	27.7	22.8	24.1	9.91
Zn	3.06	20.2	57.1	38.3	*	42.7	*	33.3	*
Al	21.5	22.7	103	38.5	143	60.1	56.5	43.8	90.6
V	13.0	21.8	64.5	27.8	61.9	49.0	67.7	24.7	35.4
Cr	8.35	20.6	45.2	44.6	74.2	35.1	84.0	25.6	74.7
Co	8.85	9.94	5.37	21.0	5.13	22.0	4.39	13.9	6.94
Ni	8.80	23.8	62.4	189	16.9	30.2	13.1	16.8	11.3
As	4.38	12.9	28.6	23.7	44.5	16.5	55.2	10.5	24.4
Mo	13.6	132	75.8	17.7	60.2	18.8	16.0	14.8	38.5
Cd	8.19	36.1	11.5	27.4	*	37.5	*	26.2	*
Pb	9.77	13.5	21.5	32.5	71.4	29.0	33.9	24.4	73.8

cal cluster analysis (VARCLUS procedure) was performed separately for shell and soft tissue data in order to derive groups of samples from the raw data set according to their similarity. This analysis was intended to assess the possibility of reducing the number of trace elements to be quantified when biomonitoring using *Unio pictorum*. The Euclidean distance was selected as the measure of similarity and the clustering algorithm used was the Ward method. The maximum number of clusters was not defined. The dendrograms for both shell and soft tissue were derived using the TREE procedure.

3. RESULTS

The coefficients of variation (CV%) calculated for the elements quantified in both the mussel tissue CRM 278 and in the shell and soft tissue samples from Lake Maggiore are reported in table 3, where *Unio* samples are divided by size class. For a comparison, means of metal concentrations in shell and mussel of the 43 samples under investigation are shown in table 2.

CV values appear to be the highest in the case of juvenile and adult stage (51-58 mm) shell samples. Soft tissue variability is comparable to that of the certified samples just for Ca and Co. For the elements analyzed, the range of data variation in shells was largest for Al in the juvenile stage and the 51-58 mm group, for soft tissues it was largest for Ni in the 51-58 mm group and for Mo in the juvenile stage. If we compare the soft tissue size classes by ranking the element CVs, the Wicoxon signed rank test revealed a significant difference (p <0.05, values greater than the critical value of 3.84) in the variability between all the paired size groups, except for the 51-58 and 58.5-65.5 mm samples. For all size classes, the variability resulted significantly different and much greater than in the certified sample.

In the case of shell samples, the variability between all the paired size groups resulted instead not significantly different according to the Wicoxon test (p <0.05, all values lower than the critical value of 3.84).

The results obtained by the signed rank test mean that element concentration variability in general depend on the shell length just for soft tissues, not for shells, and that sampling variability is significantly different to that of analytical method (CVs of CRM sample). It could be very important to know the concentration variability of different individual groups for two main reasons:

i) the sampling design should include a preliminary analysis to determine the number of samples necessary to ensure a specified level of precision;
ii) this value may be combined with the value of analytical precision (BCR) to obtain an estimate of the "experimental" (sampling + analysis) precision. The use of CV instead of variance plays an important role when datasets are characterized by different units or widely different means.

Table 4 shows the calculated CV% for the elements investigated in mussel tissue CRM 278 and in shell and soft tissue samples classified by the number of rings.Variability appeared to be higher for most of the elements in soft tissue samples with 4 or 5 rings and in the smallest individuals. Data variation was very large for Al in shells with no rings or 6 rings, for Cr in shells with 3 or 5 rings, for Ni in the soft tissues of individuals with 3 rings and for Mo in the soft tissues of 4-ring or very small mussels. As for size classes, a comparison between paired ring groups by Wicoxon test (p <0.05) revealed that:

i) variability for soft tissues is significantly different and much greater than in the certified sample;
ii) variability of shell is not significantly different.

The signed rank test showed that element concentration variability depend on the age just for soft tissues and just in some pairs (e.g., 4-6, 4-1, 5-1 and 5-3 ring classes), and that sampling variability is significantly different to that of analytical method (CVs of CRM sample).

Tab. 4. Coefficient of variation (CV%) calculated from mean and standard deviation for each element quantified in BCR (Community Bureau of Reference) mussel tissue CRM 278 (11 measurements), and mussel shell and tissue samples (4 measurements at least). *Unio pictorum mancus* samples were divided into classes by number of rings (<1, 3, 4, 5, 6). * = values not detectable.

