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Search for triorganotins along the Mar del Plata (Argentina) marine coast: finding of tributyltin in egg capsules of a snail *Adelomelon brasiliana* (Lamarck, 1822) population showing imposex effects

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Cases of imposex were clearly identified in *Adelomelon brasiliana* living in the Mar del Plata (Argentina) coastal area; percentages as high as 50.0% were determined among the samples studied. These were the first reported cases of ocurrence of imposex in this type of gastropod. Since this is one of the known tributyltin (TBTs) effects, and no previous reports of determination of TBTs in gastropods eggs were found, methods were developed for the speciation and quantitative determination of organotins in *A. brasiliana* egg capsules. Determination of organotins in samples collected in the Mar del Plata area showed contents of tributyltin chloride (TBT) as high as 400 ng l^{-1} in water and 6.50 μg g^{-1} in sediments of areas of intensive boat traffic. The results showed the presence of TBT in the egg capsules of *A. brasiliana* at three different instars (range 0.264–1.86 μg per egg). As far as we know, this is the first report of the finding of TBT in gastropod egg capsules. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: antifouling additives; tributyltin (TBT); biological effects; imposex; Argentine gastropods; organotins; gastropods eggs; *Adelomelon brasiliana*

INTRODUCTION

Since the commercialization in the early 1960s of triorganotin (TOT) compounds as antifouling paints, and especially after the introduction of the self-polishing copolymer (SCP) formulation in the 1970s, organotins have been heavily used; by the mid-1980s they were used on over 80% of the world's commercial fleet. However, severe damage to some marine aquatic organisms has been reported, and the use of TOT as an antifouling additive in boat paints is being limited, and is even banned in several countries. A recent review[1] thoroughly covers most of the more important features reported in the literature. Some organic biocidal compounds, termed organic boosters, were proposed as alternative antifoulants after the ban on tributyltins (TBTs).[2,3] Nevertheless, the long-term potential risks of these compounds are mostly unknown yet, and contaminations by organic boosters in the coastal waters of Greece,[4] the UK[3,5] and the USA[6] have recently been reported.

Some of the most frequent and acute effects of TBTs were observed in gastropods: chronic toxicity is shown by endocrine disruption, leading to effects such as imposex, intersex and masculinization of females.[7,8] Recent reviews report more than 140 different species of gastropod that have been observed to be affected by imposex.[1,9,10] (Argentine species are not among the cited species.) The bioaccumulation potential of TBTs by top trophic-level organisms had

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been considered to be low until a recent report on the contamination by TOTs of cetaceans and pinnipeds in various regions of the world;[11] TBTs has also been found in the liver of Beluga whales (*Delphinapterus leucas*) from Canada.[12] On the other hand, we have previously reported the deleterious effect of tributyltin chloride (TBT) on *Euglena gracilis* and *Chlorella* sp., two freshwater microorganisms.[13] The observed effects (measured by growth rate and chlorophyll content) were concentration dependent. Further research using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) confirmed a TBT concentration dependence of the cell damage (A. Nudelman *et al*., unpublished results). More recent studies carried out with TBT-dosed *E. gracilis* showed some restoration-promoting effects of iron-encaging–zeolite-processed water.[14] The deleterious effects of triphenyltin chloride (TPTCl) on the alga S*pirulina subsalsa* were recently shown by SEM and TEM.[15]

Novel organotin compounds have recently been synthesized and fully characterized.[16–18] Some of these are proposed to be used as insecticides,[17] especially those effective for the control of insects in their larval instar.[18] Some of these recommendations[17,18] are based on the early determinations of TOT stabilities that established that toxic TOT easily degraded in the environment to non-toxic tin species.[19] The presumed biodegradability should made them advantageous alternatives to the organophosphorus insecticides.[18] Nevertheless, more recent determinations of the half-lives of TBT have shown them to be as high as 8.7 years in sediments[20] and 4–17 years in the bivalve *Venerupis decussata*.[21] The stabilities of TOTs in natural media being surprisingly higher than the data previously reported strongly argues against their introduction into the environment as insecticides.

