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An intertidal limpet species as a bioindicator: Pollution effects reflected by shell characteristics

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ABSTRACT

Mollusc shells have been widely used for monitoring the bioavailability of contaminants in the aquatic environment. The present work examined malformations among the shells of the limpet *Siphonaria lessoni* from heavily polluted, light polluted and unpolluted sites in Argentina. Data on shell shape, thickness, dry weight, microstructure and semi-quantitative elemental composition was evaluated as well as soft tissue dry weight. Shells from the heavily polluted site were significantly ($p < 0.001$) thicker than those from other areas. SEM (scanning electron microscopy) analysis of thickened shells revealed the presence of globular malformations on inner shell surfaces. On heavily polluted shells, elemental composition analysis by EDS (electron dispersive spectroscopy) of such malformations indicated concentrations three times higher of carbon and four times lower of calcium and oxygen than the control. Light polluted shells presented concentrations two times lower of calcium. In addition, soft tissues were lighter at the heavily polluted site ($p < 0.001$). These data demonstrate the sensitivity of this abundant and widely distributed intertidal limpet to aquatic pollutants, and support the use of this limpet as a potential biomarker.

1. Introduction

Hard structures of marine organisms contain considerable information about their own growth history, including the changing conditions of mineralization and environmental stress or disease (Okoshi and Sato-Okoshi, 1996). Mollusc shells contain not only a record of their life history but also information of environmental changes preserved through structural, morphological, and chemical changes within the shell.

The presence of malformations (Frazier, 1976; Phillips, 1977, 1978) as well as histopathological injuries on molluscs have been linked with environmental chemical pollution (Gold-Bouchot et al., 1995; Najle et al., 2000). Moreover, other contaminants effects, such as shell thickening of bivalves (Almeida et al., 1998a; Waldock and Thain, 1983), differential size in limpets (Espinosa et al., 2007), inhibition of shell growth (Cunningham, 1976) and shell chambering (Bayen et al., 2007) in oysters are well described.

Species with high sensitivity, low motility and wide distribution are preferred for monitoring since they facilitate location of high polluted areas and comparison among wide separated places. This is the case of siphonarids, which are distributed around the world, and inhabit both South American coasts. De Pirro and Marshall (2005) found that siphonarids tolerate polluted environments better than patellogastropod limpets, and suggested them as pollutant indicators. In fact *S. lessoni* is the dominant intertidal mollusc species in two polluted areas of the South West Atlantic coast, Mar del Plata and Quequén ports. Meanwhile in other nearby less polluted intertidal zones *S. lessoni* cohabits with large population of mussels.

Observations of *S. lessoni* anatomy, development and spatial distribution have been reported from natural areas of rocky shore in Mar de1 Plata (38°03′ S, 57°33′ W) (38″03′ S, 57″33′ W) (Olivier and Penchaszadeh, 1968), and its growth and feeding habits were studied in populations growing on rafts within the port of Mar de1 Plata (Bastida et al., 1971). Tablado et al. (1994) and Tablado and López Gappa (2001) reported morphometric differences observed in this limpet from different habitats as a result of differential growth rates in response to environmental pressures: tidal level, wave exposure, food availability, and intraspecific competition. However, sensitivity to pollution has never been evaluated.

The aim of the present study is to determine whether pollution affects *S. lessoni* shell deposition, structure and morphology to evaluate whether this species can be used as a bioindicator. In order to achieve this objective *S. lessoni* population from three differently polluted areas are compared.

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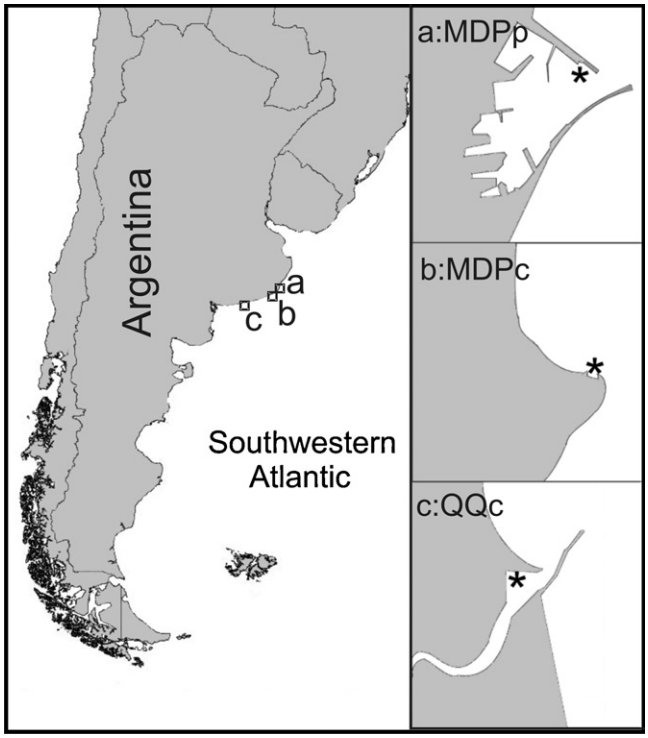

Fig. 1. Sampling sites. Study sites showing location where individuals of *S. lessoni* were collected. Sites codes: high polluted sites MDPp (Mar del Plata port), QQp (Quequen port) and less polluted site MDPc (Waikiki beach).