Element	CRM 278	<1		3		4		5		6	
	soft tissues	soft tissue	shell	soft tissue	shell	soft tissue	shell	soft tissue	shell	soft tissue	shell
Ca	5.17	15.6	4.67	14.6	2.34	50.5	1.26	31.8	2.10	26.2	1.55
Cu	4.65	15.3	11.1	26.8	24.1	54.2	32.8	34.9	28.0	25.7	23.7
Fe	5.04	18.4	19.2	20.2	32.7	23.7	22.8	26.8	34.3	16.2	49.7
Mn	9.26	20.6	16.4	17.6	3.27	35.3	18.6	20.4	16.4	24.0	11.5
Zn	3.06	20.2	57.1	18.4	*	45.7	*	30.0	*	41.7	*
Al	21.5	22.7	103	53.1	53.0	39.4	33.4	67.1	75.4	48.7	101
V	13.0	21.8	64.5	48.4	66.1	31.4	*	47.0	82.0	39.6	49.0
Cr	8.35	20.6	45.2	42.6	110	27.8	60.4	48.6	114	39.7	58.9
Co	8.85	9.94	5.37	12.4	3.50	26.1	4.01	15.6	5.65	20.8	5.30
Ni	8.80	23.8	62.4	158	20.7	28.6	10.9	35.3	15.2	6.78	15.8
As	4.38	12.9	28.6	7.09	27.4	21.2	56.7	16.4	56.1	18.8	42.8
Mo	13.6	132	75.8	10.8	53.3	20.6	142	15.5	30.6	14.6	33.3
Cd	8.19	36.1	11.5	50.2	*	27.5	*	31.8	*	21.0	*
Pb	9.77	13.5	21.5	18.3	22.4	35.9	82.2	18.6	38.6	33.6	69.2

If we classify shells either by size class or ring number, weight increases as shell size rises (8.41, 11.34 and 14.86 g respectively in the size classes considered and 9.81, 10.82, 11.03 and 14.16 for shells with 3, 4, 5 and 6 rings respectively). In tables 5 and 6, the CV% is shown for shells grouped by size and by ring class respectively, after multiplication of the element concentrations by the shell weight. In both tables, it can be seen that variability for total content is generally higher than variability for concentration, especially for those elements which have very low concentrations. Therefore, the "weight factor" is characterized by a huge range of values, not proportional to the element concentration, and enlarges the variance of the results. The variability between the paired size classes resulted not significantly different according to the Wicoxon test, except for the pair 51-58 / 58.5-65.5 mm (p <0.05). For ring classes, variability was significantly different only for 3-5, 3-6 and 4-6 pair classes.

Tab. 5. Coefficient of variation (CV%) calculated from mean and standard deviation for the total content (concentration × weight) of each element quantified in shell samples of *Unio pictorum mancus* as divided into size classes (51-58 mm, 58.5-65.5 mm and 66-73 mm, 9 replicates at least). Samples at juvenile stage were not weighed. * values not detectable.

Element	Size class		
	51-58 mm	58.5-65.5 mm	66-73 mm
Ca	14.3	20.3	13.9
Cu	33.6	34.7	29.8
Fe	16.4	36.0	52.8
Mn	22.9	37.3	22.7
Zn	*	*	*
Al	190	76.3	104
V	165	174	208
Cr	86.0	86.0	84
Co	13.8	21.6	14.6
Ni	20.4	21.3	13.7
As	46.2	61.4	21.7
Mo	53.4	148	129
Cd	*	*	*
Pb	70.2	36.8	87.5

Tab. 6. Coefficient of variation (CV%) calculated from mean and standard deviation for the total content (concentration × weight) of each element quantified in shell samples of *Unio pictorum mancus* as divided into classes by number of rings (3, 4, 5, 6). Samples with less than 1 ring were not weighed. * = values not detectable.

Element	Ring number			
	3	4	5	6
Ca	23.2	17.7	21.3	40.9
Cu	16.7	42.5	39.1	74.3
Fe	29.5	22.8	45.6	78.9
Mn	23.4	32.0	26.7	41.6
Zn	*	*	*	*
Al	53.8	70.6	124	111
V	101	*	222	131
Cr	121	62.6	139	80.6
Co	19.7	20.3	20.4	40.2
Ni	27.9	25.3	15.9	35.1
As	44.4	68.7	68.2	79.6
Mo	75.6	139	120	92.4
Cd	*	*	*	*
Pb	5.01	65.4	53.3	80.6

