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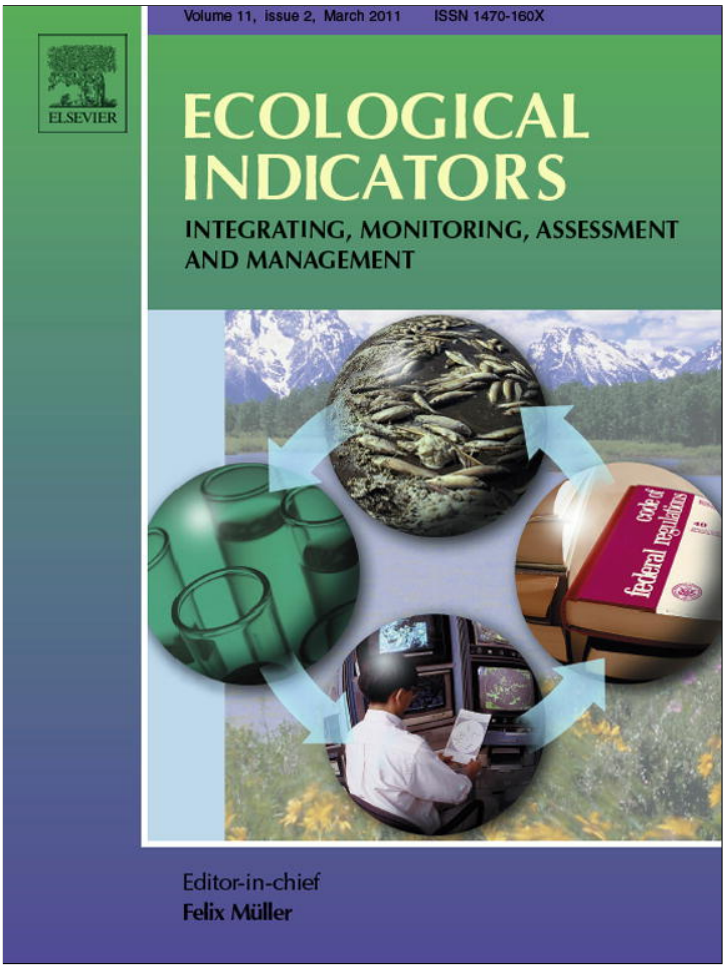

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Original article

Combined methods to detect pollution effects on shell shape and structure in Neogastropods

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ABSTRACT

Tributyltin (TBT) has been widely used as antifouling in marine environments, producing imposex in gastropod females (i.e. neoformation of a vas deferens and/or a penis) and shell malformations in bivalves. However, effects of TBT and other pollutants from high marine traffic zones on the shell of gastropods have been little explored. Shell shape in volutids *Odontocymbiola magellanica* from a harbor polluted site (P) has been compared with that of animals from a non-polluted location (NP) using 3D geometric morphometrics. Also, the microstructure and density of shells from both populations were analyzed. Prior studies made in the same area (Golfo Nuevo, Patagonia, Argentina) based on traditional multivariate morphometrics showed some differences in size but was unable to detect differences in shell shape among *O. magellanica* from P and NP areas. Departing from 3D geometric morphometrics, scanning electron microscopy (SEM) and computed tomography (CT) techniques, we have registered the presence of patent differences on shell shape and structure in animals from polluted (P) and non-polluted (NP) areas. In 100% of shells from the NP area we register three calcium carbonate layers (prismatic, cross lamellar and amorphous) and higher densities, while in 50% of the shells collected at the P area the external layer (amorphous) was lacking. Furthermore, a body weight loss of around 30% and a shell weight loss of 20% were registered in animals from the P area. Our combined results suggest that the coordinated use of 3D geometrics morphometrics, CT scan and SEM could be of great utility in order to detect the effect of environmental variables on Neogastropods shell shape and structure.