2. Materials and methods

2.1. Study sites

Siphonaria lessoni was sampled in two areas of the Mar del Plata shore (MDPc and MDPp) and one area in Quequén port (QQp) (Fig. 1). The three chosen areas have similar environmental and ecological characteristics but different contaminant concentrations. The first zone, MDPc (Waikiki beach 38°04′50 S, 57°30′08 W) is located within the southern limit of Mar del Plata city. This zone is protected by a jetty and considered to be slightly polluted (Laitano, unpublished data). The second zone, MDPp, was set within the MDP port, 5 km northwards to the first zone (38°02′10 S, 57°31′28 W). It is considered to be highly polluted: 728.3 ng/g of Tributyltin (Bigatti et al., 2009; Cledón et al., 2006), 6.78 ng/g of PCB's, 8.75 μg/g of hydrocarbons and 30.59 ng/g of organochlorine pesticides (Colombo et al., 2005). The third zone QQp (38°29′23 S; 58°48′59 W) is more contaminated than MDPc, but less than MDPp: 1.4 ng/g TBT, 5.77 μg/g of hydrocarbons, 2.51 ng/g of PCB's and 4.91 ng/g of organochlorine pesticides.

2.2. Morphometric

During April 2008, 30 individuals of *S. lessoni* were collected at each site. Only adult specimens (>6 cm) were included in all morphometric analyses. The shells were photographed from the right side and were always positioned in the same orientation. Digital images were taken against a white illuminated background in order to maximize the contrast of shell outlines. All the images were binarized and then processed using the SHAPE software (Iwata and Ukai, 2002).

The shape of the shell of these limpets is rather simple with very few homologous points that can be used as landmarks; moreover they are difficult to localize because they correspond to landmarks type 2 (maximum of curvature along the boundary or outline of a specimen) (Bookstein, 1991). Therefore, the shell shape variation between *S. lessoni* specimens from MDPp, MDPc and QQp was measured using outline analyses based on the Elliptic Fourier analysis (EFA) on the outline coordinates (Rohlf and Archie, 1984).

Elliptic Fourier coefficients were mathematically normalized in order to avoid biases in results caused by different size, location, rotation and starting position of shells (Rohlf and Archie, 1984). The closed curve of each shell was decomposed into a sum of 15 harmonically related ellipses. These 15 harmonics represent 99.99% of the total Fourier power spectrum (Crampton, 1995). With the PrinComp module, we performed the principal components analyses (PCA) on the variance–covariance matrix of the normalized Elliptic Fourier coefficients. PCA is effective in summarizing the information regarding the variation contained in these coefficients (Rohlf and Archie, 1984), which were estimated using PAST v.1.77 (Hammer et al., 2001). Finally, multivariate analyses of variance (MANOVA) were performed with PAST in order to evaluate the importance of between-group differentiation relative to within-group variation. A test to detect if there are any significant morphological differences (Wilk's Lambda test) was performed and *post hoc* Hotelling pairwise comparisons (Bonferroni corrected and uncorrected) were also performed using PAST to detect the significant differences.

2.3. Morphological variables

A pool of 100 individuals of *S. lessoni* was collected at each of the three sites around a randomly determined point. Shell length, was measured to the nearest 0.1 mm with a micrometer eyepiece under a binocular microscope and individual shell thickness was measured under stereomicroscope to the nearest 0.01 mm. To determine the dry weight of shells and soft tissues, limpets were frozen at −20 °C for 24 h prior to dissection. Then, they were thawed and whole tissue was removed from shell using a scalpel. They were placed into pre-weighted aluminium pans, dried for 12 h at 75 °C, and weighed to the nearest 0.001 g.

Data normality (Shapiro–Wilk test) and homogeneity of variances (Cochran's test) were tested and when necessary transformed (Underwood, 1997; Zar, 1999). The slopes and elevations of the regressions of the shell thickness, dry weight of shells and soft tissues (dependent variables) were tested with analysis of covariance (ANCOVAs) to assess the pollution effects (difference between zones) using limpet shell length as the covariate. ANCOVA can be used to compare elevations of regression lines if their slopes are not statistically different (Zar, 1984). When slopes were heterogeneous, we used Tukey multiple comparison tests (Zar, 1984) to determine which combinations of slopes differ. In these cases, we applied the Johnson–Neyman test (Huitema, 1980) to identify the depth range over which elevations are not significantly different.