The shell/soft tissue ratio, as calculated for each measured element (Tab. 7), indicates that partition between shell and soft tissue is roughly the same in the juvenile and adult stages for the following elements: Fe, Al, Co, As, Pb. In general, during the adult stage, the accumulation of Ca, Cu, Mn, Ni and Cd. V, Cr in shell is lower and that in the soft tissue is not proportionally lower, or the increase in the soft tissue is higher and not proportional to that in the shell. Concentrations of the same order of magnitude were measured in the shell and in the soft tissue for Co and Ni, while Ca concentrations were about 10 times greater in the shell (as expected). Concentrations were more than 10-fold higher in soft tissue for Fe, Mn, V, As, and Mo and more than 100 times higher in soft tissue for Cd.

For shells, the correlation of number of rings and length with element concentrations (Tab. 8) was significant (at p <0.01) for V, Cr and Mo (positive coefficient) and (at p <0.05) for Co (negative coefficient). The cor-

Tab. 7. Shell/tissue ratio for the mean concentrations of the investigated elements calculated for both the juvenile and the adult stage (* = values not detectable).

	Ca	Cu	Fe	Mn	Zn	Al	V	Cr	Co	Ni	As	Mo	Cd	Pb
juvenile stage	12.9	0.25	0.05	0.10	0.02	0.20	0.03	0.09	0.79	3.30	0.04	0.01	0.07	0.05
adult stage	6.08	0.14	0.04	0.04	*	0.18	0.10	0.23	0.58	1.57	0.03	0.05	0.02	0.05

Tab. 8. Comparison between the effect of ring number and shell length on element concentrations in a) shell and b) soft tissue. Correlation coefficients (*r*) and their significance: * = significance at $p < 0.05$, ** = significance at $p < 0.01$ levels, n.s. = not significant.

	Ca	Cu	Fe	Mn	Zn	Al	V	Cr	Co	Ni	As	Mo	Cd	Pb
a) shell														
ring number	n.s.	n.s.	n.s.	n.s.	-	n.s.	0.51**	0.38**	-0.31*	n.s.	n.s.	0.52**	-	n.s.
shell length	n.s.	n.s.	n.s.	n.s.	-	-0.38**	0.40**	0.35*	-0.31*	n.s.	n.s.	0.62**	-	n.s.
b) soft tissue														
ring number	n.s.	n.s.	0.48**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.30*	n.s.	0.37*	n.s.	n.s.
shell length	0.52**	0.45*	0.49**	0.63**	0.57**	-0.71**	-0.53*	n.s.	0.46*	n.s.	0.63**	n.s.	0.67*	n.s.

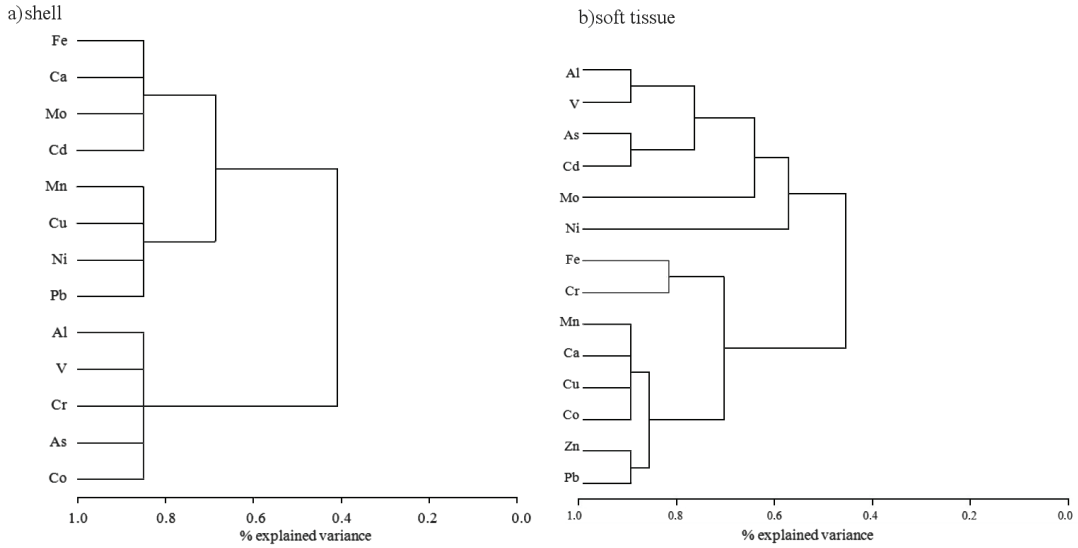

Fig. 2. Horizontal hierarchical tree plot of the metals analyzed in a) shell and b) mussel samples of *Unio pictorum mancus*.