All the chemical studies on the toxicity effects of TOTs and on TBT determinations in the environment recorded in the literature refer to developed countries in the Northern Hemisphere, where there is usually legislative control. To the best of our knowledge, only one recent study monitoring butyltin contamination in green mussels collected from various Asian developing countries has been reported.[22] The study shows that polluted areas, such as Hong Kong, Malaysia, India, the Philippines and Thailand, revealed levels comparable to those in developed nations. Only one study of environmental butyltin determinations in the Latin American region has been reported. Recent determinations in surface sediments from the São Paulo State coast (Brazil) showed high concentration levels of TBT (360–670 ng g^{-1}) in zones of intensive boat traffic.[23] The first cases of imposex in marine species living along Argentine marine coasts, namely *Adelomelon brasiliana* and *Buccinanops monilifer*, were recently reported by Penchaszadeh *et al*. As this is one of the known TBT effects, it was of interest to carry out a search for TBT in water and sediments of this coastal area and in biological samples (egg capsules) of *A. brasiliana.* Methods for the quantitative determination of TBT in gastropod egg capsules have to be developed, since no methods were found in the literature. The present paper reports the finding of TBT in *A. brasiliana* egg capsules in different stages of development, as well as in water and sediments from the Mar del Plata area.

EXPERIMENTAL

Materials

Tributyltin chloride (TBT) was purchased from Aldrich Chem. Co. (USA) and used as received. Triphenyltin (TPT) was freshly synthesized by an adaptation of a previously reported procedure.[25] Samples of butyl tin trichloride 95% (MBT, Aldrich) and dibutyltin dichloride (DBT, Aldrich) were generously provided by Professor L. Ebdon (University of Plymouth, UK). Certified samples of TBT and of TPT, (standard stock solution in toluene), both Cica reagents from KANTO Chem. Co., Inc. (Japan), were generously provided by Dr Kazuko Mizuishi (Ministry of Public Health, Tokyo, Japan). Silicagel Merck, grade 60, 230–400 Mesh, 60A° was obtained from Aldrich Chem. Co., Inc. High-performance liquid chromatography (HPLC)-grade hexane was bi-distilled. Since this solvent is used for the sample treatment, the purification was repeated until no impurities were detected by gas chromatography (GC) under the splitless system.

A HP 5890 Series II Plus gas chromatograph (Agilent Technologies, Avondale, PA) equipped with a flame ionization detector (FID) system and a 30 m × 0.25 mm HP-5 (phenyl-methylsilicone 5%) capillary column (coated with a 0.25 μm thickness film) and an FID was used. High-purity nitrogen was the carrier gas; the column head pressure was controlled at 4 psi (i.e. 0.273 84 atm = 206.867 mmHg). The temperature program used was: 60 °C for 2 min, 10 °C min^{-1} to 250 °C, hold for 15 min. The injector and detector temperatures were 200 °C. To obtain maximum sensitivity, the chromatographic analyses were made under *splitless* conditions.

In some cases, GC–mass spectrometry (MS) was also carried out under conditions similar to the GC analysis and the typical clusters of tin-containing ions were observed. GC–MS spectra were recorded on a BG-Trio-2 spectrometer. In the TBT mass spectrum listed below (only the most abundant peaks are given), clusters corresponding to the molecular ion, and to the loss of 57, (2 × 57) and (3 × 57) mass units are shown between brackets. MS m/z (relative intensity) [292, (1.59), 289, (1.2) (M^+)]; [235 (67), 234 (37.5), 233 (56.7), 232 (32.1); 231 (35.4) (M^+-57)]; [183 (29) 181 (29.6), 180 (8.3), 179 (100), 178 (57.1), 177 (100), 176 (59.3); 175, (88.5) (M^+-(2 × 57)) (M^+-1-(2 × 57)]; 121 (42.0), 120 (25.5), 119 (35.8), 118 (19.3), 117 (18.7) (M^+-(3 × 57)].