2.4. Microstructure and shell elemental composition

A detailed observation of the inner surface of the shell of three individuals of each place was done through Scanning Electron Microscopy (SEM). Samples were metallized with Ag/Pd in a Denton Vacuum Desk II metallizer. The analyses were carried out with a Jeol JSM 6460LV microscope in the Laboratorio de Microscopía Electrónica at the Universidad Nacional de Mar del Plata, Argentina.

Simultaneously, Energy Dispersive Spectroscopy analysis was performed with an EDAX GENESIS V5.11 connected to the microscope to determine the elemental composition of the shells. Elements analysed were C, O, Na, Mg, Al, Si, Cl and Ca. Energy

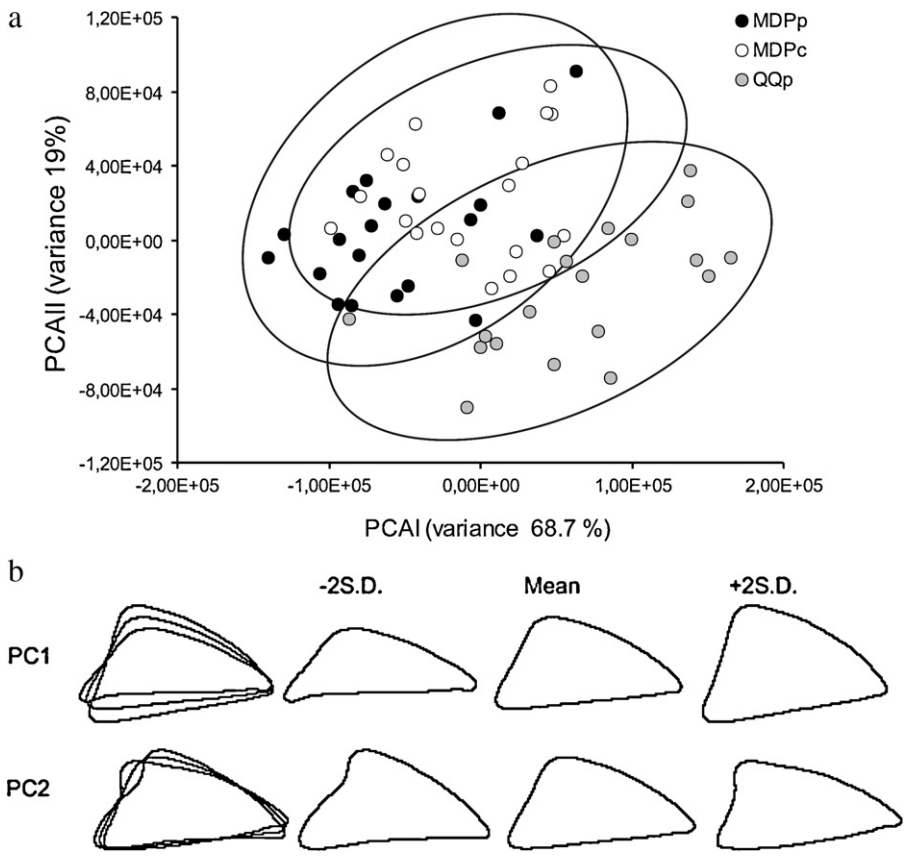

Fig. 2. Shell shape. (a) Multivariate principal components analysis of the *S. lessoni* shell shape variation. Site codes: high polluted sites MDPp (black circles), QQp (grace circles) and less polluted site MDPc (white circles). (b) Mean shape, +2 and −2 standard deviation (SD) along each principal component.

dispersive spectroscopy (EDS) is a method based on the quantification of photons released by the sample when it is bombed with electrons. The software transforms the peaks of photons into counts to determine the elemental composition which is semi-quantitative due to it is approximated from data into the software but not from a pattern sample (for further examples see Adams and Shorey, 1998; Chou et al., 2010; Yoon et al., 2003).

3. Results

3.1. Morphometrics

Principal components (PCA) and multivariate analyses (MANOVA) detected significant differences between the shapes of *S. lessoni* among all zones. PCA indicated that PC1 and PC2 together explained the 87.65% of the variance (Fig. 2a). PC1 explained 68.7% of the variance and mainly consisted of shell height change, while PC2 (19%) corresponded to small changes in shell length (Fig. 2b). Hotelling pairwise comparisons detected significant levels of differentiation among places (Wilk's lambda = 0.216; $F = 12.19$; $p < 0.05$). In general, pairwise comparison results indicated that the QQp differences were several orders of magnitude higher than MDPc and MDPp results (Table 1).