relation between shell length and Al was significant at $p < 0.01$, with a negative coefficient. On the contrary, for soft tissues, the correlation of the number of rings and/or the shell length with the element concentration was significant only for Fe, with a positive coefficient (at $p < 0.01$). The regression coefficient between length and concentration was significant (at $p < 0.05$ at least) and positive for half the elements, except Cr, Ni, Mo and Pb (not significant) and Al and V (negative values). The correlation with the number of rings was significant only for Fe and Mo (at $p < 0.01$ and < 0.05 respectively and positive) and Ni (significant at $p < 0.05$ and negative).

As can be seen in the dendrogram obtained using PROC TREE (Fig. 2), elements in the shell samples can be placed in three groups explaining over 70% of the variance: i) Al, V, Cr, As and Co; ii) Fe, Ca, Mo, Cd; iii) Mn, Cu, Ni and Pb. Zn was not included because all measured values were below the instrument's detection limit. According to the dendrogram for soft tissue samples (Fig. 2b), the elements can be divided into four groups, which explain over 70% of the variance: i) Al, V, As, Cd and Mo; ii) Ni only; iii) Fe and Cr; iv) Mn, Ca, Cu, Co, Zn and Pb.

4. DISCUSSION AND CONCLUSIONS

Notwithstanding the trueness and precision of the analytical method (Tabs 2 and 3), whether the *Unio* samples were divided by size or by number of rings, in general the variability was significantly larger than it was for the certified mussel sample (Tabs 3 and 4). By selecting the same location and collection period for adult specimens, the study was able to reduce the influence of seasonal variations and trophic level on element concentrations (Ravera *et al*. 2007b; Ravera *et al*. 2009). The minimum number of collected samples was 9 and 4 according the partition in size classes and the ring classes respectively. The accumulation was influenced not only by mussel size (Ravera *et al*. 2003b), but also by growth rate and the accumulation rates (Beone

et al. 2003; Ravera *et al*. 2009), the physiological needs of each specimen (Ravera *et al.* 2007a) and the interactions among these various elements (Daka *et al*. 2006). For example, Unio can modify its metabolism depending on the temperature, like all heterotherm animals, and depending on oxygen availability: it can survive for a long period of time buried in hypoxic or anoxic sediments by reducing its metabolic rate, living anaerobically and producing succinic acid as a by-product (Campanella *et al*. 2005). Its metabolic rate can increase heavy metal accumulation and the acidity can release the heavy metals bound to the organic matter of the shell and to calcium phosphate granules (Bauer & Wächtler 2001). In tables 5 and 6, CVs of total content (concentration × weight) in shells appear very heterogeneous among the various size and ring classes. In general, there is a negative relationship between means and variances, which results in higher CVs for metals with lower means. This is observed in the present study, but the use of CV instead of variance could be crucial to compare datasets characterized by different units (i.e., Ca) or widely different means and variances (e.g., Co, Cu and Zn). The sign test on the obtained size or ring classes after having ranked the element CVs, confirms a limited variability between paired groups.

In general, soft tissues at the juvenile stage exhibit low CV% values, except for Mo (Tab. 3). If we consider the burrowing lifestyle of *Unio* in the early adult stage, the variability of juvenile shells can be explained not only by the total concentration, but also by the available concentration in the sediments, which is strictly connected to changes in the depth of the overlying water column and, correspondingly, to the redox status (Fox & Doner 2003). In fact, the Lake Maggiore sampling station used for this study frequently shows the combined effects of a decrease in water level and a gently sloping coastal zone (Ravera *et al.* 2009). In a previous work, Ravera *et al.* (2003b) found no relationship between metal concentrations in the soft tissues and in the shell. This is because, as reported by Ravera *et al*. (2007a), the shell composition represents the situation throughout the life of the specimen, while the chemical composition of the soft tissue only reflects the recent environment situation. This also provided a rationale for the coexistence of low and high variability of the same elements in the shell and the soft tissue, which was not only observed in the smallest individuals.