Sampling

Living specimens of adult *A. brasiliana* were collected by bottom trawling from two sampling stations: one located at 1 km north from Mar del Plata harbour in a high boating-activity area, and the other in front of the restricted Marine

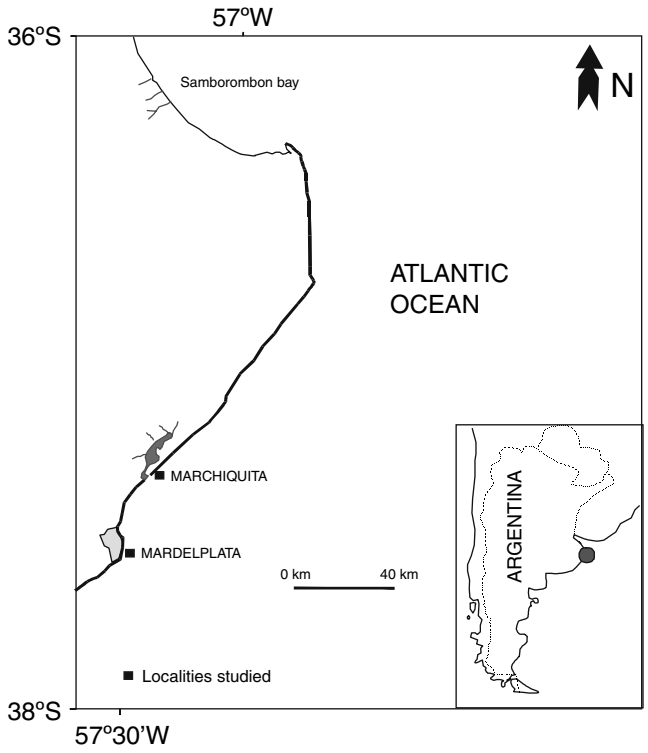

Figure 1. Sampling location. Shaded zones indicate the areas where samples of water, sediments and specimen were collected.

Reserve of Mar Chiquita, a very low boating-activity area (see the map in Fig. 1). Samples of water and sediments were collected from the beach, the coast, the North Pier and the harbour of Mar del Plata, including a very crowded boating area. Additional samples were also collected in Mar Chiquita and in Valeria del Mar, a resort area with low boat traffic, located about 100 km north from Mar del Plata. The egg capsules of *A. brasiliana* were collected near the Mar del Plata coast.

Methods for the imposex determinations were those previously reported.[24] The percentage of females with imposex, average female penis length and relative penis size index calculated for each species in each location.

Chemical analysis

Determinations of TBTs were carried out by GC after derivatization with sodium borohydride. Water and sediment samples were processed following methodologies reported elsewhere.[26] To determine the detection limit of the whole procedure, standards solutions containing known amounts of TBT, TPT, MBT and DBT were processed as the environmental samples. The minimum quantitatively detectable concentration (defined as the signal equal to three times the standard deviation of the baseline noise value) was of 0.08 ± 0.01 μg l^{-1} for TBT.

Derivatization with sodium borohydride

To processed standard solution of TBT in hexane (5 ml) or to the sample solution (5 ml) in a 50 ml round-bottomed flask, 5 ml of $NaBH_4$ solution (0.08 g in 5 ml ethanol) were added; the mixture was allowed to react for 1 h. The reaction mixture was washed with 10 ml of 10% aqueous NaCl solution; the organic layer was dried with anhydrous sodium sulfate and then purified through a silicagel column. The eluate was gently concentrated under vacuum at room temperature up to 0.2 ml; it is very important to avoid heating and also to complete solvent distillation, since losses of TBT hydride (TBTH) can occur. It was checked that under the controlled conditions described complete TBTH is recovered. 0.5 μl of the solution was injected for the CG analysis. Decaline (*cis,trans*-decahydronaphthalene) was used as subrogate. The chromatogram of the subrogate showed two signals, one at 7.4 min and the other at 8.1 min retention time. The latter exhibits the larger area, and this was taken as the reference.

