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Shell alterations in the limpet *Bostrycapulus odites*: A bioindicator of harbour pollution and mine residuals

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ABSTRACT

Molluscs are commonly used as bioindicators because of their abundance, low motility and the information their shells record. Although in harbour areas gastropod shell deposition would be affected at an endocrinal level, which may increase their vulnerability, studies on the shell of gastropods are scarce. *Bostrycapulus odites* is a limpet species that possess those characteristics as well as a wide distribution. Limpets were collected in 2001 and 2011, in a channel polluted by both, harbour activities and leaching mine residuals, to compare to a 2011 sample from an unpolluted area within San Antonio Bay. The sensitivity to pollution of this species and the possibility of it use to detect changes in the environmental situation of an area in a 10 years period were investigated. Soft body wet weight and shell morphological variables were measured while shells were also analyzed through scanning electron microscopy and energy dispersive spectroscopy for microstructure and elemental composition, respectively. Maximum likelihood ratio test showed shells from the polluted channel were thicker as well as the same shells presented microstructure malformations and changes in elemental composition (lower Ca and O levels, higher C and Fe levels). The present results indicate that *B. odites* can be considered a useful bioindicator species to study these kinds of pollution and the potential processes implicated in shell alterations are discussed.

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1. Introduction

Molluscs are widely studied as sentinel bioindicators due to their ecological and economic importance and their abundance and life habits, among other characteristics. Several sublethal effects on the shells of these organisms have been reported and related to different pollutants. In bivalves, longer shells, "ball-shaped", shell thickening and lower shell weight have been related to Tributyltin (TBT) pollution (Almeida et al., 1998a; Higuera-Ruiz and Elorza, 2009). Moreover, alterations of the bivalve shell inner layer and shell composition occur after exposure to heavy metals (Lopes-Lima et al., 2012). The limpet *Siphonaria lessoni* also develops shell organic malformations which were related to TBT and organochlorine pesticides (Nuñez et al., 2012). The integration of the available information about sensitivity of many species in different degrees of pollution, along with bioassays, contributes to the development of monitoring and remediation programmes. In this sense, bivalves are the main studied group while studies on the shell of gastropods are comparatively scarce. However, in harbour areas gastropods are affected at an endocrinal level by TBT (Carter, 2005; Abidli et al., 2012; Tallmon, 2012), which may increase their vulnerability to pollutants.

Although morphological variables are an indicator of pollution effects on molluscan shells, they are also susceptible to variation due to other environmental features (Giraldo-López and Gómez-Schouben, 1999; Tablado and López Gappa, 2001). The scanning electron microscopy (SEM) is a tool used to detect such effects but at a microstructure level (Márquez et al., 2011; Nuñez et al., 2012; Zuykov et al., 2012). The knowledge of micro-scale alterations can also help to conduct future more specific studies correctly.

In this work we study three populations of the limpet *Bostrycapulus odites* (*sensu* Collin, 2005; formerly *Crepidula aculeata*, Gmelin) from San Antonio bay in the Argentinean Patagonia. *B. odites* originally inhabits the Atlantic coasts of South America, from São Paulo, Brazil to Puerto Madryn, Argentina, and South Africa, from Cape Town to Port Elizabeth and north to northern Natal (Natal Museum) (Collin, 2005). However, Collin et al. (2010) have identified the invasive limpet in Alicante Harbour, Spain as this species and it had been introduced in other places expanding the species distribution (Coles et al., 1999). These limpets live at the