It is commonly accepted that calcium is the most abundant metal in both shells and soft tissues: it has a key role in the physiology of bivalves, as the main component of the shell and the calcium carbonate granules in the soft tissues (Förstner & Wittmann 1983; Byrne 2000; Byrne & Vesk 2000). Furthermore, its low affinity with chelating substances increases its availability to the organism. The result is that the calcium concentration in the aquatic environment is reflected in the amount accumulated by *Unio* specimens. In this study, we found that calcium was the main element in the shell (Tab. 2), followed by Mn, Al, Fe, Ni and Cu (from highest to lowest concentration). However, as can be seen in table 7, only Ca and Ni are stored in the shell. Nyström *et al*. (1996) pointed out the well-known capacity of *Unio* to accumulate manganese in its shell by binding it to organic matter encircling aragonite crystals in the nacreous layer, Ravera *et al*. (2003b) highlighted a similar chemical behavior in iron. It is plausible that Mn, Al, Fe and Cu are mostly bound up in the carbonate granules in the specimens investigated. Aluminum does not play a physiological role and is typically present in the sediment compartment. The probable reason for its high concentration in shells and soft tissues (ranging from 7 to 140 mg kg^{-1} in shells and approximately 5 times more concentrated in soft tissues) is that divalent and trivalent nonessential trace metals should follow the same metabolic pathway as calcium from the water to the mussel's gills (Markich & Jeffree 1994). Ni and Cu are also accumulated (in shells and soft tissues respectively). However, our data highlight the fact that the accumulation of Ca, Ni and Cu in shells is not proportional to size, although Al accumulation decreases significantly with length (Tab. 8). In soft tissue, Ca accumulation clearly tends to increase with mussel size, whereas Al accumulation decreases with size. The findings of Markich & Jeffree may also explain the data concerning Cu and Zn, which are essential only in small amounts. As these elements are more concentrated in soft tissues than in the shell (Tab. 7), they are almost certainly detoxified by *Unio* as previously discussed (Ravera *et al*. 2003a), for the most part without being transferred to the mantle and consequently to the shell. The remaining elements were represented more in the soft tissue (Tab. 7) as well. Our observation concerning distribution between the shell and the soft tissue partly contradicted what has been reported in the literature (Ravera *et al.* 2003a). These results may depend on the more or less marked ability to discriminate between elements based on the physiological needs of individuals, which should be considered in the environmental context. Unfortunately, we do not have any data concerning concentration in the water and sediments. It is plausible, however, that these elements could be more concentrated in the mussels than in the water, and in some cases more than in the sediment, due to the filtering and accumulating nature of *Unio*.

It is interesting to note that V, Cr and Mo, which are essential elements (Yarsan *et al*. 2007), tend to accumulate in the shell progressively over the years (Tab. 8), whereas Co and Al are "diluted" during growth, as if the growth rate is faster than the accumulation rate (Boyden 1977) or as if they are absorbed mainly during the juvenile stage, (from the sediment), with the absorption rate steadily decreasing during the adult stage. The results obtained by Koretsky *et al*. (2006), Gimeno-Garcìa *et al*. (1996) and Gao *et al*. (2007) confirm that Co and Al

are more prevalent in sediment than in the water in lentic environments.

The relationship between growth and accumulation in both the soft tissues and in the shell is similarly based on a positive correlation in the case of Mo only and on a negative correlation in the case of Al only. Most elements in the soft tissues of mussels are present in greater concentration as the mussels increase in size and age, with a significant correlation. In the case of Al and V, we observed a "dilution" by the larger mass. Our results concerning Mn, Fe, Zn and Ca are in agreement with the study realized by Ravera *et al*. (2003b). The accumulation of Cd in soft tissue, due to repeated biological detoxification by metallothioneins and the glutathione system (Belcheva *et al*. 2006), causes the size-dependent cadmium concentration observed. For Cu, Fe and Zn, which are characterized by an analogous increment, the sequestration and toxicity limitation mechanism is similar (Cosson 2000).

The size correlation was not clear for the other elements investigated. These could be due to mutual independence between the variables. In general, shell length appears to be more strongly linked to element concentrations than the specimen age. As stated by Kwan *et al*. (2003), the independence of element concentrations from size classes may not be real, but could be smaller than the smallest one of the size class immediately above. According to our results, a size class may contain animals of different ages, reflecting the environmental conditions of different years. As reported by Negus (1966), Peterson & Beal (1989) and Adam (1990), bivalve growth can depend on population density, site temperature, organic matter availability and the alternation between wet and dry periods. Our sampling station was affected by all of these factors except the first, and especially by the last. The number of rings, if correctly determined, corresponds to the age (Negus 1966). Therefore, if growth is not homogeneous for all the samples investigated, the number of rings could probably be used to highlight differences in the aquatic pollution of the environments over the recent past. For the older specimens, these differences could be due to higher bioavailable concentrations of the elements during their first year of life or a higher capacity for accumulation due to metabolic modifications in their last year of life. As it can be seen in the dendrogram obtained by PROC TREE (Fig. 2), elements in the shell can be placed into three groups that explain more than 70% of the variance: i) Al, V, Cr, As and Co; ii) Fe, Ca, Mo, Cd; iii) Mn, Cu, Ni and Pb. Zn was not included because all measured values were below the instrument's detection limit.