Determination of TBT in *Adelomelon brasiliana* egg capsules

The giant egg capsules of *A. brasiliana* (with *ca* 100 ml of intracapsular liquid) were classified according to the embryos' development stage.[27] The eggs were cut with a knife; the liquid was separated from the solid phase by a syringe and treated separately.

For the liquid phase 40 ml of the phase were dispersed with 5 ml of 10% aqueous NaCl solution, 20 ml of hexane were added and stirred for 15 min. The material was centrifuged until net phase separation was achieved. The organic phase was transferred to a dry Erlenmeyer flask fitted with a Nalgene cap; this phase was dried with anhydrous sodium sulfate, then filtered and concentrated to nearly 5 ml at room temperature under reduced pressure. A similar procedure as described before was followed for the derivatization and chemical analysis of TBT.

The solid phase was treated consecutively with 5, 5, and 2.5 ml of hexane. The organic phase was worked up as described above and TBT was determined as described previously.

For both phases, the efficiency of the extraction of TBT was controlled in the preliminary runs by using five hexane aliquots and processing separately both the 1–3 and the 4–5 aliquots. No signals for TBT were found in the analysis of the second extraction batch.

RESULTS AND DISCUSSION

Imposex is clearly identified in *Buccinanops monilifer* from the Mar del Plata area; the percentage of imposex varies from 33.3 to 85.7% among the studied samples collected at different times in several areas of Mar del Plata.[24] The *A. brasiliana* specimens collected from the same area also showed imposex occurrence with a percentage variation from 38.9 to 50%.[24] *B.*

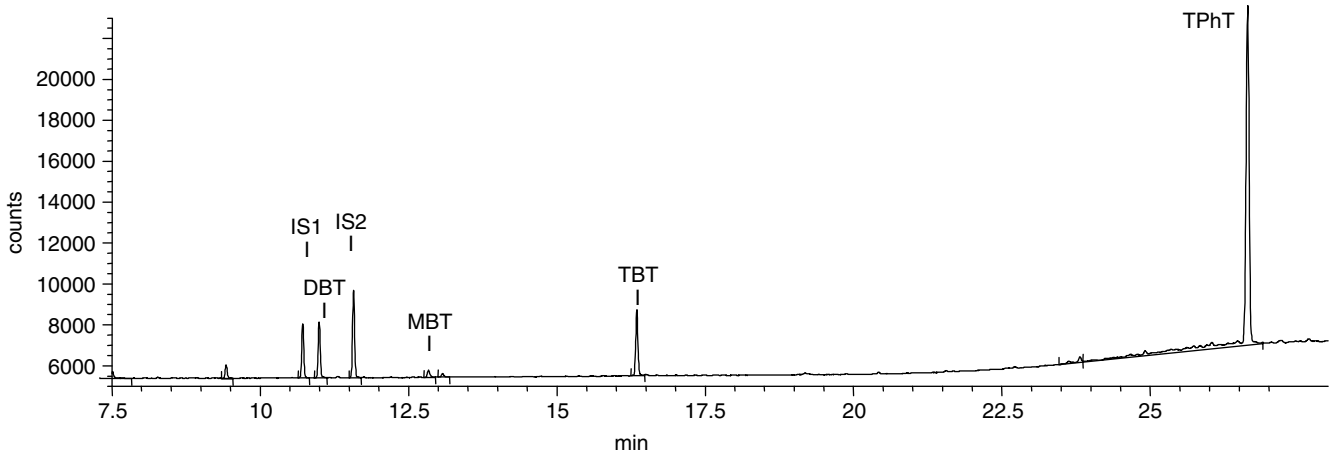

Figure 2. Chromatogram of a typical mixture of standards processed as described in the Experimental section. Amounts of each TOT in this mixture: DBT: 17 μg; TBT: 22.5 μg; MBT: 28 μg; TPT: 250 μg.

