Bull Environ Contam Toxicol (2009) 83:223–229
DOI 10.1007/s00128-009-9693-9

Trace Metals in Oysters, *Crassotrea* sps., from UNESCO Protected Natural Reserve of Urdaibai: Space-Time Observations and Source Identification

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Abstract The seasonal and spatial variability of trace metal concentrations of oyster tissues (*Crassostea* sps.) was studied. The samples were collected between 2002 and 2004 along the Urdaibai estuary (Murueta, Kanala and Arteaga). The seasonal influence on some metal concentrations (higher values in spring–summer season than in autumn–winter season) is more pronounced than the local site-specific influence. Furthermore, within each location a significant time-specific dependence on the trace metal concentration variations can be detected. Substantially higher concentrations of Zn (445.5–2,403.3 mg kg^{-1}) and Mn (29.4–169.6 mg kg^{-1}) were recorded in oysters inhabiting estuarine locations, possibly indicating a markedly higher natural bioavailability at these locations. Principal component analysis demonstrated that oysters could be separated depending on the inhabiting areas: Sn for Murueta, essential metals for Kanala, and Co and Cd for Arteaga sampling points, respectively.

Keywords Oysters · Trace metals · ICP-MS · Biomonitoring · Urdaibai estuary

Metals are a group of the most important pollutants which cause environmental degradation in coastal areas. Trace metals are introduced into the aquatic ecosystems in a number of ways. These chemicals accumulate in the tissues of aquatic organisms at concentrations many times higher than concentrations in water and may be biomagnified in the food chain to levels that cause physiological impairment at higher trophic levels and in human consumers.

A conventional monitoring system of environmental metal pollution includes the measurement of the level of selected metals in the whole organism. It is well known that, compared to bottom sediments, marine organisms are characterised by a greater spatial ability to accumulate some metals. In the literature, several critical reviews have been published on the comparative evaluation of molluscs as quantitative monitors of metal pollution (Phillips and Rainbow 1993; Rainbow 1995; Szefer 2002). Oysters (*Crassostea* sps.) have already been proposed as sentinel organisms for marine ecotoxicological tests for the purpose of assessing coastal water quality, because they are very sensitive to pollutants and provide rapid responses (His et al. 1999). Some studies have shown significant amounts of trace metals in the soft tissues of oysters (Quiniou et al. 2007; Sidoumou et al. 2006; Mora et al. 2004). Despite the importance of the previous results, few studies of the heavy metal contents in oysters have been carried out to date in the Urdaibai estuary. Relatively low levels of industrial activities are found in this area (Biosphere Reserve since 1984 by UNESCO). However, there is a growing need to control the quality of coastal waters and marine organisms that may be influenced by waste discharges from different sources into the estuary. However, quantitative extrapolation of environmental exposures from metal body concentrations still remains with a big uncertainty. This is mainly due to several biological and environmental factors that influence accumulation and concentrations of trace metals in oyster tissues.

Multivariate analytical methodologies to treat contaminant data are presently available (Mardia et al. 1989; Deming and Morgan 1993; Wackernagel 2003) and they

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are useful tools for decomposing mixed data assemblages into their individual components. In order to reduce a relatively large number of variables to a smaller number of orthogonal factors, metal concentration data are treated by multivariate statistical methods, i.e., principal component analysis (PCA). The advantages of using multivariate methods in the evaluation of environmental and biological data with respect to mineral composition of the object matrix studied are well known. The applicability of these techniques and how they can be used to interpret complex environmental data have been reported previously (Astley et al. 1999; Bechmann et al. 2000; Romeo et al. 2003). Moreover multivariate methods are recommended for use in monitoring studies since they can help to reduce the costs of carrying out further environmental surveys (Szefer 2002).

In this work, PCA has been used to quantify distribution patterns of trace elements in oyster from different subareas of the Urdaibai estuary (northern Spain). A comparative study was designed to evaluate the adequacy of oysters as biomonitors for the Urdaibai estuary in the monitoring of metallic contamination, using oysters collected from different geographical locations.