According to the dendrogram for soft tissue (Fig. 2b), the elements can be placed into four groups, that explain more than 70% of the variance: i) Al, V, As, Cd and Mo; ii) Ni only; iii) Fe and Cr; iv) Mn, Ca, Cu, Co, Zn and Pb.

Three factors influence metal specificity *in vivo*:

1) differences in metal affinity;
2) differences in intracellular metal concentrations (for example, presence of certain uptake or efflux systems);
3) coordination geometry preferences of various metals/ability to trigger the desired conformational change (Harvie *et al.* 2006; Rensing 2005; Dutta *et al*. 2006; Liu *et al*. 2006).

In 2001, Markich confirmed that extracellular granules are the main deposition sites for Mn, Co, Cu, Zn and Pb and that hard and borderline metals, such as Mn, Co and Zn, appear to have a higher affinity for these granules. In the case of Pb, we know that the transporters responsible for zinc uptake and efflux are also responsible for transporting other metal ions, such as Pb(II) (Rensing 2007).

Cr (III) is less mobile and less toxic and usually binds to organic matter in soil and in aquatic environment (Shanker 2008). The same behaviour is observed in Fe (Ravera *et al*. 2003). These elements are classified as hard and borderline metals and probably behave similarly to other hard metals as regards extracellular granules.

The elements V, Cd and As are frequently associated with the metallothionein (MT) system. MT concentrations were significantly correlated with both total and soluble concentrations of V (Amiard 2009). Induced MTs can bind nonessential metals (including Cd), reducing their potential toxicity (Roesijadi 1992, 1996; Zaroogian & Jackim 2000).

In the mussel *Perna viridis*, Cd is a strong MT inducer, as reflected by MT concentrations (Shi & Wang 2005). Metallothionein levels are usually correlated with cadmium levels (Geffard *et al*. 2002); in some cases exposure to cadmium and the subsequent induction of metallothionein-like proteins affected cadmium uptake (Blackmore & Wang 2002).

Arsenite is extremely thiol-reactive. Metallothioneins are thought to have a protective effect against arsenic toxicity and may be responsible, at least in part, for self-induced tolerance (Shanker 2008). Nikel ions have a high affinity for cysteine (Costa *et al*. 1994) and MT induction by Ni has been reported in the cod *Eleginus navaga* (Eriksen *et al*. 1990). However, although MT induction has been demonstrated in the copepod *Tigriopus brevicornis* after exposure to Ni, this element was far from the most potent of a series of metals tested in the same set of experiments (Barka *et al*. 2001).

The correlation values obtained from a cluster analysis of various elements is due to differential binding of metals to ligands on the surface of detoxification system. Hard metals have a high affinity with oxygen donor ligands (phosphate is the major anion in granules), whereas soft metals have high affinity to sulphur or nitrogen donor ligands (thiol groups or metallothioneins).

The clustering pattern of elements is less distinct in the soft tissues of mussels than in shell. The different clustering pattern in shell probably results from the different chemical processes involved and from its characteristics as a storage matrix for toxic metals (Walsh *et al*. 1995). Moreover, the shells of long-living, slow-growing mussel species could serve as records of long-term variation (e.g., on an interannual scale) (Gillikin 2005; Vander Putten 2000; Lazareth 2003). In fact, we found that heavy metal accumulation in shells was not correlated with that in soft tissues (Yap *et al*. 2010).

The distinct difference between the shells and the various soft tissues in most molluscs may be due to the fact that some trace metals are incorporated into the shells of the bivalve through substitution of the calcium ion in the crystalline phase of the shell or are associated with the organic matrix of the shell (Foster & Chacko 1995; Yap *et al*. 2003a). The metals could be distributed through the various soft tissues before being biodeposited in the shell (Yap *et al*. 2003b). Shell composition is closely related to chemical mineralogy (which involves metals accumulated from the environment); this may be one of the reasons why metal concentrations in the shell tended to be similar to the concentrations in the organism's environment.

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