3.2. Morphological variables

Shell thickness and dry weight of shells slopes were heterogeneous, while soft tissue slopes were homogeneous among sites (Table 2). Multiple comparison of shell thickness showed that the slopes of MDPc vs MDPp do not differ. Regression line elevation of MDPp exceeds MDPc. In comparisons where slopes differ significantly, MDPp and QQp present a larger slope than MDPc. Johnson–Neyman tests showed that in the cases where slopes differ, the elevations were significantly different within the size range (Fig. 3).

Multiple comparison of the dry weight of shells showed that the slopes of two combinations did not differ: MDPc vs MDPp and MDPp vs QQp (Table 2). The elevation of MDPc and QQp exceeded that of MDPp. In comparisons where slopes differed significantly, the biggest difference was that the QQp increased with size faster than the MDPc. Johnson–Neyman test showed that in the cases where slopes differed, the elevations were significantly different within the size range (Fig. 4).

Soft tissue dry weight multiple comparisons showed that slopes of all combinations did not differ (Table 2). The elevations in degree of increase were MDPc > QQp > MDPp (Fig. 4).

MDPc vs MDPp and MDPp vs QQp did not differ (Table 2). The elevations for MDPc and QQp exceeded that of MDPp. In comparisons where slopes differed significantly, the biggest difference was that the QQp increased with size faster than the MDPc. Johnson–Neyman test showed that in the cases where slopes differed, the elevations were significantly different within the size range.

Table 1
Morphometric pairwise comparisons. *p*-Values of hotelling pairwise comparisons between *S. lessoni* morphotypes of MDPp, MDPc and QQp. Bonferroni corrected (above diagonal) and uncorrected (below diagonal).

	MDPp	MDPc	QQp
MDPp		0.017	$1.62E^{-11}$
MDPc	0.521		$1.10E^{-7}$
QQp	$4.86E^{-11}$	$3.30E^{-7}$	

Table 2
Morphological variables analysis. ANCOVAs for *S. lessoni* soft tissues dry weight and shell thickness and dry weight against size of highly polluted zones MDPp (Mar del Plata port), QQp (Quequen port) and less polluted zone MDPc (Waikiki beach). The dependent variables and covariate are expressed as logarithm. Inequality signs indicate significant differences ($p < 0.05$) and the direction of the difference. For comparisons where slopes differ, the Johnson–Neyman (JN) test provides the depth range (m) over which the compared regression lines did not differ significantly in elevation.

ANCOVA	df	*F*
Shell thickness		
Slope	2	29.84***
Elevation	2	391.4***
Dry weight of shells		
Slope	2	8.43*
Elevation	2	28.9***
Soft tissues		
Slope	2	n.s.
Elevation	2	121.6***

Multiple comparisons	Slope	Elevation	J–N test
Shell thickness			
MDPc vs MDPp	n.s.	MDPc < MDPp	–
MDPc vs QQp	MDPc < QQp		0.89–1.59
MDPp vs QQp	MDPp < QQp		1.78–2.60
Dry weight of shells			
MDPc vs MDPp	n.s.	MDPc > MDPp	–
MDPc vs QQp	MDPc < QQp		1.23–1.72
MDPp vs QQp	n.s.	MDPp < QQp	–
Soft tissues			
MDPc vs MDPp	n.s.	MDPc > MDPp	–
MDPc vs QQp	n.s.	MDPc > QQp	–
MDPp vs QQp	n.s.	MDPp < QQp	–

* $p < 0.05$.
*** $p < 0.001$.
n.s., not significant.

3.3. Microstructure and shell elemental composition

The SEM images revealed the presence of globular malformations on the inner surface of the shells from the MDPp and QQp, but MDPc shells showed a smooth inner surface without any evidence of alterations. These malformations were confined to a shell zone faraway from the edge. In addition, the elemental composition of the malformations was different depending on the sampling zone. Compared with MDPc, MDPp presented concentrations three times higher of carbon and four times lower of calcium and oxygen while QQp shells presented concentrations two times lower of calcium and the presence of aluminium (Table 3).