Materials and Methods

The Urdaibai estuary drains into the Bay of Biscay (Fig. 1). It has an extension of 220 km^2 along 12.5 km of the mouth of Oka River and other minor rivers, covering 1.9 km^2 with an average depth of 3 m and a maximum width of 1.2 km at the mouth. In 1984, it was declared Biosphere's Reserve

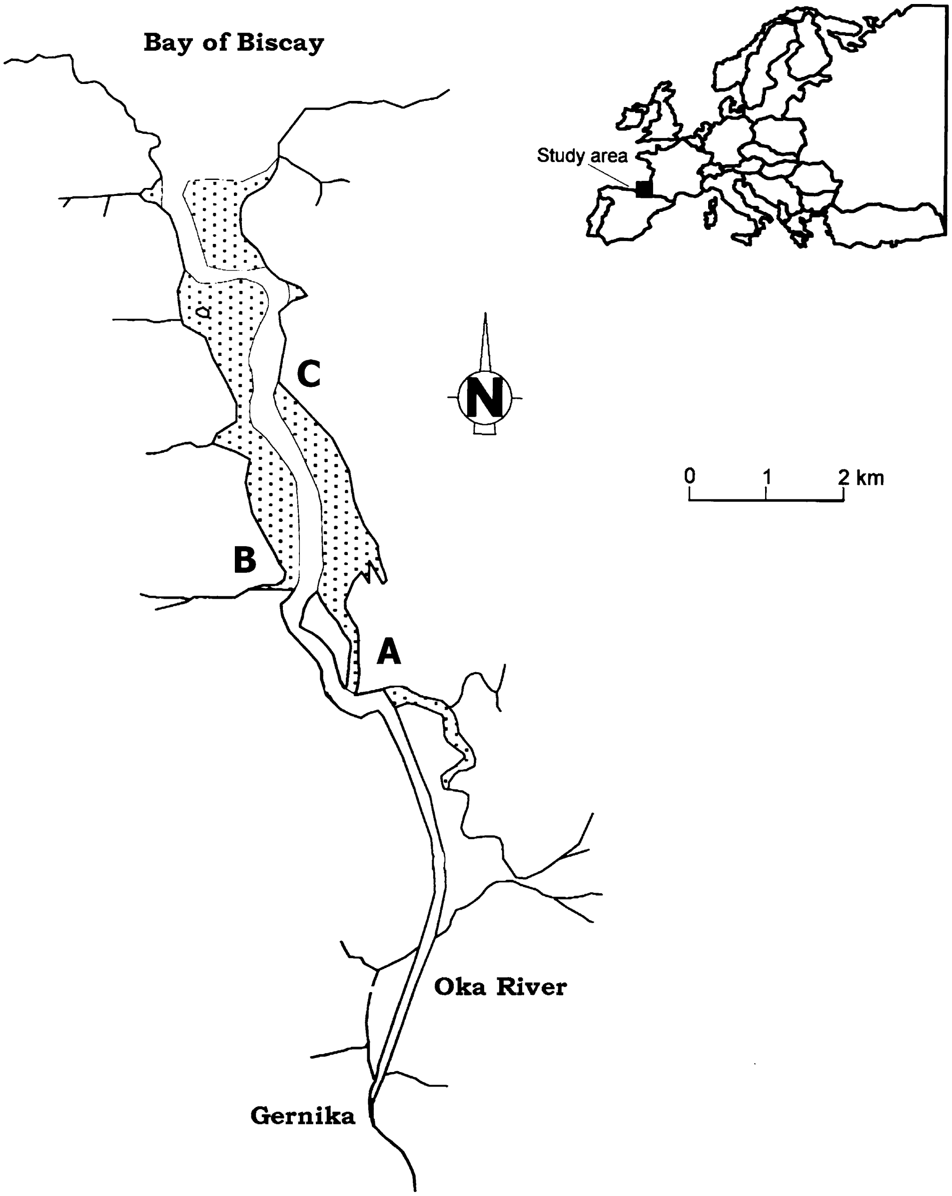

Fig. 1 Sampling points in the Urdaibai estuary: *A* Arteaga, *B* Murueta and *C* Kanala. There is no available sample in Jun-02 for Arteaga sampling point

by UNESCO. River discharges are dominant in the upper reaches and tidal inflow in the lower zone. The upper segment is partially mixed during low river regime and well mixed during enhanced river flows. The lower estuary is always well mixed by tidal flushing. A population of about 45,000 people living in several towns is situated at the source of the Oka River.