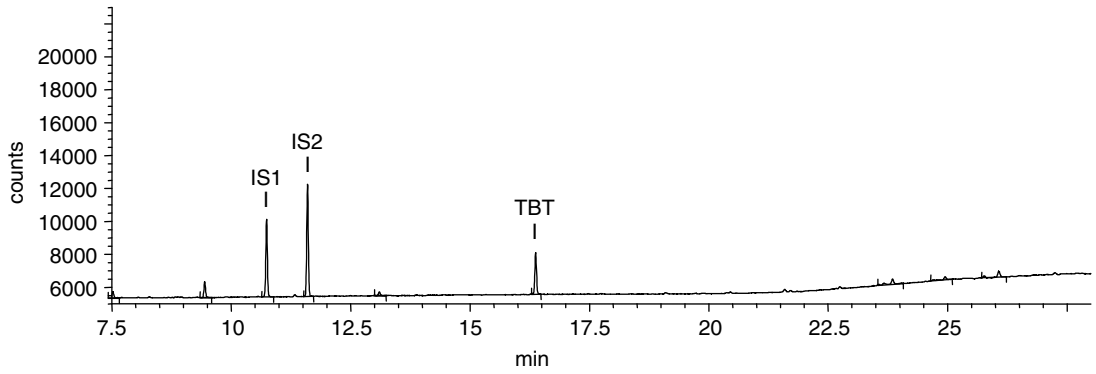

Figure 3. Chromatogram of a sample of water from the Mar del Plata coast (sample 4 of Table 1) processed as described in the Experimental section.

monilifer and *A. brasiliana* from the Mar Chiquita area showed no sighs of imposex and no TBTs were detected in water or sediments from that area. Mar del Plata harbour contains the most important coastal fishery fleet, constituting about 200 boats; it concentrates 82.5% of the coastal catch landings from the Province of Buenos Aires; the harbour also has about 100 boats in the offshore fleet.[28]

TBT determination in water and sediments

Various analytical techniques for organotins and their degradation products in environmental matrices have been reported.[29] GC after chemical derivatization is one of the most popular techniques for butyltin[30–34] and methyltin species[35] analysis. GC analysis is currently being performed utilizing one of many suitable detection methods.[36–39] Several HPLC determinations of TOTs have been published;[40,41] nevertheless, since in HPLC separations for organotin applications the peaks are generally broader than those encountered with GC, this technique was chosen as the most suitable for the purposes of the present work.

The methods currently used for the determination of environmental TOTs involve various analytical steps, such as extraction, derivatization, separation, and final detection, and this multiplies the risks of analytical errors.[42,43] In order to improve and ensure good quality control of tin speciation analysis, a series of interlaboratory studies have been organized in the past few years;[44] we have tried to follow most of the recommendations given. The analysis of each sample was made at least by individual triplicate, especially in the cases of samples with minor contents, to ensure that the products detected were present in the sample and not just artefacts of the analytical procedure.

Figure 2 shows a typical chromatogram of a standard solution containing TBT, DBT, MBT, TPT and the subrogate (called IS1 and IS2 in Figs 2 and 3). MBT is highly hygroscopic and the actual concentration in the mixture could not be defined with the same precision as the rest of the TOTs. Figure 3 shows the chromatogram obtained for a sample of water from the Mar del Plata coast (sample 4), working under splitless conditions. For some cases, the identity of the peak

Table 1. Concentrations of TBT determined in water and sediment samples

	Sample	Concentration of TBT[a]		Total tin[b]	
		Water ($ng\ l^{-1}$)	Sediment ($ng\ g^{-1}$)	Water ($ng\ l^{-1}$)	Sediment ($ng\ g^{-1}$)
1	Mar del Plata beach	180	160		
2		190	240		
3	Mar del Plata coast	200	Not determined		
4		400	Not determined	405	Not determined
5		300	Not determined		
6	Harbour[c]	4800[d]	4300		
7		8000[d]	6500	7900	6400
8		7500[d]	5000		
9	Mar del Plata North Pier	4800[d]	1300	4820	1250
10		4000[d]	1100		
11		4500[d]	1400		
12	Mar Chiquita	0	0		
13		0	0		
14	Valeria del Mar[e]	0	2.5		
15		0	0		

[a] Concentrations are reported as Sn, according to Ref. 12. Detection limit for quantification: 80 $ng\ l^{-1}$.
[b] Total tin determined by HGAAS. Error ±5%.
[c] Very crowded boating area of Mar del Plata harbour.
[d] Supernatant liquid of the deep sediment.
[e] Samples were collected after storm with winds from the south, named 'Sudestada'.

at the retention time of TBT was confirmed by GC–MS of the derivatized samples as giving TBTH. In all cases, the clusters corresponding to the molecular weight of TBTH (292 for ^{120}Sn), as well as the ions of m/e (M^+-1), (M^+-1-57), [M^+-1-(2 × 57)] and [M^+-1-(3 × 57)], were clearly shown.

The results obtained in the analysis of water and sediment samples for TBT and total tin are summarized in Table 1. As can be observed, TBT was not detected in samples of water from the Mar Chiquita area (samples 12 and 13 in Table 1), nor in Valeria del Mar (samples 14 and 15), a neighbouring open beach. In samples of water collected near the Mar del Plata (MdP) beach, (samples 1 and 2) and also in the MdP coastal area (samples 3–5), TBT was found in significant amounts. Small leisure boats arrive at the MdP coasts, and these might be the cause of the increased [TBT] with regard to the beach area. Interestingly, determination of the total tin concentration in the water and sediment samples by hydride generation atomic absorption spectrometry (HGAAS) indicated that the TBT made up mostly 100% of the tin, suggesting that anthropogenic organotins represent the major source of tin in these areas (data are given in the last column of Table 1). The observed results for [TBT] in the range 180–400 $ng\ l^{-1}$ in the waters of these two zones are higher than those reported, for example, by Suzuki *et al*.[45] i.e. 242 ± 30 $ng\ l^{-1}$ (72.6 $ng\ l^{-1}$ expressed as tin), for depuration experiments in Moroiso Bay (Japan), but they are comparable to the higher value of 480 $ng\ l^{-1}$ determined in December 1994 in the Aburatsbo Bay (Japan).[46] Nevertheless, more recent determinations at more than 100 sampling points in Japan showed important decreases of organotins in seawater: the maximum observed value was 9.8 $ng\ l^{-1}$,[1,47] and the concentrations of TBT in the open sea and in deep-sea locations are lower (a maximum of 0.1 $ng\ l^{-1}$).[48]

The sedimentary reservoir of TBT is important, given its apparent persistence. In contrast to the more rapid rates of degradation observed in the water column in laboratory studies (about 6–10 days) and in seawater (several weeks to several months according to recent determinations),[49] TBT has been proved to have a half-life in sediments in the range 0.9–5.2 years;[50] and a value as high as 8.7 years has recently been reported.[20] Therefore, the observed concentration ranges of TBT in sediments are usually higher than those in water. In the present study, the [TBT] in sediments of the MdP beach are not much higher than those observed in the seawater, suggesting that the inputs of TBT in this area could be relatively recent (probably arising from TBT coming from the more contaminated, crowded areas). On the other hand, at the MdP North Pier (where the boat traffic is intense and the area was restricted water exchange) and in samples coming from the MdP harbour (where big commercial vessels operate), the TBT concentrations determined in sediments are very large. The values found in the supernatant liquids of the sediment samples were also very high; we have found no reports on such high [TBT] in the recent literature. These values show the accumulation of TBT since probably more than 15 years ago. The highest reported values we have found

were those of Amouroux *et al*.,[51] who found concentrations of TBT of 3340 ng g^{-1} in sand and 600 ng g^{-1} in silt collected in the vicinity of the shipyard in Arcachon Harbour (France). A recently published range of measured TBT in different aquatic environments shows values within the range 1–1000 ng g^{-1} for harbour sediments.[52] The highest concentration levels observed in recent determinations of TBT in São Paulo State (Brazil) show 360 ng g^{-1} in Santos harbour and 670 ng g^{-1} in Guaruja marina, which seem to be related to intensive boat traffic.[23]

TBT determination in egg capsules of *Adelomelon brasiliana*

Interest in the effects of TBT on eggs of different species is currently being shown. Toxicity studies of TBT in fertilized eggs of gilthead seabream *Sparus aurata*[53] and also in eggs of *Ascidia malaca*[20] have recently been published, and the results confirm the acute toxicity of TBT; they showed that the fertilization process is affected greatly and the reproduction of ascidians under unfavourable environmental conditions is prevented. These studies were carried out by incubation of the eggs in solutions of TBT of variable concentration, and the different degrees of damage were evaluated. No determinations of TBT in the eggs were reported.

In the present study, egg capsules of *A. brasiliana* at different stages of development were collected near the more contaminated areas and methods were developed for the determination of TBT concentration in the liquid phase and in the solid phase. Table 2 shows the results obtained for egg capsules at different stages. It can be observed that, even in early stages of embryogenesis, TBT is present in the liquid, as in the dispersed phases; in fact, the total amount of TBT/egg capsule present in the liquid phase is much greater than that in the dispersed phase. In early stages of development, the amount of TBT is increasing, although it is still relatively low; the content/egg capsule of the liquid phase is also relatively higher than in the solid phase. Contrastingly, at stage 3, the TBT content in the solid phase is very high and the content/egg is higher than in the liquid phase.

It is probable that, if the egg matures, the new born gastropod will show a high degree of imposex, masculinization of the females and other known disorders caused by organotins. Although the egg capsules are permeable to TBT, the concentration observed even in the stage 0 of *A. brasiliana* eggs, which is much higher than that determined in the water of the more contaminated areas, suggests that TBT could be transferred from the gastropod females to the eggs.

To the best of our knowledge, this is the first report of the finding of TBT in the egg capsules of gastropods. TBT has been found in a neonate Beluga whale; since the necropsy revealed that the neonate had no milk in its stomach, the only explanation for the presence or butyltin compounds in the liver is via *in utero* transfer from maternal blood to the foetus.[12]

Table 2. Concentrations of TBT determined in eggs[a]

Development stage	Solid phase (ng/ovicapsule)	Liquid phase	
		ng ml^{-1}	ng/ovicapsule
0	14.2[b]	3.2	250
		2.9	240
0	41.8[b]	5.6	437
		4.2	328
1	390[c]	7.1	618
		2.4	213
		5.3	460
3	1180[d]	6.8	683
		5.3	484
3	800	6.0	548
		5.0	456

[a] Concentrations are reported as tin, according to Ref. 12.
[b] Dispersed solid.
[c] The solid phase was not abundant.
[d] Both phases had sharp limits.

CONCLUSIONS

Our results indicate that high concentrations of TBT in water and sediments in the Mar del Plata area, especially in the harbour and North Pier, are responsible for the high level of imposex observed in *B. monilifer* and also in *A. brasiliana*. The results also show that the TBT content made up mostly 100% of the tin present in the area, confirming that anthropogenic organotins represent the major source of tin there. The observed decrease in the population of *B. monilifer* and *A. brasiliana* in the Mar del Plata area make clear the need for controlling the environmental risks associated with the release of TBT, and the urgent ban of tin-based antifouling paints, at least for leisure and fishing boats. In view of the present results, fishermen's practices of cleaning and re-painting their boats along the Mar del Plata coasts should also be controlled.

The first reported finding of TBT in *A. brasiliana* egg capsules showed the accumulation of TBT. The observed concentrations, which are much higher than the environmental TBT concentration (surprisingly high in the liquid phase of early states of embryogenesis), likely suggest that it could also partially arise by transferability of TBT from the contaminated mother.

Acknowledgments

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