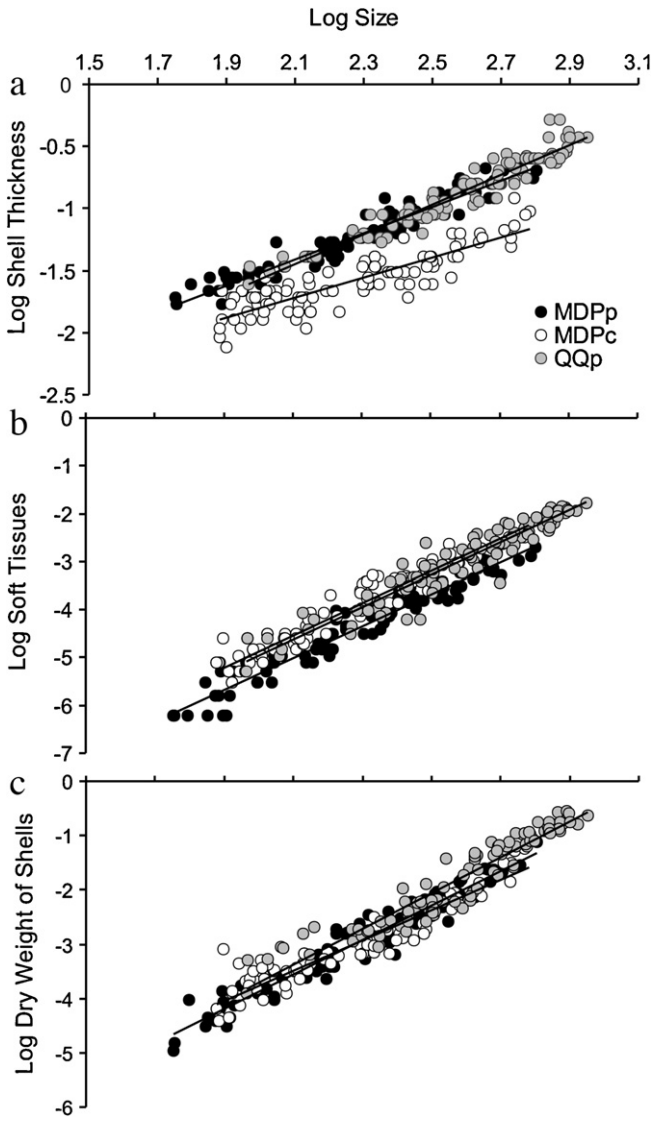

Fig. 3. ANCOVA analysis. (a) Plot showing linear increase in shell thickness related to shell length at high polluted sites MDPp (black circles), QQp (grace circles) and less polluted site MDPc (white circles). (b) Plot showing linear increase in soft tissues dry weight related to shell length. (c) Plot showing linear increase in shell dry weight related to shell length.

4. Discussion

The present results demonstrate that pollution produces changes in *S. lessoni* soft tissue dry weight and shell thickness, dry

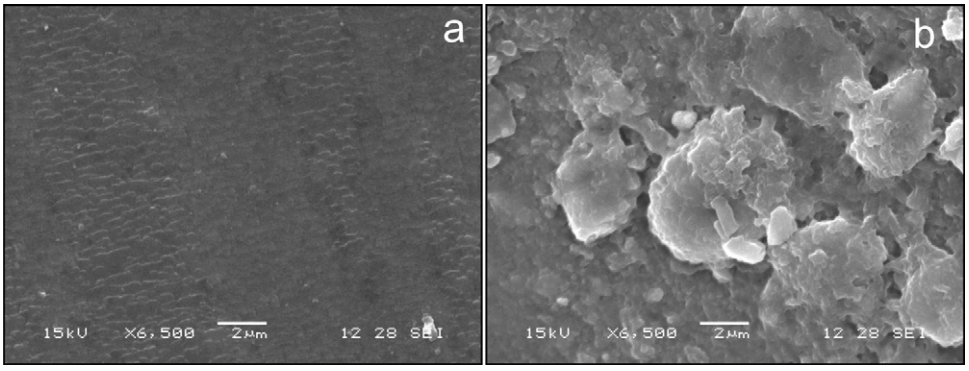

Fig. 4. SEM images of the inner side of the shell. (a) Shell of an individual from the less polluted site (MDPc). (b) Shell of an individual from the high polluted area (MDPp).

Table 3
Semi-quantitative elemental composition analysis. Elemental shell composition (in percentage) of individuals from high polluted zones MDPp (Mar del Plata port), QQp (Quequen port) and less polluted zone MDPc (Waikiki beach). Entries in italics indicate conspicuous alternation from MDPc reference of elemental composition.

Element	Treatments					
	MDPc		QQp		MDPp	
	Wt%	At%	Wt%	At%	Wt%	At%
C	15.8	34.29	*17.41*	*33.34*	*52.86*	*84.81*
O	26.65	43.42	*32.65*	*46.93*	*6.83*	*8.22*
Na	0.5	0.5	0.4	0.4	*0.12*	*0.1*
Mg	0	0	*0.4*	*0.4*	0	0
Al	0	0	*2.17*	*1.85*	0	0
Ca	26.83	18.31	*19.12*	*10.97*	*7.05*	*3.39*
Cl	0	0	0.31	0.2	0	0
Si	0	0	*3.17*	*2.59*	*0.15*	*0.11*

weight, shape, microstructure and elemental composition. Soft tissues dry weight in the limpets of both ports was lower in average in relation to individuals of less polluted site. This coincides with the effect reported on the intertidal snail *Nerita atramentosa* when exposed to oil spills (Battershill and Bergquist, 1982).

