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Monitoring of Organotin Pollution in Bizerta Channel (Northern Tunisia): Temporal Trend from 2002 to 2010

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Abstract Imposex and tributyltin (TBT) body burden were quantified in the gastropod *Hexaplex trunculus* collected from the Bizerta channel between 2002 and 2010. Except for the imposex frequency that remained maximal (100%), all the other imposex indices decreased throughout the study period. Similarly, TBT levels also decreased over time, being the less frequent compound among butyltins, with a proportion of 22.2%, against 42.9% for dibutyltin (DBT) and 34.9% for monobutyltin (MBT). These findings reflect the effectiveness of new generation of TBT-free antifouling paint introduced in the Tunisian market and global ban of TBT on reducing the environmental impact of this biocide.

Keywords *Hexaplex trunculus* · Imposex · TBT · Southern Mediterranean

Tributyltin (TBT) is a biocide that has been widely used in antifouling paints for ships. It has been considered one of the most toxic substances and most potent androgenic compounds deliberately introduced into the marine environment (Morcillo and Porte 1998). Imposex in marine gastropods is one of the best documented examples of chemically-induced endocrine disruption in wildlife, with effects at all organizational levels, from molecular to population levels (Davies and Minchin 2002). Investigations on organotin pollution are well documented, mainly those focusing on spatial gradients. The evaluation of temporal trends in \pollution and gastropod population recovery from imposex are the primary targets of recent studies after legislative measures prohibiting the use of TBT. The application of antifouling paints containing TBT was banned in the late 1980s and early 1990s on ships less than 25 m in total length in Europe, USA, Canada, Australia, New Zealand, Japan and Hong Kong (Horiguchi et al. 1994; Ko et al. 1995; Evans et al. 1996; Stewart 1996). This partial ban revealed itself ineffective in reducing TBT pollution in many locations (Barroso et al. 2002; Santos et al. 2002). Thus, attention is now focused on the TBT global ban adopted in 2001 by the International Maritime Organization (IMO) and that entered into force in September 2008.

In the Tunisian market, TBT-based antifouling paints were discarded since 2003 and replaced by new TBT-free products. This step taken voluntarily by local paint producer reflects awareness against harmful effects of TBT on aquatic organisms, although no restrictions on its use were imposed locally. This was due to the fact that Tunisian antifouling paint producer has a partnership with an international company that complies with the legislation on the use of TBT (AkzoNobel® in Tunisia since 1993). This reflects that the Tunisian fleet already accomplishes the changes adopted by the IMO. Besides, most shipping traffic along the Tunisian coast comes from foreign countries that have already adopted TBT restrictions, which further supports a probable decline in TBT pollution starting from the date of banning. To verify this suspected recovery progress of the marine environment from TBT pollution,

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imposex was monitored in *Hexaplex trunculus* collected in the most TBT-polluted site in Tunisia (Bizerta channel), from 2002 (a year before replacing old paint) to 2010 (2 years after the global ban entered into force in European neighbouring countries). The present study constitutes a baseline after the IMO global ban in 2008, which might be very useful for future monitoring programmes in the Mediterranean, especially in the southern countries where no restrictions on the use of TBT-based paints were yet adopted.

Materials and Methods

Samplings of *H. trunculus* were performed in 2002, 2003, 2004, 2005, 2007 and 2010 at the same station in the Bizerta channel (northern Tunisia) (Fig. 1). This channel, of 1,500 m in length, 240 m in width and with 12 m in depth, is characterized by intense shipping traffic (more than 500 commercial boats per year). The individuals collected were originally subjected to studies on the species biology (mainly growth and reproduction), and then were preserved in the freezer for subsequent analyses aiming to detect changes in imposex and TBT body burden before and after the introduction of the new generation TBT-free paint. A number of 48–93 adult *H. trunculus* (50–60 mm in shell length) belonging to the sample of July from each year were thawed and analysed. Specimens were measured for shell length (SL) using a vernier calliper to the nearest 0.1 mm and then de-shelled using a bench vice. The soft-body was extracted from the shell and the sex was determined under a binocular microscope, based on the presence of a capsule gland and a vagina in females and the presence of a big penis in males. Penis length (PL) was also measured under a dissecting microscope using a calibrated eyepiece. It is well known that freezing and thawing increases penis measurements in dead specimens compared