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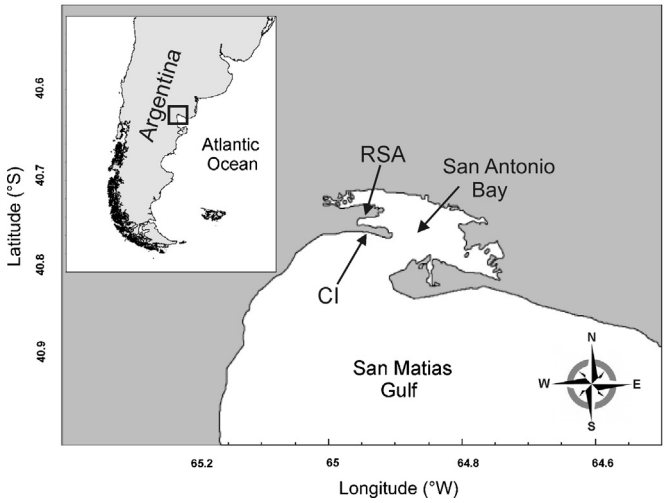

Fig. 1. Sampling sites in San Antonio Bay, North Patagonia, Argentina. Sampling sites are indicated with black arrows. *B. odites* individuals were collected at Ría San Antonio channel (RSA) and Canal del Indio channel (CI).

intertidal and subtidal zones at depths of at least 40 m. They had not ever been used in a pollution study but other species of the genus had been reported to present imposex (Carter, 2005).

The San Antonio Bay is located at the northeast of the Patagonia in Argentina within the San Matías Gulf (Fig. 1). Although it was declared a Natural Protected Area by the province of Río Negro, it is threatened by diverse sources of anthropogenic pollution. San Antonio Oeste city is located at the northwest edge of the bay and is one of the principal sources of pollution. The nearby Gonzalito mine operated during 20 years until 1980 while an electrochemical plant produced lead and silver within the town itself, piling the wastes around the city in the open air and in the bay edge. Gil et al. (1999) and Vázquez et al. (2007) recorded high levels of heavy metals in sediments and mussels and demonstrated that waste piles from the abandoned mine are still leaching various metals to the environment. There is also a port in the city where Bigatti et al. (2009) found high levels of TBT and imposex in the snail *Buccinanops globulosus*. Although there are not exact records of the pollutant concentrations within the period studied here, given the global trends, we could expect an increase of pollution levels in the area every year.

Within the bay there are two channels which constitute a good model to study pollution effects: Ría San Antonio channel and Canal del Indio channel (Fig. 1). The first one is located at the northwest of the bay and receives the pollution produced on the San Antonio Oeste harbour and the remaining residuals of the Gonzalito mine. The second channel is located at the southwest of the bay and it can be considered free of those pollution sources.

In this context, the purpose of the present study is to determine whether harbour pollution and abandoned mine residuals affect morphological characteristics and shell elemental composition and microstructure of the limpet *B. odites*. This would contribute to the information available about the effects of these kinds of pollution in gastropods. A second objective is to assess whether relevant differences of the pollution state of this area could be detected in a 10-year period, using this limpet species as a bioindicator. This, eventually, would provide valuable information about the usefulness of this widely distributed species for monitoring programmes.

By the foregoing, we propose the following two hypotheses: (i) shells of *B. odites* from Ría San Antonio channel should present any different morphological variable, irregular microstructure or elemental composition when compared to those from Canal del Indio channel and (ii) the shells of this species from Ría San Antonio collected in 2001 should present any different morphological variable, microstructure and elemental composition in relation to those collected in 2011.

2. Materials and methods

2.1. Samples collection

In 2011, limpets *Bostricapulus odites* Collin, 2005 were sampled from the intertidal zone of the two channels within San Antonio bay, Ría Storni and Canal del Indio (Fig. 1). Fifty individuals of *B. odites* collected in 2001 at Ría Storni channel were also studied to get a temporal comparison of pollution effects. All individuals were fixed in formaldehyde 4% and transferred to ethanol 96% after 1 week.

2.2. Morphological variables

Between 50 and 70 individuals of each site and year (Ría Storni 2001, Ría Storni 2011 and Canal del Indio 2011) were slightly dried with tissue paper and cleaned with a pin to remove adhering organisms or detritus. Then, each animal was excised and their soft body and shell wet weight (BWW and SWW, respectively) were determined to the nearest 0.001 g. Shell length (SL) and width (SW) were measured to the nearest 0.1 mm with a digital calliper. Finally, shells were cut in half with a small saw and shell thickness (ST) was measured with a micrometre eyepiece under stereomicroscope to the nearest 0.01 mm.

2.3. Statistical analysis

A set of models of morphological data from different sites and years were fitted using maximum likelihood. First, the association degree between SL, SW and SWW was analyzed through maximum likelihood ratio test (LRT) (Kimura, 1980; Cerrato, 1990) to evaluate which variables to use as independent. This method allows testing several hypotheses to compare two curves by analyzing one or more growth parameters simultaneously as detailed in the results section. Such variables showed an exponential relation with statistically equal parameters among the sites/times studied (no difference in slopes or intercept). Thus, to avoid redundancy in results, only SWW was used as independent variable in further analyses.

We used BWW and ST as dependent variables, and site and year as factors. The lineal (BWW vs. SWW) and the logarithmical (ST vs. SWW) equations fitted the data best. The equation parameters (slope and intercept) were compared using LRT.

Statistical analyses were conducted in R 2.13.0 (R Development Core Team, 2011). We used the library bbmle (Bolker, 2011) to find parameter values maximizing the likelihood.

2.4. Shell microstructure and composition

Three shell fragments and three transverse sections (randomly chosen) of each site and year where prepared for the microstructure study. Samples were first washed with distilled water and then placed in NaClO overnight and rinsed again with distilled water. Finally, they were dried at ambient temperature and metallized with Ag/Pd in a Denton Vacuum Desk II metallizer. The inner surfaces and transverse sections of the shells were examined with a scanning electron microscope Jeol JSM 6460LV in the Laboratorio de Microscopía Electrónica at the Universidad Nacional de Mar del Plata, Argentina.

Simultaneously, Energy Dispersive X-ray microanalysis was performed with an EDAX GENESIS V5.11 connected to that microscope to determine the elemental composition of the shells. Elements (C, O, Na, Cl, K, Ca and Fe) were analyzed in a reduced area