The sampling points considered are shown in Fig. 1. Samples were manually taken from June 2002 to September 2004 in the same sampling point immersed 10 cm of river-bed surface, rinsed with the natural water, introduced into plastic bags and frozen until sampling preparation. Arteaga (A), Murueta (B) and Kanala (C) are representative sites of the middle estuary. All reagents were of analytical-reagent grade and Milli-Q 18 MΩ pure water was used. The volumetric glassware was grade A and was calibrated at laboratory temperature. 1,000 mg L^{-1} Alfa Aesar (Karlsruhe, Germany) Specpure stock solutions of As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Se, Sn, V and Zn were used. Nitric acid (69%, Tracepur) was provided by Merck (Darmstadt, Germany).

For the validation of the analytical method, the SRMs 2977 and 2976 (mussel tissue) were purchased from NIST (Gaithersburg, USA). All ICP-MS measurements were repeated three times for each element and made with an Elan 9000 (Perkin Elmer, Ontario, Canada) instrument using a Ryton cross-flow nebulizer, a Scott-type double pass spray chamber and standard nickel cones. The instrument operating parameters and data acquisition details are listed in Table 1. A 3000S microwave-assisted system (Anton Paar, Graz, Austria) with Teflon vessels was used in the acid digestions of the mussel tissues.

Rigorous cleaning procedures of all the laboratory ware and other equipment that comes in contact with samples have been employed in order to avoid contamination of samples. All glassware and plastic ware were washed with a common detergent and thoroughly rinsed with Elix quality water (Millipore, Bedford, MA, USA). After that, all the laboratory ware was soaked into a clean dilute HNO_3 (15%) bath for 24 h. Afterwards, the material was rinsed with Milli-Q water (Milli-Q Element A10, Millipore, Bedford, MA, USA) quality water.

Table 1 ICP-MS operating and acquisition parameters

RF power (W)	1,000
Plasma gas flow (L min^{-1})	15
Auxiliary gas flow (L min^{-1})	1.0
Nebulizer gas flow (L min^{-1})	0.91
Sample flow rate (mL min^{-1})	1.0
Data acquisition	(Dwell time, 250 ms)
Sweeps per replicate	10
Replicates	3
Detection mode	Peak hopping
Isotopes	^{75}As, ^{114}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{202}Hg, ^{55}Mn, ^{56}Ni, ^{208}Pb, ^{51}V, ^{66}Zn

Frozen samples were freeze-dried at 10^{-1} mBar and −48°C in a Cryodos T-50 (Telstar, Barcelona, Spain) system. Then, samples were ground in a Pulverisette 6 ball mill (Firstch, Quebec, Canada) and kept in the refrigerator at 4°C until analysis. Then, 0.2 g of this material was accurately weighed and transferred into the extraction vessel together with 15 mL of HNO_3 (7.0% v/v). The extraction vessel was placed in the microwave oven and the digestion program was run ($p = 450$ w, $t = 18$ min). All the acid extracts were filtered through Whatman filters (0.45 µm) and diluted to 25 mL with Milli Q water. Finally, the solutions obtained were analysed by ICP-MS. This analytical procedure was optimised and validated in a previous work (Bartolomé 2007).

Percentages of lipids of the oyster tissues were also measured. Lipid content was operationally described as the weight of sample extracted using dichloromethane according to the procedure defined by the NOAA (Wade et al. 1993). In addition, water content was also calculated as the weight loss of a heated sample (0.5 g) at 110°C.

Results and Discussion

The accuracy of the analytical method was checked using two different certified reference materials, mussel tissues (NIST 2976 and NIST 2977) for heavy metal determination. These CRMs were considered because no commercial oyster certified material was available at the monitoring study time and the similar matrix effects between mussel and oyster samples on the trace metal analysis could be assumed. The results (Table 2) are in good agreement with the certified values.

The precision of the analysis were evaluated using standard stock dilutions (10 µg L^{-1}) and the measurements were done at the same day (n = 10) and in different days (n = 10). The relative standard deviation (% R.S.D.) of all determinations was minor than the 3% in all cases.