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1. Introduction

Shell morphology of snails was classically studied using distances and ratio variables as the input of classical multivariate analysis. However, such approach to measuring shell variation can confound size and shape, and the results are very sensitive to the particular distances and ratios chosen in the study (Bookstein, 1991; Zelditch et al., 2004). Furthermore, these variables present serious statistical drawbacks (Sokal and Rohlf, 1995). Conversely, geometric morphometrics (GM) techniques present some relative advantages in order to detect subtle shape changes. First, they are the most effective way to capture information about the shape of an organism, especially when combined with multivariate statistical procedures (Rohlf and Marcus, 1993; Marcus et al., 1996; Rohlf et al., 1996). Another important advantage of GM is that it is possible to visualize the results of any statistical multivariate analyses graphically: the geometric nature implicit on the concept of shape is never lost during the analyses. This is possible because the geometric shape is preserved throughout the analysis (Adams et al., 2004).

Some previous studies on shell shape changes in marine snails applied GM tools to discriminate between taxonomic or sympatric ecotypes (Caravajal Rodríguez et al., 2005; Urra et al., 2007), and phenotypic plasticity and evolution of adaptation (Hollander et al., 2006a,b). All of them used two-dimensional GM. However, caenogastropods' shells are a patent example of three-dimensional specimens, making 3D GM approaches more accurate and proper when analyzing within and between-group morphological variability.

Odontocymbiola magellanica (Gmelin, 1791) is a conspicuous neogastropod from the Pacific and Atlantic benthic communities of Southern South America (Weaver and Dupont, 1970; Bigatti et al., 2008). It is being exploited as a fishery resource in Chile (Osorio, 2002), and is consumed locally in northern Patagonia (Bigatti and Ciocco, 2008). As an edible resource, it must be studied regard-

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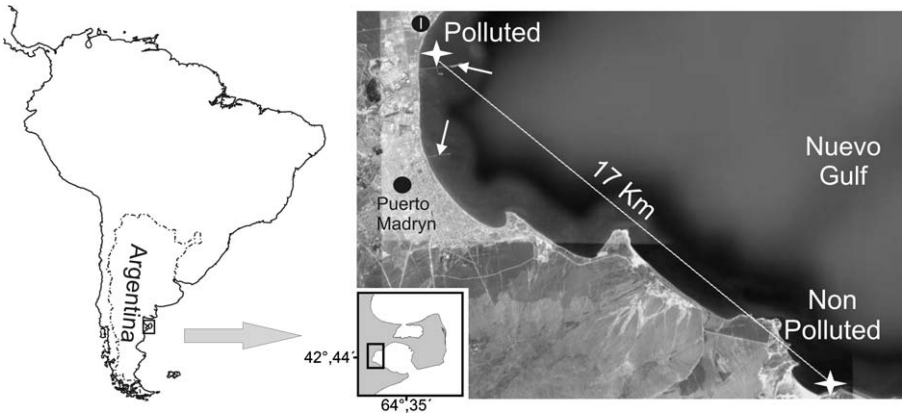

Fig. 1. Sampling sites at Nuevo Gulf, Patagonia. Site polluted (high marine traffic) is situated near the commercial piers (arrows) and industry area (I) in Puerto Madryn city, and 17 km far the non-polluted site (low marine traffic).

ing its general biology and the effects that the contaminants could cause on it. Bigatti and Penchaszadeh (2005) registered an imposex incidence between 85 and 100% for *O. magellanica* inhabiting near the piers of Puerto Madryn (Golfo Nuevo, Argentina) during 2000–2004. This result was interpreted as related to the high marine traffic, while in zones with low marine traffic imposex was null. Contamination by TBT due to marine traffic was corroborated by Bigatti et al. (2009) in the same zone. Negative effects of this compound were detected in hundreds of gastropod species around the world (Gibbs and Bryan, 1994 among others). In addition, the harbor of Puerto Madryn showed low to moderate hydrocarbon levels in sediments (Commendatore and Esteves, 2007) and heavy metals contamination in sediments and in the bivalve *Aulacomya atra* (Gil et al., 2006) but the specific effects of this pollutants on the biota are not clear yet. Bigatti and Carranza (2007) also studied the communities of *O. magellanica* from Golfo Nuevo by means of uni- and multivariate morphometrics, registering size differences (but not in shell shape) and body weight losses in females affected by imposex.