Moreover, while shells from the highly polluted areas are thicker than those from the less polluted one, dry weight of the shells indicate the opposite. Therefore hardness in polluted areas decreases. Besides pollution, other environmental and biological factors that affect shell thickness in molluscs are predator presence (Appleton and Palmer, 1988; Cheung et al., 2004; Palmer, 1990; Trussell, 1996) and coastal energy (Giraldo-López and Gómez-Schouben, 1999). Predators are present all along the study coasts; consequently they are not likely to be a factor causing differences among areas. Coastal energy was intended to be as similar as possible for the three selected zones. However it is likely that port sites are more protected. Thus, if some effect is to be expected on shell thickness due to environmental energy, the contrary should have been observed (i.e. shells from MDPc should be thicker). In this context, if environmental conditions could be controlled under laboratory conditions, it is likely that differences in shell thickening due to pollution would be even more conspicuous.

Shells from Quequén individuals were higher than the other populations. Previous studies have shown that shell height can be influenced by several environmental variables. For example it was found that *siphonsrids* shells height changes at sites with lower environmental energy (Tablado et al., 1994) as well as due to thermal stress (Harley et al., 2009).

SEM images revealed the presence of globular malformations in the microstructure of the inner surface of the shells. These malformations were deposited principally in a specific zone of the shell that coincides with the layer M-1 described by Fuchigami and Sasaki (2005) which in patellogastropods is characterized by a crossed lamellar structure. Such particular structure would be more sensible to pollution than the others. However this assumption cannot be confirmed since the way in which these layers are formed and differentiated is still not described (Suzuki et al., 2010).

The elemental composition of shell malformations was different between the ports sites as well as in comparison to the less polluted site. These results indicate alterations on the organic matrix secretion and/or on the calcium carbonate deposition (which remains to be tested) as shell thickening is the anatomical expression of such alterations. Similar results were reported for *Crassostrea gigas* by Almeida et al. (1998b), where an altered shell amino-acid composition together with lower Ca, Na, Co and higher Cl were observed due to lead pollution.

On the other hand, Héral et al. (1989) suggested that organotins can affect shell deposition through the ATP synthesis inhibition (decrease the concentration of the Ca-ATP chelate which reacts with HCO_3) and the reduction of respiration rate (decrease in CO_2 necessary for the formation of $CaCO_3$). Furthermore, the exposure to TBT has been related to alterations in the main shell organic matrix amino-acids (Krampitz et al., 1983; Almeida et al., 1998a) and to the inhibition of the amino-acid transport (Singh and Bragg, 1979).

The difference of the malformations compositions found between the ports shells could be originated by unequal concentrations of some pollutant on both ports. TBT has been reported as a mollusc shell thicker (Alzieu et al., 1986; De Fur et al., 1999; Márquez et al., 2011; Waldock and Thain, 1983) and shell chambering inductor (Chagot et al., 1990) in bivalves at levels as low as 0.05 mg/L in seawater. In the case of the ports studied here, TBT was followed in a series of previous reports in the area during the last decade (Bigatti et al., 2009; Cledón et al., 2006; Goldberg et al., 2004) revealing a gradual increase in TBT pollution at MDPp but very low values at QQp. Organochlorine pesticides (OCP) present the same pattern as TBT (Colombo et al., 2005). For this reason we are not able to asseverate which of both pollutants is causing the malformations. Other pollutants present on both ports are PCB's and hydrocarbons, but their concentrations in sediment do not differ so much among ports (Colombo et al., 2005).It is clear that *S. lessoni* is sensitive to certain pollutants. It remains to perform bioassays to determinate the sensitivity of *S. lessoni* to organic pesticides and TBT. Beyond this, the present study demonstrates that siphonariids are useful for pollution biomonitoring.

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References

Adams, S.M., Shorey, C.D., 1998. Energy dispersive spectroscopy of granular concretions in the mantle of the freshwater mussel *Hyridella depressa* from Lake Burragorang as a technique to monitor metals in aquatic systems. Aquat. Toxicol. 44, 93–102.

Almeida, M.J., Machado, J., Moura, G., Azevedo, M., Coimbra, J., 1998a. Temporal and local variations in biochemical composition of *Crassostrea gigas* shells. J. Sea. Res. 40, 233–249.

Almeida, M.J., Moura, G., Pinheiro, T., Machado, J., Coimbra, J., 1998b. Modifications in *Crassostrea gigas* shell composition exposed to high concentrations of lead. Aquat. Toxicol. 40 (4), 323–334.

Alzieu, C.L., Sanjuan, J., Deltreil, J.P., Borel, M., 1986. Tin contamination in Arcachon Bay: effects on oyster shell anomalies. Mar. Pollut. Bull. 17, 494–498.

Appleton, R.D., Palmer, A.R., 1988. Water-borne stimuli released by predatory crabs and damaged prey induce more predator-resistant shells in a marine gastropod. Proc. Natl. Acad. Sci. U.S.A. 85, 4387–4391.