Absolute detection limits of 13 ng g^{-1} for As, Cd, Co, Cr, Cu, Ni, V; 26 ng g^{-1} for Sn and Se, 0.1 µg g^{-1} for Pb, 0.5 µg g^{-1} for Mn and 2.0 µg g^{-1} for Zn were estimated, calculated as the concentration corresponding to the blank signal plus three times the standard deviation of the blank.

In order to perform a quantitative extrapolation of the external environmental exposures from the metal concentrations, a clear understanding of several biological and environmental factors (season, location or physiological condition) is critical. However, as trace metal concentrations can vary with mass, age, estuary position, and

Table 2 Results obtained (mg kg^{-1}) in the heavy metal analysis of the certified reference materials

	NIST 2976		NIST 2977	
	Cert.	Exp.	Cert.	Exp.
As	13.3 ± 1.8	19.9 ± 1.1	8.83 ± 0.91	14.5 ± 2.3
Cd	0.82 ± 0.16	0.77 ± 0.02	0.179 ± 0.003	0.19 ± 0.01
Co	0.61 ± 0.02	0.64 ± 0.06	0.48 ± 0.13	0.44 ± 0.03
Cr	0.5 ± 0.16	2.4 ± 0.4	3.91 ± 0.47	4.59 ± 0.53
Cu	4.02 ± 0.33	4.23 ± 0.05	9.42 ± 0.52	12.8 ± 3.5
Hg	0.061 ± 0.004		0.101 ± 0.002	0.116 ± 0.091
Mn	33 ± 2	34 ± 2	23.9 ± 0.3	23.1 ± 1.6
Ni	0.93 ± 0.12	1.04 ± 0.06	6.06 ± 0.24	6.20 ± 0.38
Pb	1.19 ± 0.18	1.25 ± 0.03	2.27 ± 0.13	2.04 ± 0.35
V			1.1	1.12 ± 0.08
Zn	137 ± 13	148 ± 1	135 ± 5	163 ± 21

temporally, care must be taken to collect individual organisms of similar mass and age, and to minimise variability, and from similar consistent positions and times to allow for seasonal changes in environmental conditions. Apart from that, when studying oysters collected from different habitats or seasons, variations in the average of lipid concentration is the most predictable variable. The relationship between lipid percentage and metal concentration is often described using power functions or their logarithm transformations (Boyden 1974). However, within a narrow range of body size, linear equations are most appropriate (Newman 1995) and the influence of lipid content can be minimised. The percentage ranges of lipid (w/w%) were (7.0–15.6) for Arteaga, (4.2–10.3) for Kanala and (4.7–11.5) for Murueta, respectively. However, there were few significant relationships ($R < 0.1$) between trace metal concentrations and mass (lipid percentage) and no significant differences in trace metal concentrations with the lipid contents were finally obtained. The mean metal concentrations (dry weight) are shown in Fig. 2. The greatest metal concentrations found were 2,403 mg kg^{-1} for Zn and 169 mg kg^{-1} for Mn in Arteaga (Sep-04).

Inter annual variability of trace metal concentrations was considerable and trace metal concentrations also fluctuated throughout an annual cycle with clear seasonal trends. Higher levels were found in summer–autumn seasons while minimum contents occurred in wet seasons (biogeochemical cycle). These general differences in mean concentrations between months were most likely due to inherent trace metal variability associated with differences in food availability and changes in metabolic rates associated with changes in the location parameters during the study period.

Comparison of trace metal concentrations at the three sites within the Urdaibai Estuary showed a significant difference depending on the geographical conditions of each particular location, indicating that the Arteaga sampling point has elevated biologically available metal concentrations. On the other hand, trace metal concentrations in oyster tissues were lower in Kanala and Murueta.

Essential trace metals as Cu (29.6–143.7 mg kg^{-1}), Mn (29.4–169.6 mg kg^{-1}), or Zn (445.7–2,403.3 mg kg^{-1}) showed similar concentration levels with low individual variability and not significant differences in the accumulation patterns were observed among all the three sampling. From these data, we cannot deduce any anthropogenic source for these trace metals.

Arsenic concentrations were ranged from 8.2 to 25.2 mg kg^{-1}. Trace metal concentration variability is higher for Kanala and Murueta, with maximum concentrations in Sep-02, Sep-03 and Sep-04. Similar time accumulation pattern was found in Arteaga with lower trace metals concentrations.

Cadmium concentrations were significantly higher in Arteaga than at the other two sampling sites, where similar patterns were found. The concentration values obtained varied from 0.41 mg kg^{-1} in Kanala (Dec-03) to 3.0 mg kg^{-1} in Arteaga (Sep-04).

In the case of Cobalt (0.26–0.79 mg kg^{-1}), no significant differences were found between the location site and the accumulation pattern. From these concentration profiles, the only remarkable data was obtained in Arteaga in Sep-04 (1.32 mg kg^{-1}) with twice the general concentration level obtained for the other points.

Similar mean concentrations were obtained for Chromium (2.3–9.8 mg kg^{-1}) in the three sampling points considered. In this case, the profiles do not reveal any external source, assuming only natural variation.

Nickel concentrations increased from Kanala to Arteaga sampling points (1.62–9.2 mg kg^{-1}). No clear trends were obtained for Kanala and Murueta but Arteaga showed the same pattern obtained in the general tendency with maximum concentration value for Sep-03.

The pattern obtained for Lead (0.63–3.81 mg kg^{-1}) was significantly different with respect to the other trace metals. In this case, maximum levels are obtained in winter seasons (Dec-03) for the 3 sampling sites. This fact could be explained by the same anthropogenic input source affecting the sampling points.

Vanadium concentrations (0.6–2.8 mg kg^{-1}) showed the same time pattern obtained in here. However, the highest concentrations were found in the furthest site (Kanala).

The profiles obtained for Tin (0.04–0.5 mg kg^{-1}) were significantly similar for the three sampling sites. Only Kanala (Feb-03) and Arteaga (Dec-03) broke down the general time pattern with a concentration peaking.

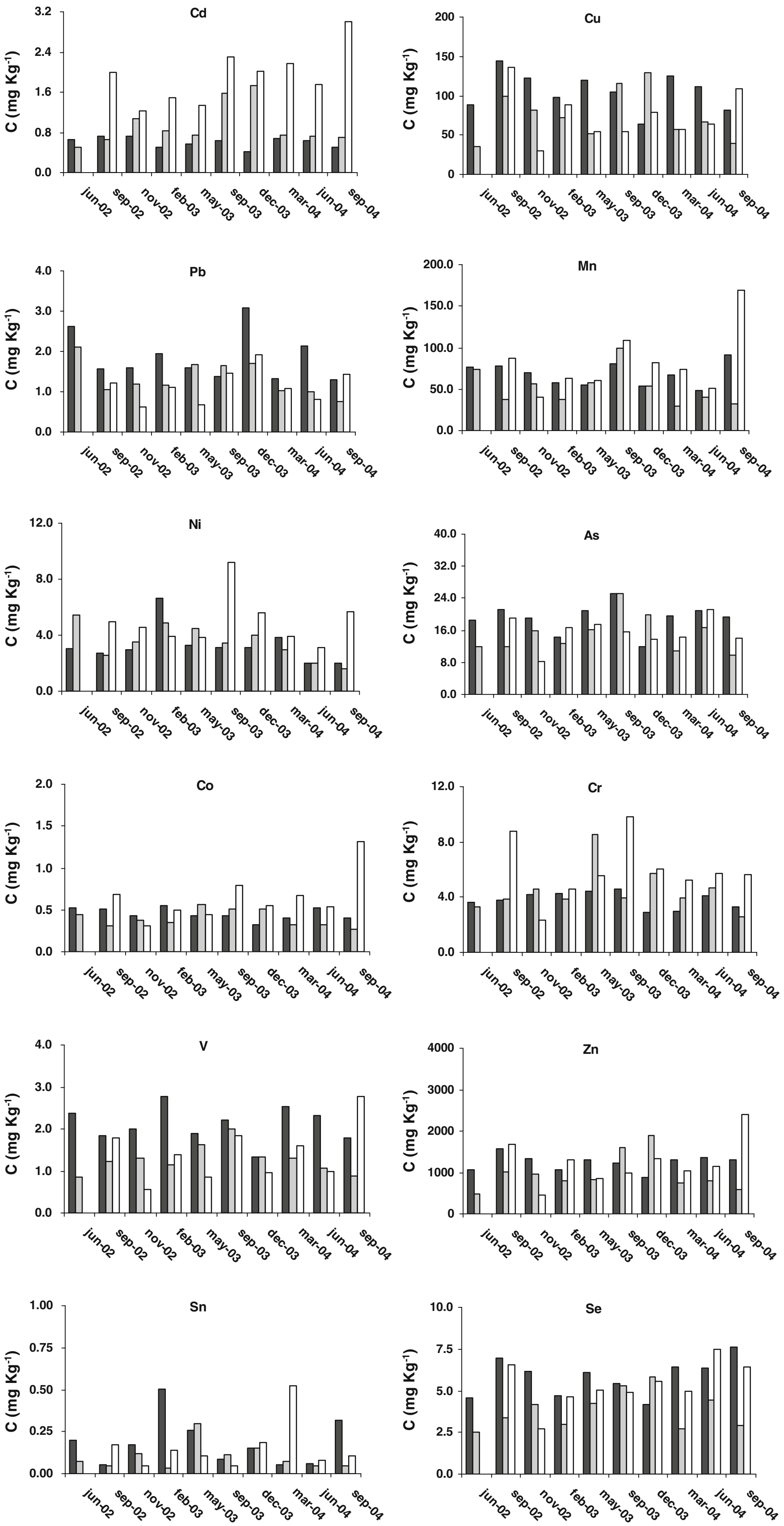

Fig. 2 Metal concentrations (mg kg^{-1}) for each sampling point. ☐ Arteaga, ▨ Murueta, ■ Kanala

Finally, Selenium concentrations (2.7–7.6 mg kg^{-1}) showed no time pattern and no relationships were found between mean concentration and sampling season. This profile was similar for the three sampling sites considered.

In order to see any temporal trend in the trace concentrations obtained in this work, the Mann–Kendall correlation coefficient was used. This coefficient is an association measurement that allows the performance of a non-parametric contrast and it does not require the normality of the variables that is compulsory in regression models. The use of this coefficient as a trend test was proposed by Mann (Hollander et al. 1999) to detect monotonic downward trends, and according to the advice of ICES Working Group on Statistical Aspects of Environmental Monitoring (Swertz 1996), we have used it in year wise data. In this work, we have used the MULTMK/PARTMK add-in macro for Excel (Libiseller C., http://www.mai.liu.se/~cllib/welcome/welcome.htm) to carry out the test.

Some temporal trends were statistically obtained in here for a 95% confidence level ($p < 0.05$). In that sense, concentration increasing was obtained for Cd, Co, Mn and V in Arteaga. For Murueta site, only Mn had downward and finally Co and V showed a decreasing pattern for Kanala, while most of the other data are at the same level in the whole sampling period.

From the experimental results, a statistical treatment (correlation analysis and principal component analysis) was carried out by means of The Unscrambler ® program (Esbensen 1994). In order to get a normal distribution of data, we have performed the logarithm transformation according to $C' = \log (C)$, where C' is the transformed value and C is the mean metal concentration. Once this transformation was done the data were centred and scaled to assure an equal weight to all the variables.

From the correlation analysis, the different patterns of chemical variables (trace metal concentration) were concluded along the seasonal campaigns considered. The correlation values with $r > 0.6$ were considered as an important tendency among the chemical variables studied. The main correlations obtained for the chemical variables considered are collected in Table 3. In this correlation matrix, the chemical similitude and bioaccumulation pattern among all the trace elements could be extracted from the positive correlation of their mean concentrations in the oysters. Only the Cd and Sn correlations broke down the general tendency and no significant correlation was obtained for these trace metals.

From the PCA, different location patterns and contamination sources could be defined considering the scores and x-loadings plot (Fig. 3). The new analysis indicated that 3 PCs explained up to 78% of the total variance. These values may appear modest, probably because animals used in the study did differ so widely in regard to a heterogeneous condition. Therefore, any changes in these processes due to a variety of factors such as tidal exposure, sex, reproductive status or nutritional status may contribute to variability among individuals. From the scores plot (Fig. 3), the second and third PCs seem to be useful to group the stations according to their spatial position and to the sampling season. As an example, from those figures we can clearly see how samples from Arteaga (A), Murueta (B) and Kanala (C) are clustered depending on different trace metals: (1) Arteaga sampling point is highly influenced by Cd, Co, Cr and Ni, (2) Murueta samples with Sn and (3) Kanala by essential trace metals and Pb. Finally, and considering both scores and x-loadings plot simultaneously, different contamination sources could be defined. Distribution of samples in Kanala site is explained by natural variability observed among the individuals within the same habitat. On the other hand, clustering of Murueta sampling point is explained by the presence of shipyard explaining the Sn pollution. Finally, urban and industrial activities could be the main pollution sources in Arteaga due to the trace metal influence (Cd, Co, Cr and Ni).