Due to the complexities of the putative shape changes derived from the effect of pollution, the main objective of this work is to combine modern techniques of geometric morphometric analysis with computed tomography scans and scanning electron microscopy tools to assess morphometric alterations on *O. magellanica* inhabiting polluted and non-polluted environments in Golfo Nuevo, Argentina.

2. Materials and methods

2.1. Sampling and weight assessment

The study sites comprised a polluted (P) and a non-polluted area (NP) separated by 17 km in the Nuevo Gulf (Fig. 1) as previously assessed (site 13 and site 17, respectively, in Bigatti et al., 2009): individuals from P area exhibited 100% imposex and a RPLI of 26.31 (with TBT sediment dry weight concentrations of 1.7 ng Sn g^{-1}), while in the animals from NP area the imposex % and the RPLI were null.

Sixteen individuals of *O. magellanica* were collected by SCUBA diving from P and 25 from NP subtidal zones (a subsample of individuals studied by Bigatti et al., 2009). All individuals collected were mature according to length measures (i.e. >70 mm) determined by Bigatti et al. (2008). Specimens were classified as males from P (MP), female from P (FP), male from NP (MNP) and females from NP (FNP). Total body weight was recorded to the nearest 0.1 g on fresh individuals. After dissection, we register the weight without shell (body weight) and the weight of the shell separately (shell weight). To evaluate differences on body weight and shell weight among sex and area we used a two-way ANOVA with interactions. For significant results ($p < 0.05$) Tukey–Cramer test was applied (Zar, 1999). Before the analyses, data were tested for normality whit the Shapiro–Wilk test and for homogeneity of variance with the Levene test. The weights were normalized dividing by the total length of the shell, to avoid biases in results due to the different specimen sizes. Sex was determined based on the presence or absence of sexual accessory glands (albumen, capsule, and pedal glands in females).

2.2. 3D geometric morphometrics

Analyses of snails' shell shape from P and NP areas were performed using geometric morphometrics (Rohlf and Marcus, 1993; Adams et al., 2004; Zelditch et al., 2004) using the software MorphoJ version 1.00j (Klingenberg, 2008). The forms of individuals were captured by the Cartesian coordinates of a three-dimensional configuration of 13 anatomical landmarks (Fig. 2). All specimens were measured by one observer (FM) using a Microscribe G2X digitizer. Landmark configurations were superimposed by generalized Procrustes analysis (Rohlf and Slice, 1990; Slice et al., 1996). This procedure translates and rotates the landmark configurations to a common origin and scales them to unit centroid size.

To assess and control putative allometric effects, we computed the multivariate regression of shape (Procrustes coordinates used as dependent variables) on size (independent variable). Klingenberg (2008) suggested that such regression is of utility to detect and remove the effect of size, age or environmental factors before shape comparisons between groups. To evaluate the independence between the shape and size variables, we carried out a permutation test with 10,000 rounds.

The principal components of shape were calculated from a principal component (PC) analysis of the variance–covariance matrix of the Procrustes coordinates to display the major features of shape variation along the axes. The variable used for size normalization was the centroid size, calculated for each specimen as the square root of the sum of the squared deviations of landmarks from the centroid (Bookstein, 1991; Zelditch et al., 2004). To test the change in size due to the exposure to the pollutant, we performed a *t*-test for non-paired samples on centroid size, classifying the samples by origin area.

Finally, we have computed a canonical variate analysis (CVA) on the first PCs in order to recover an axis of maximum discrimination between P and NP groups. Only the first, most informative, PCs were used in order to accomplish the "rule of thumb" of discriminant analysis which indicates that the sample size of the smallest group (P, $n = 16$) needs to exceed by large the number of predictor variables (13 3D landmarks = 32 shape variables or PCs). To eval-

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uate differences on shells shapes among sex and area we used a MANOVA. For significant results ($p < 0.05$) Hotelling–Bonferroni test was applied (Cuadras, 2008).

2.3. Shell structure and density

To explore individual differences in *O. magellanica* shell composition, transverse sections of the shell of six snails (randomly chosen) from P and 6 from NP areas were made on the same part of the shell analyzed for CT scans density studies (see below); sections were observed with scanning electron microscopy (SEM, Philips XL 30) at MACN-CONICET and (Jeol JSM-6460LV) ALUAR S.A. Examination of microstructure and density by CT scans were performed on the shell's lip because this is the shell zone that is continuously secreted. Lip's area was preferred over other structures in order to estimate structure and density since it is free from the noising effect of erosion and fouling/attachment of epibionthic fauna that characterizes the more aged structures of the shell. Computed tomography (CT) scans were made for one individual (randomly chosen) from P and one from NP areas. Sagital and transverse sections of the shell were studied in order to visualize shell density variations in localized structures. CT scan image was analyzed using the software packages COMPUDENT NAVIGATOR. Note that the CT analysis is presented not as a statistical proof of density differences between P and NP areas, but as a description of the variation on density within a single shell. In this context, it should be viewed as supportive data for the SEM results, which include more specimens into the analysis and enable more solid statistical conclusions.

3. Results

3.1. Weight and size

The body weight related to shell length was significantly different ($p < 0.05$) between areas (P and NP), but not between sexes.

Table 1
Analyses of variance (ANOVA) for effect of the sex and area on the body and shell weight related to shell length. FP = female from polluted area; MP = male from polluted area; FNP = female from non-polluted area; MNP = male from non-polluted area.

Source	GL	MS	*F*	*p*-Value
Body weight/total length				
Area	1	1.31	23.65	<0.0001*
Sex	1	0.07	1.29	0.26
Area × sex	1	0.00005	0.01	0.92
Error	37	0.06		
FP: a; MP: a; FNP: b; MNP: b				
Shell weight/total length				
Area	1	0.16	8.97	0.005*
Sex	1	0.08	4.68	0.03*
Area × sex	1	0.0008	0.05	0.82
Error	37	0.02		
FP: a; MP: ab; FNP: ab; MNP: b				

Tukey–Kramer tests were used to test for differences between shell and body weight when the factors sex or area were significant. Different letters indicate significant differences ($p < 0.05$) in Tukey–Kramer test.

* $p \leq 0.05$.

In addition, we found significant differences ($p < 0.05$) between areas and sexes on shell weight related to shell length (Table 1). Furthermore, males from NP (mean: 0.66 g/cm, SD = 0.14, $n = 13$) presented a tendency to have heavier shell than females from P (mean: 0.43 g/cm, SD = 0.11, $n = 8$).

The shell and body weight for both sexes together as well as the centroid size presented significant differences ($p < 0.05$) between groups from P and NP areas. Specimens from the NP area (both sexes) were heavier in total body weight (mean: 1.93 g/cm, SD = 0.32, $n = 25$) than those from the P (mean: 1.39 g/cm, SD = 0.33, $n = 16$) area. Pooled individuals from the P area registered 30.53% of reduction in body weight, 22.6% of shell weight loss and 18.46%

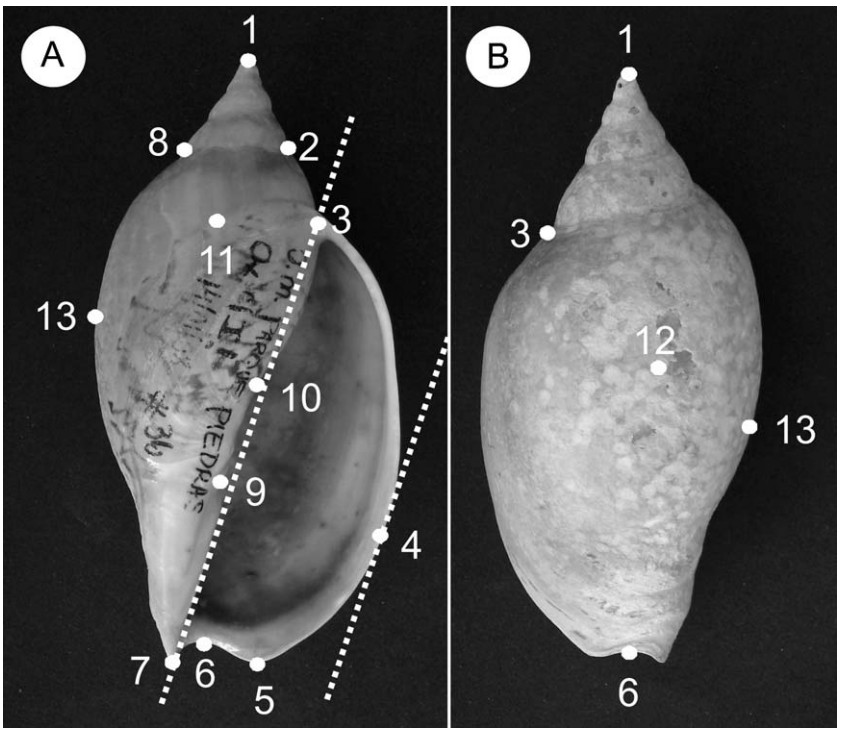

Fig. 2. Landmark configuration placements (white dots) on the shell were made using two different orientations, apertural (A) and abapertural (B). These landmarks include: (1) apex, (2) abapertural extreme of the starting point of last whorl, (3) posterior angle of the aperture, (4) tangent point in the lip, parallel to the line (dash line) formed by landmarks 3 and 7, (5) anterior most point of the lip, (6) just siphonal canal, (7) anterior tip of columella, (8) left border of suture in the penultimate whorl on a middle plane to the axis from landmarks 7 to 1, (9) aperture maximum width, (10) depression at the external border of columella upper plicae, (11) abapertural maximum height, (12) apertural maximum height, and (13) maximum width. All landmarks were painted with small points of indelible marker to aid in a more precise identification.

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centroid size difference compared with pooled individuals from NP areas.

3.2. Geometric morphometrics

Since P and NP samples significantly differed in size, we computed the pooled-within group multivariate regression of shape on size. This regression was non-significant ($p = 0.6$), thus we performed all the subsequent analyses without allometric correction. The first 10 principal components (PCs) explained 87.0% of the total variation. However, only the PC1 presented differences between specimens from P and NP zones. Two groups are evident on the ordination of specimens along the first PC, one including specimens from P (positive values) and the other including those from the NP area (negative values). Individuals from P zones present a more slender and narrower shell shape. This change in the shell shape is produced by the upward-pointing orientation of the apex and the outer inflexion point of the siphonal canal. Also, the aperture of the shell has a triangular shape much narrower than that in NP individuals (Fig. 3).

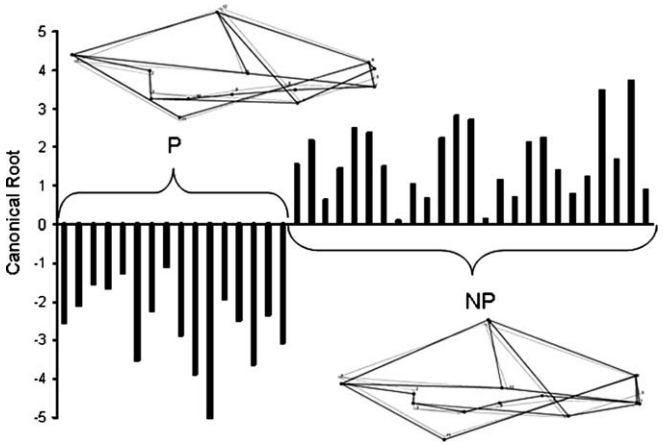

Fig. 3. Canonical variate analysis. P = polluted area specimens (negative side of Score 1); NP = non-polluted area (positive side of Score 1). Wireframe diagrams show the shape changes associated with variation along the first root.

The first eight PCs explaining collectively 80% of total variance were used in order to compute a CVA with the smallest sample size (P, $n = 16$) being twice the shape variables used as predictors. Results indicate that differences among P and NP were significant at $p < 0.001$ and that all individuals were reclassified correctly. Shape differences depicting variation across the canonical root are presented in Fig. 3. The MANOVA analysis performed on the first eight PC indicated a highly significant shell-shape differentiation between groups from P and NP areas (Wilk's $\lambda = 0.12$, $F = 3.89$, d.f. 24 and 88, $p < 0.001$). Specimens of both sexes from

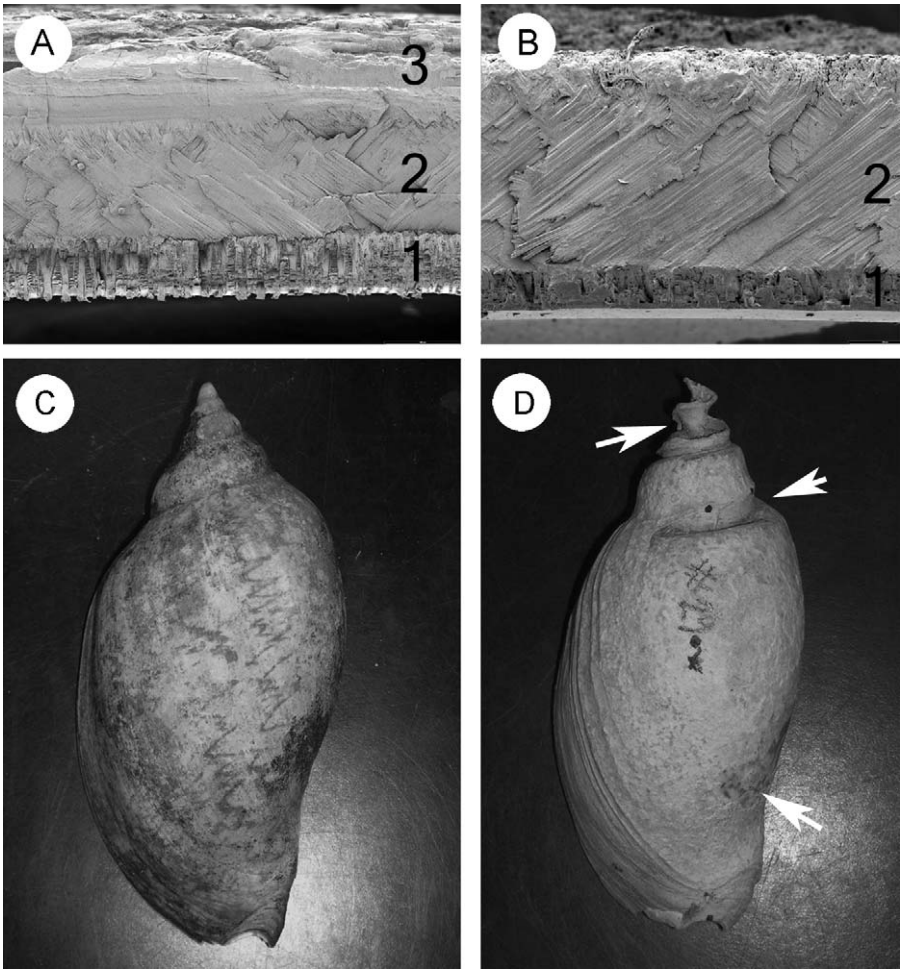

Fig. 4. Shell morphotypes from polluted (P) (right pictures) and non-polluted (NP) area (left pictures). (A and B) Transversal cross-section of the shell lip by SEM showing the constituent layers of calcium carbonate. (A) (NP) area = 3 layers. (B) (P) area = only 2 layers. (C) External view of shell morphotype from (NP) with thicker shell than fragile one from (P). (D) Shell presenting malformations in the apex and the external layer (arrows). *Abbreviations*, 1: internal prismatic layer; 2: medium cross lamellar layer; 3: external amorphous layer.

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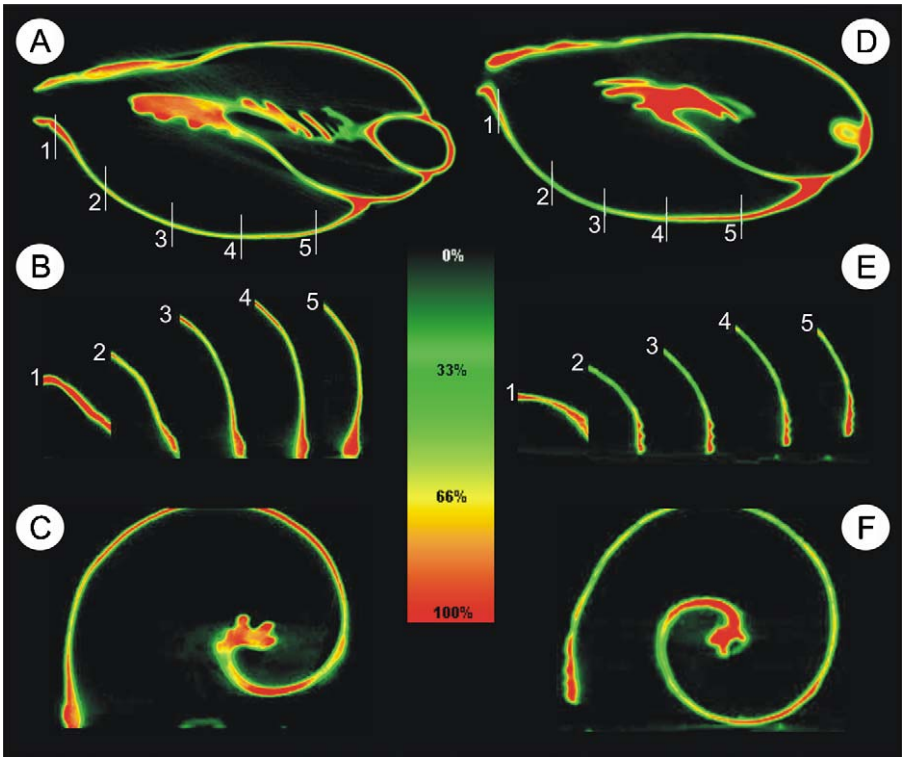

Fig. 5. Percentage densitometry analyses in shell cross-section by CT scan; from A to C: specimen from non-polluted area; from D to F: specimen from polluted area. White lines on the sagital images A and D shows the transverse section made in the lip zone. B and E detail of transverse sections of A and D. C and F images shells transversal sections. Densities are represented by a color scale (center) as 100% red and 0% black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the NP area were different in shell shape, than those from the P area.

3.3. Shell structure and density

Fragile shells (shells that can be broken by hand) were registered in 81.25% of the animals from the pier zone (P), while the same was observed only in 4% of the snails from the area with low marine traffic (NP).

In 100% of shells ($n = 6$) from the NP area three carbonate layers were registered by SEM: prismatic (internal), cross lamellar and amorphous (external). In 50% of shells from the P area ($n = 6$) we detected only 2 layers (prismatic and cross lamellar) lacking the external one (amorphous) (Fig. 4).

CT scans revealed that, in general terms, the shell density and thickness registered in animals from P area were minor than those from NP area. Shell density at the lip of the specimen analyzed from NP area was more uniform and higher than that of the individual of P area (Fig. 5).

4. Discussion

Imposex is not the only effect associated to TBT pollution. Malformation and thick increment in shells of the oyster *Crassostrea gigas* had been documented as a response to prolonged expositions to TBT and related compounds (Alzieu, 1986; Lee, 1991). Reduction in the growth rate in the bivalve *Mytilus edulis* and larvae mortality in the bivalve *Scrobicularia plana* (Ruiz et al., 1994) were also registered. Coelho et al. (2006), found that juveniles of the clam, *Ruditapes decussates*, exposed to TBT developed abnormal shell growth with variations in shell shape, from typical flattened shape to more rounded form. Sokołowski et al. (2008) suggested that the abnormal shell shape presented in the clam *Macoma balthica* under exposition of TBT among other organotin contaminants in sediment cannot be neglected. Thereby, other pollution sources from harbor of marine traffic zones could lead to physiological alterations in marine biota.

O. magellanica is sensitive to TBT contamination (Bigatti et al., 2009), a phenomenon denoted by the neoformation of secondary sexual characters as multiple penes in females, while other species in the same area do not present external imposex signs. *O. magellanica* individuals from high marine traffic zones and TBT pollution (P) presented the imposex phenomenon, while animals from the zone with low marine traffic (NP) did not. Here we show that, further than the imposex phenomenon registered in females, also variations in shell shape, differences in size and shell density as well as weight losses detected in both sexes are characters likely to be influenced by exposition to pollutants found near piers of Puerto Madryn. The estimated age of the snails used in this work ranged from 8 to 17 years (Bigatti et al., 2007), so individuals inhabiting P zone were exposed to pollutant since hatching. This is a striking result, since it suggests that the endocrine disruption caused by the TBT levels detected in the pier zone (sediment dry weight concentrations 1.7 ng Sn g^{-1}) and probably other pollutants deriving in sexual anomalies are also likely responsible of alterations on shell shape and composition and development traits on both sexes. Penchaszadeh et al. (2009) also registered in the same zone the presence of 2 penes and warts-like outgrowths anomalies in male *vas deferens* of the sympatric volutid *Adelomelon ancilla*. Several works explain the possible metabolic pathways of TBT toxicity in marine gastropods (Oberdörster and Mcclellan-Green, 2000; Santos et al., 2005; among others), although there are no general consensus about the action of the pollutant. Alterations produced by marine traffic pollutants, among them TBT, could explain the differences in shell shape and density and the body weight losses of up to 30% and around 20% in shell weight in individuals from

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P zone registered in the present work. In opposite to the result of Bigatti and Carranza (2007) (10% of reduction in body weight only in females from the pier zone), we found that the male from P area also presented a significant reduction in body and shell weight compared with NP area, so males must be also discriminate when studying phenotypic variations due to environmental pollution as TBT. Studies regarding the imposex physiology are needed to clarify the metabolic pathways of the TBT or other pollutants in Neogastropods.

Most of the classical papers of Vermeij (1977, 1982 among others), state that changes on the size of the spire and the aperture are a consequence of the selective pressure of a predator. However, we should note that both zones (P and NP) present the same predators as well as the same environmental variables (distance of 17 km between sites). In fact, we have explicitly selected such zones in order to magnify the factor under study: presence of marine traffic pollutants.

Bigatti and Carranza (2007) worked with the same community as the present work using classic morphometrics, detecting differences in two morphometric variables, but not overall effects in shell shape. The use of the classic morphometric techniques to study the change in shape has been criticized by different authors (Bookstein et al., 1985; Adams et al., 2004; Zelditch et al., 2004). To overcome the limitations of classical morphometrics, we used 3D geometric morphometric techniques, which allowed us to detect subtle changes in the shell shape of individuals from P and NP zones. No relation between size and shape was detected (no allometry), an indication that the changes in shape were independent of the changes in shell size. The main variation we found is that individuals from P zone present a more slender and narrower shell shape. Discriminant and MANOVA analyses made on these specimens indicate that the shape changes represented by variation on the studied landmark configurations could be a good predictor of the shell shape change between polluted site (imposex incidence) and non-polluted locations (imposex free), enabling correct reclassification of the specimens from polluted or non-polluted environments.

The results of this work lead us to believe that the use of these new methodologies in the study of Neogastropoda will facilitate the description and functionality of the subtle changes in the shape, structure and densities of hard structures presenting landmarks. Also, we suggest that the use of 3D geometric morphometrics will make up for one of the main drawbacks of the geometric morphometrics techniques that have been applied in mollusks so far, which consists in studying the 2D shape variation of species which present very complex 3D structures. This new approach (3D GM, SEM and CT scans) yielded the clearest results in the study of intraspecific shell shape and structures variations produced by environmental differences such as pollution. The methodology presented here brings some promising insights concerning evaluation and monitoring of natural resources without destructive or extractive methods. For instance, it could be of great utility in order to study shell shape of specimens inhabiting protected areas or long-lived species as *O. magellanica*. Even though this methodology must be tested in other Neogastropods species, we advance that it could be put into practice on living specimens, thus avoiding killing of individuals in order to establish the imposex phenomenon due to TBT pollution.

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