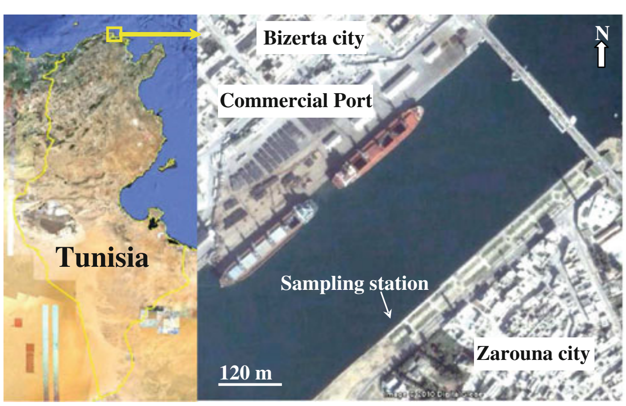

Fig. 1 Location of sampling station in the Bizerta channel (northern Tunisia)

to live individuals. However, because this is expected to occur in both sexes, it is not supposed to significantly affect the calculation of imposex indices based on penis measurements. Besides, sampling date (July) corresponds to the resting phase in the species reproductive cycle, when male PL does not exhibit significant monthly variation (Lahbib et al. 2009a). Relative Penis Length Index (RPLI), defined as the length of the female penis relative to that of the male, was calculated using the following equation: [(penis length of each female/average male penis length) $\times$100]. The degree of imposex in each individual was also quantified using the Vas Deferens Sequence Index (VDSI) following the scheme updated for *H. trunculus* by Lahbib et al. (2008) [VDSI = (sum of imposex stages of all females)/(total number of females)]. The VDSI varies from 0 to 5, with stage 0 denoting absence of imposex and stage 5 indicating female sterility (due to overgrowth of vas deferens tissues or the splitting of the capsule gland). Butyltin analyses were performed using five males and five females randomly selected from each sample. The operculum was removed and the soft-body was finely grinded in glass bottles using a T18 basic Ultra-Turrax® disperser at 6,000 rotations min^{-1}. Thereafter, tissues were freeze-dried, weighed and maintained in the dark at −20°C until analysis. Approximately 150 mg of sample (two replicates) were spiked with a diluted solution of the ^{119}Sn-enriched spike of monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT) and 4 mL of a mixture of acetic acid and methanol (3:1) (g/g) were immediately added in 7 mL glass vials with screw cap (Supelco, Bellefonte, PA). The vials were introduced in a thermostatic bath at 37°C for at least 2 h under mechanical shaking. During the *derivatization*, 2 mL of acetate buffer to adjust the pH to 5.4, 2 mL of hexane and 300 µL of a 2% w/v sodium tetraethyl borate in 0.2 M NaOH were added to 1 mL of the extractant for the ethylation of the organotin compounds. Then, the vials were centrifuged at 3,000 rpm for 5 min to allow a better phase separation. The organic layer was transferred to a 2 mL chromatographic vial with a Pasteur pipette. The hexane phase was evaporated under a gentle stream of argon until nearly dry (a few microliters). Finally, 2 µL of this final volume were injected into the GC–MS system. The analytic instrument was an Agilent Model 6890 N gas chromatograph (Agilent Technologies, Waldbronn, Germany) fitted with a splitness injector and a HP-5MS column (30 m × 250 mm i.d. × 0.25 mm) and equipped with an Agilent Model 5973 Network MSD mass spectrometric detector (Agilent Technologies, Tokyo, Japan). The detection limits in solid matrix (sediment samples, 3 replicates) were 0.09 ng Sn/g for MBT, 0.03 ng Sn/g for DBT and 0.06 ng Sn/g for TBT. Analysis of a certified reference material (mussel tissue BCR 477, 3 replicates) using this procedure resulted in the following recoveries:

116.17 ± 2.65 for MBT, 98.13 ± 1.76 for DBT and 91.43 ± 1.57 for TBT, being in agreement with the certified range. In order to ascertain the chronology of organotin contamination, the butyltin degradation index (BDI) was calculated as follows: BDI = (MBT + DBT)/TBT. Calculation of statistical tests between years was conducted using the software Sigmastat® 3.5 for Windows. The paired sample *t*-test was used to compare differences in RPLI and butyltin body burden and the Mann–Whitney rank sum test for comparing VDSI.

Results and Discussion

Imposex data for *H. trunculus* from the Bizerta channel was reported for the first time for 2002, 2003, 2005 and 2010, as compared to previous studies that only analysed samples of 2004 and 2007 (Lahbib et al. 2008, 2009a, b). Using preserved snails allowed gathering valuable data on imposex and TBT levels in samples collected before the use of the new generation of TBT-free antifouling paints. As suspected, imposex levels were invariably high in all samples collected from the Bizerta channel, which agrees with previous investigations, although in a different sampling station (Lahbib et al. 2008, 2009a, b). This is explained by the existence of the commercial harbour that receives yearly more than 500 boats of more than 100 m in length (Fig. 1). Although imposex incidence did not change between 2002 and 2010, a temporal decline in imposex intensity was registered in the present study. The VDSI, RPLI and FPL decreased progressively across the sampling years (Table 1). Significant decrease in VDSI versus 2002 was observed starting from 2004 (Mann-Withney Rank sum test, $p < 0.05$). Pairwise comparison between years showed similar values for 2002 versus 2003 and 2004 versus 2005. Imposex stages were all above VDS4 in 2002 and 2003 with stage 4.3 being the most frequent until 2005 (Table 1). Stage 4 appeared starting from 2004 with a rate of 35.1% and increased continuously until 2010, when stage 3 was recorded for the first time with 8.2%. VDS 4.7 was absent from 2007 to 2010 samples, but VDS5 (which corresponds to sterility) was still recorded in these samples at low rates (5.2% and 3.3%, respectively) (Table 1). The occurrence of sterility despite the significant global decline in imposex intensity is certainly related to the irreversibility of imposex and the relatively high longevity of the species (duration of exposure to contamination). This could also explain the very slow decrease in FPL, which was only statistically significant for the 2010 sample (Table 1). Although r values were not significant for all samples ($p > 0.05$) due to the narrow size class (50–60 mm), this parameter was positively correlated to female SL for the period 2002–2007 (data not showed). As for FPL, the RPL values decreased progressively across the years, being similar from 2002 to 2007 and becoming significantly different in 2010 (*t*-test $p < 0.05$, Table 1).

High TBT body burdens were recorded in 2002 sample, then significantly decreased across the years, except between 2005 and 2007 (*t*-test, $p > 0.05$). In terms of total butyltins, high bioaccumulation was also recorded in 2002

Table 1 Imposex and butyltin levels in *Hexaplex trunculus* collected in the Bizerta channel from 2002 to 2010

	2002	2003	2004	2005	2007	2010
Imposex indices						
%I	100.0	100.0	100.0	100.0	100.0	100.0
	62.7 ± 22.	57.2 ± 24.	56.1 ± 19.	51.7 ± 19.	39.4 ± 21.	12.8 ± 9.
RPLI	4	6	1	0	3	2
FPL (mm)	10.5 ± 3.8	9.8 ± 4.3	9.6 ± 3.8	9.5 ± 3.9	7.5 ± 4.1	2.4 ± 1.7
VDSI	4.5 ± 0.3	4.4 ± 0.2	4.3 ± 0.3	4.3 ± 0.3	4.2 ± 0.3	4.0 ± 0.3
VDS stages (%)						
VDS3	0	0	0	0	0	8.2
VDS4	0	0	35.1	31.1	57.9	82.0
VDS4.3	66.6	75	40.5	57.8	36.8	6.6
VDS4.7	16.6	14.6	13.5	4.4	0	0.0
VDS5	16.6	10.4	10.8	6.7	5.2	3.3
Butyltins (ng/g dw)						
TBT	65.2 ± 0.5	32.5 ± 0.4	24.3 ± 0.4	12.3 ± 0.1	13.2 ± 0.9	6.1 ± 0.3
DBT	101.2 ± 0.5	58.1 ± 0.1	48.5 ± 0.4	53.0 ± 0.5	20.8 ± 0.6	14.1 ± 0.2
MBT	59.1 ± 0.4	67.9 ± 0.1	48.1 ± 0.5	38.2 ± 0.3	21.1 ± 0.6	6.8 ± 0.8
BDI	2.5	3.9	4.0	7.4	3.2	3.4

Table 2 Correlations established between imposex indices and TBT/BTs in 278 *Hexaplex trunculus* during the study period (2002–2010)

Equation		R2
FPL versus TBT	$y = 2.93Ln(x) - 0.41$	0.68
FPL versus BTs	$y = 3.65Ln(x) - 8.29$	0.89
VDS versus TBT	$y = 0.2Ln(x) + 3.79$	0.91
VDS versus BTs	$y = 0.23Ln(x) + 3.24$	0.98
RPLI versus TBT	$y = 18.88Ln(x) - 8.99$	0.76
RPLI versus BTs	$y = 22.82Ln(x) - 56.73$	0.95
Sterility versus TBT	$y = 5.68Ln(x) - 7.97$	0.95
Sterility versus BTs	$y = 5.761Ln(x) - 17.32$	0.83

and then significantly decreased from a year to another (*t*-test $p < 0.05$, Table 1). It seems that this decline in TBT levels, as well as in imposex severity, reflects some effectiveness related to the introduction of the new generation paint (TBT-free) since 2003. The slow decrease observed from 2002 to 2005 is certainly explained by the slow rhythm in coating replacement, which became faster in latest years. Although Tunisia is not contracted to the AFS convention, another factor is the regulation imposed by many neighbouring European countries on the use of TBT, which started in 2003 and entered into force in 2008. Thus, the number of foreign boats coated with TBT-based paint has reduced starting from 2003 and stopped in 2008. These are the main reasons why the decline in imposex and TBT levels were significant in 2007 and 2010, reflecting the effectiveness of the entry into force of the IMO global ban.

Considering all samples, among the three butyltins compounds analysed, TBT was the least detected (153.2 ng/g), followed by MBT (181.6 ng/g) and DBT (295.3 ng/g). The BDI was above 1 in all samples, reflecting an historical pollution. Thus, the available fraction of TBT found in the studied site could have been leached by sources other than vessels. Indeed, some authors consider that after the IMO ban entry into force, TBT inputs will be maintained by the remobilization from sediments to the water column and some illegal use (Galante-Oliveira et al. 2010).

The establishment of relationships through linear regression between imposex indices and pollutant concentrations allowed detecting significant correlations between variables (r^2 values, Table 2). Relationships were better correlated using total butyltins instead of TBT concentrations (Table 2). Higher correlations were obtained for VDSI and sterility rate, whereas the worst fitting was recorded for FPL.

Monitoring the Bizerta channel (the most TBT-vulnerable site along the Tunisian coasts) for the period 2002–2010 allowed gathering data on TBT temporal trends before and after introduction of new generation TBT-free paint in Tunisia, and after the restrictions in the use of TBT-based paints imposed in neighbouring European countries. This study is be very useful by acting as baseline data for future monitoring programmes in the Mediterranean. The present results were very satisfactory in terms of imposex recovery and pollution decline. Nevertheless, imposex levels are still high in the studied site and therefore monitoring surveys should continue in the near future until final recovery.

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