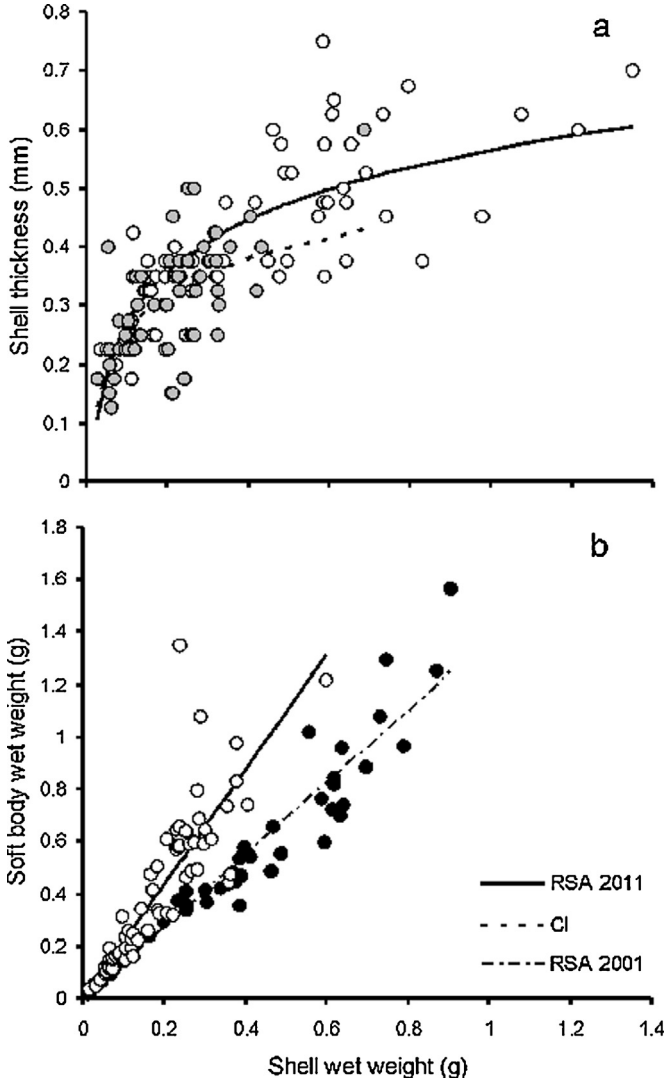

Fig. 2. Likelihood radio test comparison. (a) Plot showing the relation shell thickness-shell wet weight of shells from Canal del Indio (grey circles) and Ría San Antonio 2011 (white circles). (b) Plot showing the relation soft body wet weight-shell length of shells from Ría San Antonio 2001 (black circles) and 2011 (white circles).

of the surface (punctual measurement) and by mapping a 713 μm^2 area (map measurement). The punctual measurements in shells from Ría San Antonio (2001 and 2011) were done in a reduced area without malformation and in a reduced area with malformation.

3. Results

3.1. Morphological variables

Spatial comparison of *B. odites* morphological variables (Ría Storni and Canal del Indio, both 2011) showed some differences. ST and SWW relation differed in slopes between reference and polluted sites ($p < 0.01$) (Table 1). In general terms, shells from Ría Storni were thicker than those from Canal del Indio (Fig. 2a). The other morphological variables did not present significant differences.

Temporal comparison of *B. odites* morphological variables showed differences in the BWW and SWW relation. Slopes show significant differences ($p < 0.001$) (Table 1) between 2001 and 2011, being bodies from 2011 heavier than those from 2001 (Fig. 2b).

3.2. Shell microstructure and composition

Scanning electron microscope observations revealed malformations in the inner surfaces of the shells belonging to Ría San Antonio 2001 and 2011 (Fig. 3c–f). In general, no differences were found either in malformations or in elemental composition between shells from these years. On the other hand, shells from Canal del Indio exhibited smooth inner surfaces with a regular crystallization (Fig. 3a and b). Shells transverse sections did not show any difference in the crystallization pattern between different sites or years.

Malformations of shells from the polluted area, both 2011 and 2001, showed important differences in elemental composition compared with the unpolluted site (Table 2). Carbon content doubled the reference, Oxygen content was three and two-fold (respectively) lower and Calcium content was eight and sixteen times lower. Finally, Iron was detected in such malformations (Table 2).

Although the differences in elemental composition were more evident in the malformations, calcium was also lower in the whole shell area of both polluted sample groups. Further, polluted shells of 2001 presented excessive Carbon content in areas without malformations (Table 2). All the above results were confirmed through the elemental composition maps (Fig. 4). This qualitative technique supplemented and supported the punctual measurements satisfactorily providing evidence of the same elemental composition alteration when a major area is covered.

4. Discussion

Shells from Ría San Antonio and Canal del Indio presented differences in one morphological feature (shell thickness), in shell elemental composition and shell microstructure. On the other hand, organisms from different years differ also in one morphological variable (soft body wet weight) but did not show contrasting shell microstructure or elemental composition.

When comparing same weighted shells from control and polluted sites the last were thicker. This result was expected because it is a widely reported pollution effect although the majority of these reports are studies with oysters in which shell thickening has been observed as a consequence of shell chambering (Alzieu et al., 1986; Dyrynda, 1992; Almeida et al., 1998b). This abnormal thickening has been always related to TBT contamination, not only because it is observed in sites with high levels of such compound but also because it was experimentally tested in *Crassostrea gigas* (Alzieu et al., 1982; Lawler and Aldrich, 1987) and moreover, tin content was found in malformed chambers of *Ostrea edulis* (Medakovic et al., 2006). There are a few studies reporting shell thickening in gastropods, which related it with TBT and organochlorine pesticides (Nuñez et al., 2012). Although in Ría San Antonio (polluted site), where shells were thicker, high levels of TBT have been reported, it would be lacking experimental testes to prove this relationship in gastropods. Moreover, despite the fact that in the available bibliography, shell thickening is mainly related with TBT, other pollutants like heavy metals, coming from the abandoned mine residuals or hydrocarbons that are also present in great amounts in harbours, cannot be neglected.

Scanning electron microscopy observations revealed malformations in the inner surface of *B. odites* shells from the polluted Ría San Antonio, both 2001 and 2011 and a smooth surface in shells from Canal del Indio; further, we found an increase in C and a decrease in Ca and O content in the shells, which may be tightly related to such malformations. There are few studies where malformations in mollusc shell microstructure were observed. In a previous

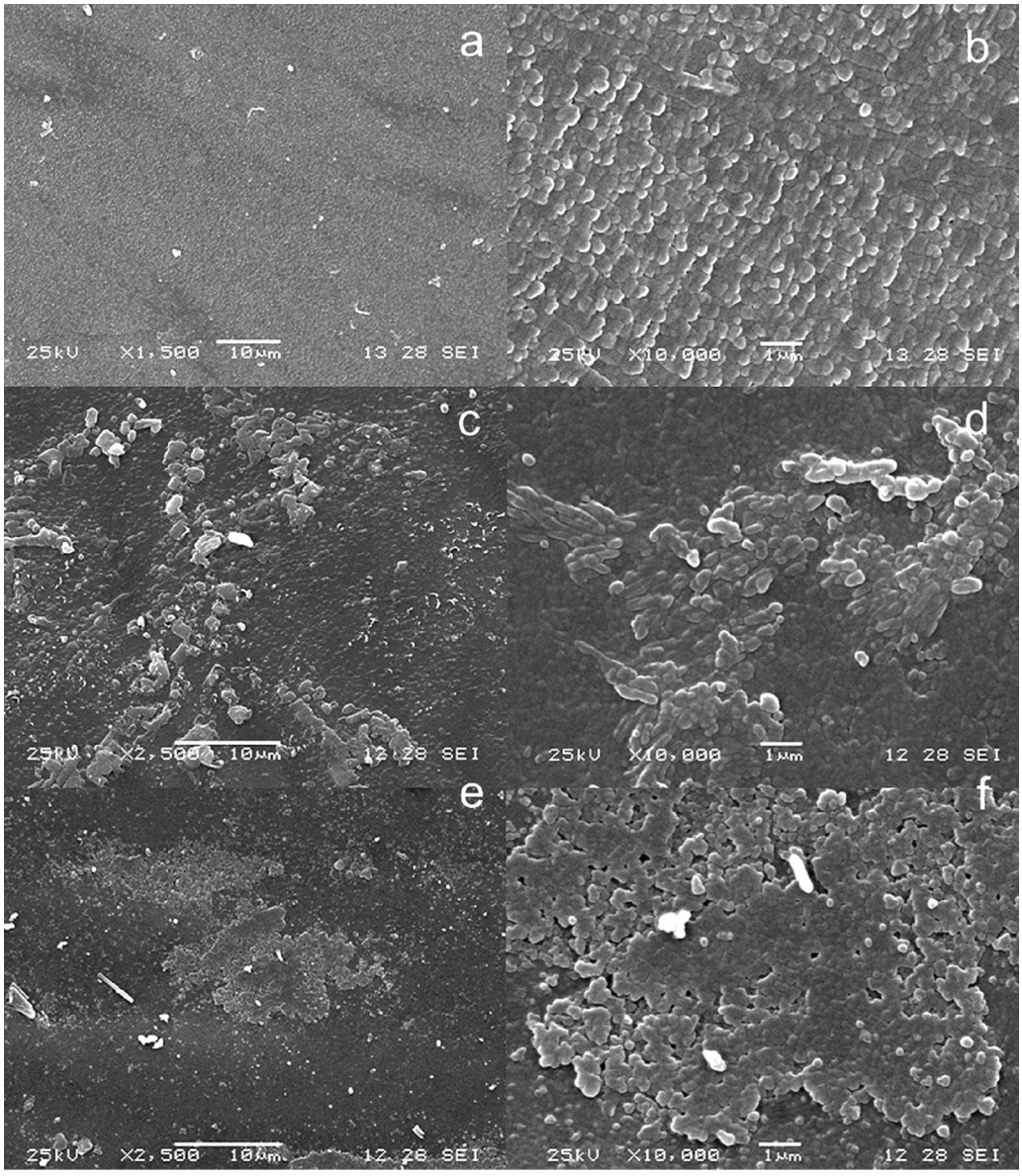

Fig. 3. SEM images of the *B. odites* inner shell layer. (a and b) shells from Canal del Indio, (c and d) shells from Ría San Antonio 2011, (e and f) shells from Ría San Antonio 2001.

study of the limpet *S. lessoni* from two ports, we observed similar malformations and related them with organotins and organochlorine pesticides (Nuñez et al., 2012). Higuera-Ruiz and Elorza (2009) suggest that the malformations that they found in the oyster *C. gigas* shell microstructure are caused by TBT and propose a denaturalization of the organic matrix by this compound, which ultimately produces loss of cohesion in the mineralized part. Further evidence of TBT toxicity in mollusc shells includes alterations of shell organic matrix amino-acids composition (Krampitz et al., 1983; Almeida et al., 1998a). Therefore, we can think that this compound could be the responsible for generating the malformation in the species studied here by altering the organic matrix.

However, malformations of the inner layer of molluscan shells due to heavy metals have been experimentally tested. Cd^{2+} exposure produced changes in shell crystallization of the inner shell layer of the freshwater mussel *Anodonta cygnea*, indicating an ion disturbance in shell calcification (Faubel et al., 2008). These authors observed SEM images similar to those presented here and suggest an accelerated precipitation of the $CaCO_3$. Further, after exposure to Cu^{2+} or Cr^{3+} the prismatic and nacreous shell layers of the

Table 1
Comparison between sites or times of morphological variables relationships through likelihood radio test. RS: Ría San Antonio (polluted site); CI: Canal del Indio (unpolluted site); BWW: soft body wet weight; ST: shell thickness; SWW: shell wet weight. *a*, slope; *b*, intercept; n.s., not significant.

Comparison	Sites or times	Relation	H_{01} $a_1 = a_2$	H_{02} $b_1 = b_2$	H_{03} $\theta_1 = \theta_2$
Likelihood ratio test	RS–CI	BWW-SWW	n.s.	n.s.	n.s.
		ST-SWW	<0.01	n.s.	<0.01
	2001–2011	BWW-SWW	<0.001	n.s.	<0.001
		ST-SWW	n.s.	n.s.	n.s.

Table 2
Elemental composition in percentage of atoms (A%) of *B. odites* shells. C: control area (without malformation) M: malformation area. In bold, conspicuous difference in shell elemental composition respect to shells from the reference site Canal del Indio.

Element A%	Sites				
	Canal del Indio	Ría San Antonio 2011		Ría San Antonio 2001	
		C	M	C	M
C	34.07	39.97	**71.94**	**50.52**	**73.54**
O	29.33	28.45	**9.49**	28.71	**14.41**
Na	0.88	0.43	0.93	1.01	0.56
Cl	0.05	0.00	0.42	0.07	0.00
K	0.00	0.00	0.13	0.14	0.09
Ca	33.03	**19.7**	**4.51**	**12.3**	**2.17**
Fe	0.09	0.34	**1.02**	0.16	**0.97**