Bastida, R., Capezzani, A., Torti, M.R., 1971. Fouling organisms in the port of Mar del Plata (Argentina). I. *Siphonaria lessoni*: ecological and biometric aspects. Mar. Biol. 10 (4), 297–307.

Battershill, C.N., Bergquist, P.R., 1982. Responses of an intertidal gastropod to field exposure of an oil and a dispersant. Mar. Pollut. Bull. 13, 159–162.

Bayen, S., Lee, K., Obbard, J.P., 2007. Exposure and response of aquacultured oysters, *Crassostrea gigas*, to marine contaminants. Environ. Res. 103, 375–382.

Bigatti, G.I., Primost, M.A., Cledón, M., Averbuj, A., Theobald, N., Gerwinski, W., Arntz, W., Morriconi, E., Penchaszadeh, P.E., 2009. Contamination by TBT and imposex biomonitoring along 4700 km of Argentinean shoreline (SW Atlantic: from 38° S to 54° S). Mar. Pollut. Bull. 58, 695–701.

Bookstein, F.L., 1991. Morphometric Tools for Landmark Data: Geometry and Biology. Cambridge University Press, Cambridge.

Chagot, D., Alzieu, C., Sanjuan, J., Grizel, H., 1990. Sublethal and histopathological effects of trace levels of tributiltin fluoride on adult oysters *Crassostrea gigas*. Aquat. Living. Resour. 3, 121–130.

Cheung, S.G., Lam, S., Gao, Q.F., Mak, K.K., Shin, P.K.S., 2004. Induced anti-predator responses of the green mussel, *Perna viridis* (L.), on exposure to the predatory