Table 3 Correlation matrix for the metal concentrations obtained (mg kg^{-1})

	Cd	Cu	Pb	Mn	Ni	As	Cr	Co	Zn	V	Sn	Se
Cd	**1.00**											
Cu	0.20	**1.00**										
Pb	0.05	0.72	**1.00**									
Mn	0.51	0.68	0.73	**1.00**								
Ni	0.42	0.35	0.60	0.69	**1.00**							
As	0.24	**0.86**	0.75	0.77	0.41	**1.00**						
Cr	0.56	0.59	0.60	0.71	0.66	0.71	**1.00**					
Co	0.59	0.66	0.71	**0.92**	0.71	0.72	**0.81**	**1.00**				
Zn	0.44	**0.94**	0.71	**0.81**	0.42	**0.86**	0.69	**0.81**	**1.00**			
V	0.18	**0.87**	**0.81**	0.79	0.52	**0.85**	0.65	**0.81**	**0.86**	**1.00**		
Sn	0.11	0.44	0.56	0.53	0.37	0.49	0.48	0.54	0.51	0.56	**1.00**	
Se	0.34	**0.86**	0.70	**0.81**	0.39	**0.92**	0.70	0.78	**0.92**	**0.83**	0.55	**1.00**

Values higher than 0.8 in bold

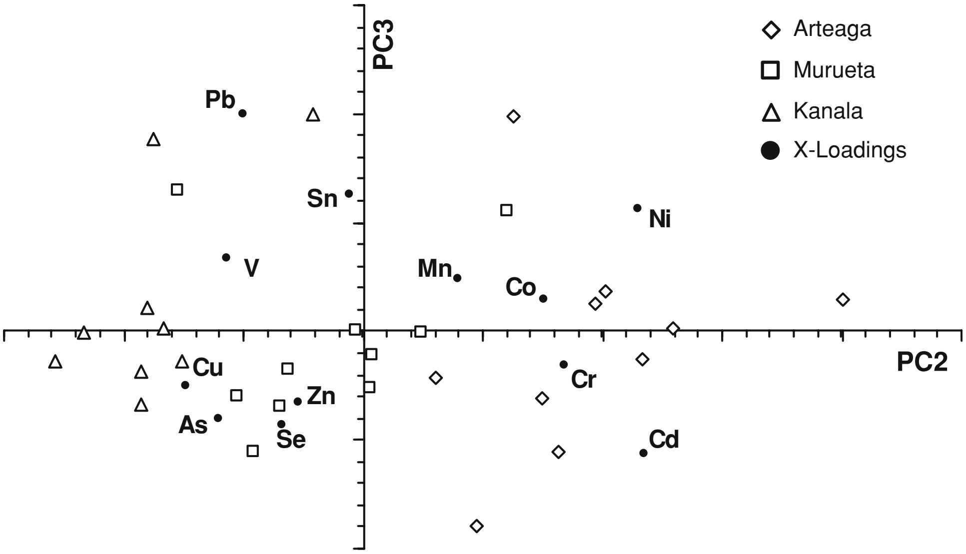

Fig. 3 Principal component analysis (PCA) biplot (PC2/PC3) for all samples and variables in all sampling points, Arteaga (◇), Murueta (☐) and Kanala (Δ)

The relationships evaluated between metal concentration and season and location sites suggest an influence of age and physiological patterns of oysters in metal uptake. This fact may condition the interpretation of the experimental data and multivariate analysis was found as an important tool to interpret the analytical data obtained. In that sense, PCA and correlation analysis confirmed that trace metals patterns vary throughout the sampling area and reflect contaminant source locations.

Acknowledgments This work was supported by the ETORTEK programme of the Basque Government through the Berrilur II project (IE06-179).

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