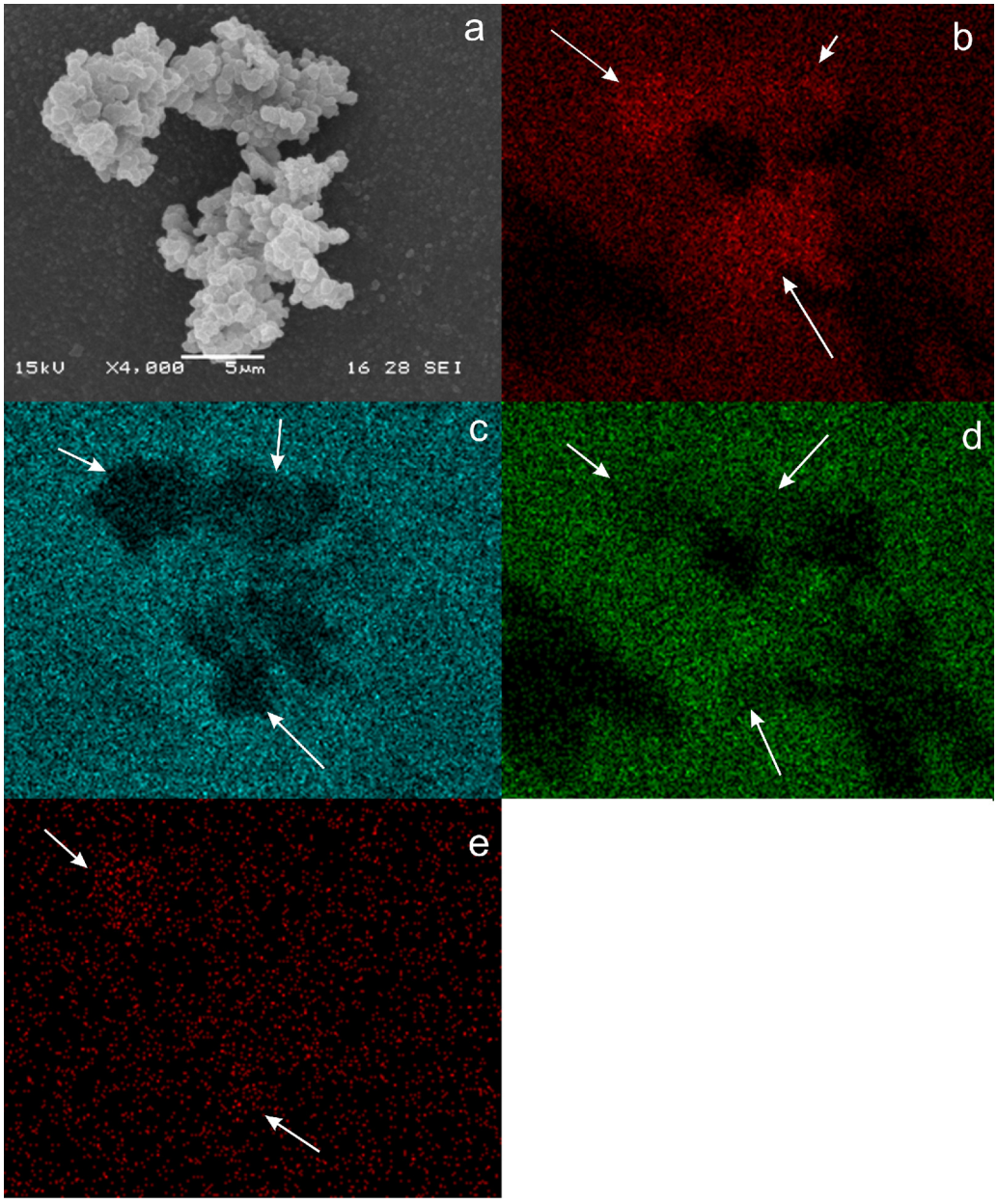

Fig. 4. Elemental composition map of a malformation in the inner shell layer from Ría San Antonio 2011 (713 μm^2). (a) SEM image of the malformation, (b) carbon content map, (c) calcium content map, (d) oxygen content map, (e) iron content map.

same bivalve species developed malformations, bearing the last the greatest effects (Lopes-Lima et al., 2012). It is also suggested that alteration occurs in the organic matrix which interferes with normal $CaCO_3$ crystal mineralization.

Heavy metals also have been proved to affect calcium metabolism and transport (Antunes et al., 2002). Their incorporation into the shell is affected by environmental and physiological factors (Kanduc et al., 2011) and some of them can be incorporated in bivalve shells through isomorphic substitution and replace calcium ions (Lingard et al., 1992). Almeida et al. (1998b) found a decrease in Ca content in the shells of *C. gigas* exposed to high concentrations of Pb and a great amount of this element content. Although we found a minor amount of calcium atoms in shells from the polluted site, we did not observe a great increase of any metal, except for a little rise in iron atoms. However, this difference could be due to metals concentrations: while Almeida et al. (1998b) obtained those results experimentally with high concentrations of that metal, we observed elemental composition differences in a natural environment metal concentration. Thus, in our case Fe could be replacing Ca atoms. Finally, carbon and oxygen content also changed, mainly within the malformations, which could be a consequence of calcium content modification. Although Energy Dispersive X-ray microanalysis is not a greatly accurate approach since it generate semi-quantitative data, given the wide differences found between the control and polluted sites, we consider we can rely on these results. Whether produced by heavy metals, TBT or another pollutant, malformations and altered elemental composition, may be a consequence of the alteration of the shell organic matrix. From empirical evidence we know that metals produce similar changes but that organotins alter the organic matrix. Thus, more studies are needed to reach certain conclusions with respect to the compounds and processes that cause such effects.

Although between years *B. odites* shells microstructure and elemental composition did not present differences, in 2011 *B. odites* samples had lighter bodies than 2001. Reduced soft tissue weights have been previously found in the gastropods *Odontocymbiola magellanica* and *Macoma balthica* and it was associated with high levels of TBT (Márquez et al., 2011; Smolarz and Bradtke, 2011). However, another factor affecting soft body weight in molluscs and other invertebrates is the reproductive stage in which the specimens are collected. *B. odites* from 2001 were caught in April, whereas those from 2011 were collected in February. Females of co-occurring calyptraeid species spawn their egg masses until March (Cledón and Penchaszadeh, 2001; Cledón et al., 2004). In this sense female gonads are ripe the whole summer, while at the end of this season re-absorption of sexual products initiates (Cledón and Penchaszadeh, 2001). To test this possibility, we analyzed the relation between gonad and somatic wet weight, being the gonad wet weight from organisms from 2011 higher than those from 2001 (see Supplementary material). In the light of this, we can deduce that the reproductive stages are causing the differences in soft body wet weight in the temporal analysis.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2013.05.022.

On the other hand, individuals of *B. odites* can reach a 5 years age (unpublished results), so population renewal of 2 generations occurred in the time period of this study. Hence, the same malformations observed both in 2001 and 2011, were generated at least at two different instances in the studied population. This means that during the time span studied these limpets were exposed to the same kind of pollution. Pollution trends in the studied area show increments in TBT levels: 3.1 ngSn/g in 2005 (Delucchi et al., 2011), 20.1–33.3 ngSn/g in 2006 (Bigatti et al., 2009) and 125 ngSn/g in 2007 (De Waisbaum et al., 2010). However, there are no records after the total ban on 2008 of the paints containing this compound. On the other hand, heavy metals presented a decrease between 1995 and 2003 (Gil et al., 1996; Vázquez et al., 2007), but there are not more recent records.

This study demonstrates that *B. odites* can be considered a sensitive bioindicator of multiple-source pollution. Shell malformations, thickening and altered elemental composition have been found in organisms from a highly polluted site in San Antonio Bay, Argentina. However, in contrast to our expectations, from the features of the limpet species studied in this work, we are not able to deduce changes in the environmental situation of the area in a 10-year period. Therefore, either pollution could have remained stable along these years or, more probably, the effects in these limpets of an increase of such pollution could be evident in a larger period of time than 10 years.

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