gastropod, *Thais clavigera Kutser*, and the swimming crab, *Thalamita danae Stimpson*. Mar. Biol. 144, 675–684.
Chou, J., Clement, G., Bursavich, B., Elbers, D., Cao, B., Zhou, W., 2010. Rapid detection of toxic metals in non-crushed oyster shells by portable X-ray fluorescence spectrometry. Environ. Pollut. 158, 2230–2234.
Cledón, M., Gerwinski, W., Theobald, N., Penchaszadeh, P.E., 2006. Organotin compounds (OTs) in sediments and commercial gastropods of Mar del Plata, Argentina. J. Mar. Biol. Assoc. UK 86, 751–755.
Colombo, J.C., Barreda, A., Cappelletti, N., Migoya, C., Skorupka, C., 2005. Organic pollutants in waters and sediments of littoral afluents in Argentina. Final report. Transnational Diagnostic Analysis of the Rio de la Plata and its Oceanic Front. ADT document and background information, 24 pp.
Crampton, J.S., 1995. Elliptic Fourier shape analysis of fossil bivalves: some practical considerations. Lethaia 28, 179–186.
Cunningham, P.A., 1976. Inhibition of shell growth in the presence of mercury and subsequent recovery of juvenile oysters. Proc. Natl. Shellfish. Ass. 66, 1–5.
De Fur, P.L., Crane, M., Ingersoll, C., Tattersfield, L., 1999. Endocrine disruption in invertebrates: endocrinology, testing, and assessment. In: Workshop on Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment, 1998, Noordwijkerhout, The Netherlands. SETAC Press, Pensacola, FL.
De Pirro, M., Marshall, D.J., 2005. Phylogenetic differences in cardic activity, metal accumulation and mortality of limpits exposed to copper: a prosobranch-pulmonate comparison. J. Exp. Mar. Biol. Ecol. 322, 29–37.
Espinosa, F., Guerra-García, J.M., García-Gómez, J.C., 2007. Sewage pollution and extinction risk: an endangered limpet as a bioindicator? Biodivers. Conserv. 16, 377–397.
Frazier, J.M., 1976. The dynamics of metals in the American oyster *Crassostrea virginica*. II. Environmental effects. Chesap. Sci. 17, 188–197.
Fuchigami, T., Sasaki, T., 2005. The shell structure of the recent Patellogastropoda (Mollusca: Gastropoda). Paleontol. Res. 9, 143–168.
Giraldo-López, A., Gómez-Schouben, C., 1999. Variación en la concha de *Siphonaria gigas* (Sowerby, 1825) como respuesta al efecto de la intensidad de las olas. Cienc. Mar. 25, 213–224.
Gold-Bouchot, G., Sima-Alvarez, R., Zapata-Pérez, O., Güemez-Ricalde, J., 1995. Histopathological effects of petroleum hydrocarbons and heavy metals on the american oyster (*Crassostrea virginica*) from Tabasco, Mexico. Mar. Pollut. Bull. 31, 439–445.
Goldberg, R.N., Averbuj, A., Cledón, M., Luzzato, D., Nudelman, N.S., 2004. Search for TOTs in Mar del Plata (Argentine) marine coast. Finding of TBT in egg capsules of a snail *Adelomenlon brasiliana* (Lamarck, 1822) population showing imposex effects. Appl. Organomet. Chem. 18, 117–123.
Hammer, O., Harper, D.A.T., Ryan, P.D., 2001. PAST: palaeontological statistics software package for education and data analysis. Palaentol. Electron. 4, 9, http://folk.uio.no/ohammer/past.
Harley, C.D.G., Denny, M.W., Mach, K.J., Miller, L.P., 2009. Thermal stress and morphological adaptations in limpets. Funct. Ecol. 23, 292–301.
Héral, M., Alzieu, C., Deslous-Paoli, J.M., 1989. Effect of organotin compounds (TBT) used in antifouling paints on cultured marine molluscs – a literature study. In: De Pauw, N., Jaspers, E., Ackefors, H., Wilkins, N. (Eds.), Aquaculture: A Biotechnology in Progress. European Aquaculture Society, Bredene, Belgium, pp. 1081–1089.
Huitema, B.E., 1980. The analysis of covariance and alternatives. New York.
Iwata, H., Ukai, Y., 2002. SHAPE: a computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. J. Hered. 93, 384–385.
Krampitz, G., Drolshagen, H., Deltreil, J.P., 1983. Soluble matrix components in malformed oyster shells. Experientia 39, 1105–1106.
Márquez, F., González-José, R., Bigatti, G., 2011. Combined methods to detect pollution effects on shell shape and structure in Neogastropods. Ecol. Indic. 11, 248–254.
Najle, R., Elissondo, M., Gentile, S., Gentile, M., Vacarezza, G., Solana, H., 2000. Histopathology of the digestive gland of an Antarctic limpet exposed to cadmium. Sci. Total Environ. 247, 263–268.
Okoshi, K., Sato-Okoshi, W., 1996. Biomineralization in molluscan aquaculture Growth and disease. Bull. Inst. Oceanogr. Monaco. 14, 151–169.
Olivier, S.R., Penchaszadeh, P.E., 1968. Observaciones sobre la ecología y biología de *Siphonaria* (Pachysiphonaria) *lessoni* (Blainivlle, 1824) (Gastropoda, Siphonaridae) en el litoral rocoso del Mar del Plata (Buenos Aires). Cah. Biol. Mar. 9, 469–491.
Palmer, A.R., 1990. Effect of crab effluents and scent of damaged conspecifics on feeding, growth, and shell morphology of the Atlantic dogwhelk *Nucella lapillus* (L.). Hydrobiologia 193, 155–182.
Phillips, D.J.H., 1977. The common mussel *Mytilus edulis* as an indicator of trace metals in Scandinavian waters. I. Zinc and cadmium. Mar. Biol. 43, 283–291.
Phillips, D.J.H., 1978. The common mussel *Mytilus edulis* as an indicator of trace metals in Scandinavian waters. II. Lead, iron and manganese. Mar. Biol. 46, 147–156.
Rohlf, F.J., Archie, J.W., 1984. A comparison of Fourier methods for the description of wing shape in mosquitoes (Diptera: Culicidae). Syst. Zool. 33, 302–317.
Singh, A.P., Bragg, P.O., 1979. The action of TBT CL on the uptake of proline and glutamine by intact cells of *E. coli*. Can. J. Biochem. 57, 1376–1383.
Suzuki, M., Kameda, J., Sasaki, T., Saruwatari, K., Nagasawa, H., Kogure, T., 2010. Characterisation of the multilayered shell of a limpet, Lottia kogamogai (Mollusca: Patellogastropoda), using SEM-EBSD and FIB-TEM techniques. J. Struct. Biol. 171, 223–230.
Tablado, A., López Gappa, J.J., Magaldi, N.H., 1994. Growth of the pulmonate limpet *Siphonaria lessoni* (Blainville) in a rocky intertidal area affected by sewage pollution. J. Exp. Mar. Biol. Ecol. 175, 211–226.
Tablado, A., López Gappa, J., 2001. Morphometric diversity of the pulmonate limpet *Siphonaria lessoni* in different coastal environments. Sci. Mar. 65 (1), 33–41.
Trussell, G.C., 1996. Phenotypic plasticity in an intertidal snail: the role of a common crab predator. Evolution 50, 448–454.
Underwood, A.J., 1997. Experiments in Ecology. Cambridge University Press, Cambridge.
Waldock, M.J., Thain, J.E., 1983. Shell thickening in *Crassostrea gigas*: organotin antifouling or sediment induced? Mar. Pollut. Bull. 14, 411–415.
Yoon, G.L., Kim, B.T., Kim, B.O., Han, S.H., 2003. Chemical–mechanical characteristics of crushed oyster-shell. Waste Manage. 23, 825–834.
Zar, J.H., 1984. Biostatistical Analysis, second ed. Prentice-Hall, Englewood Cliffs, New Jersey.
Zar, J.H., 1999. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ.