

RESEARCH ARTICLE

## TBT pollution in Tunisian coastal lagoons as indicated by imposex in *Hexaplex trunculus* (Gastropoda: Muricidae)

Y. Lahbib, S. Abidli, N. T. El Menif\*

Department of Biology, Laboratory of Environment Biomonitoring, Group of Applied and Fundamental Malacology (LBE/MAF), University of Carthage, Faculty of Sciences of Bizerta, 7021 Tunisia

\*Corresponding author: Telephone/Fax: +21672590566; E-mail address: najoua.trigui.elmenif@gmail.com

### Abstract

- 1 - Most of the shipping traffic in Tunisia comes from foreign countries that already enforced tributyltin (TBT) regulation.
- 2 - Local and imported antifouling paints in Tunisia did not contain TBT.
- 3 - Actual TBT contamination in Tunisia is the result of an old/historical pollution events.
- 4 - Here we report data on imposex in *Hexaplex trunculus* from three Tunisian lagoons. This gastropod, which locally has fishery great commercial value, is currently used mostly for monitoring TBT effects in transitional and marine waters in the Mediterranean Sea.
- 5 - Our results showed a decrease of imposex in Bizerta and in Northern Tunis lagoons, whilst in the southernmost lagoon of Boughrara imposex has significantly increased.
- 6 - The effect of specimen size and reproductive activity on penis length variation in males and on the relative penis length index was validated.
- 7 - Overall, we found that TBT is still a significant pollutant in the Tunisian waters, which requires further studies on the contamination causes.

**Keywords:** Coastal lagoons, TBT, Imposex, Southern Mediterranean, Tunisia

### Introduction

The imposex deformity of *Hexaplex trunculus*, i.e. the masculinization of females, is currently studied in relation to tributyltin (TBT) contamination, TBT being used in the past as an antifouling agent. The suspected effects of imposex in *H. trunculus* are mainly somatic growth in females (Axiak et al., 1995; Rilov et al., 2000), the sterility recorded in some sites in Malta, Italy and Tunisia (Axiak et al., 1995; Terlizzi et al., 1999; Trigui El Menif et al., 2006; Lahbib et al., 2008), and decrease in fecundity (Trigui El Menif et al., 2006; Lahbib et al., 2009a).

Several indices have been developed to characterize imposex intensity, among which the most used are the Vas Deferens Sequence Index (VDSI), the Relative Penis Length Index (RPLI) and the Relative Penis Size Index (RPSI) (Gibbs et al., 1987). VDSI gives an indication of the reproductive competency of the population, while both RPLI and RPSI express the relative development of the penis in males and females, either in terms of length or bulk. Recently, the use of RPSI has been questioned, because it has been found to vary along with the reproductive activity and size of some gastropod species

(Oliveira et al., 2010; Vasconcelos et al., 2010). Imposex has been also found in Tunisian lagoons and coastal waters (Trigui El Menif et al., 2006; Lahbib et al., 2007; 2008; 2009a,b). Tunisia is still not a contracted country to the Antifouling System (AFS) convention and no local legislations have been enforced so far to restrict the TBT use. However, the shipping traffic comes mainly from foreign countries that already banned TBT use. Besides, local and imported antifouling paints available in Tunisia do not contain TBT. Therefore, we hypothesised that TBT pollution is presently due to former contamination and that the available TBT likely comes from sediment stocks.

This study aims at assessing possible effects of the residual TBT bulk, through the analysis of imposex in *H. trunculus* from three Tunisian lagoons that are suspected to have different TBT contamination levels. The effects of gastropod size and reproductive activity on the relative penis length index were also studied taking into account data from previous works in the same studied sites.

**Materials and methods**

*Study area and target species*

Six coastal lagoons are located along the Tunisian coastline, accounting for about 1000 km<sup>2</sup>. They are exploited for fishery and shellfish farming, mainly in the Bizerta (150 km<sup>2</sup>), Tunis (39 km<sup>2</sup>) and Boughrara (500 km<sup>2</sup>) lagoons. The banded murex *H. trunculus* is a gastropod distributed mainly in the Mediterranean Sea and adjacent Atlantic Ocean, from the Portuguese south coast, southward to Morocco and to the Madeira and Canary Archipelagos (Houart, 2001). It is a commercially valuable species in several Mediterranean countries. This species occurs in the inter-tidal and infralittoral zones down to 120 m depth (Poppe and Goto, 1991), being more frequent down to 30 m depth (Houart, 2001). *H. trunculus*

inhabits both hard and soft substrates, from rocky shores (Houart, 2001) to sandy, sandy-muddy and preferentially muddy bottoms (Poppe and Goto, 1991). In Tunisian lagoons, *H. trunculus* is a very common gastropod and usually fished at low tide especially in spring, either by hand harvesting or with fishnets in the deeper zones.

Individuals of *H. trunculus* (35-78 mm shell length) at resting stage of reproduction were sampled in July 2007 in 18 stations: seven in Bizerta, six in Northern Tunis and five in Boughrara lagoons (Fig. 1). These lagoons have variable boating traffic. The Bizerta lagoon showed the highest traffic in terms of number and type of Boats (~ 700 fishing boats: trawlers, sardine boats, coral boats, inshore fishing boats, and ~ 550 commercial

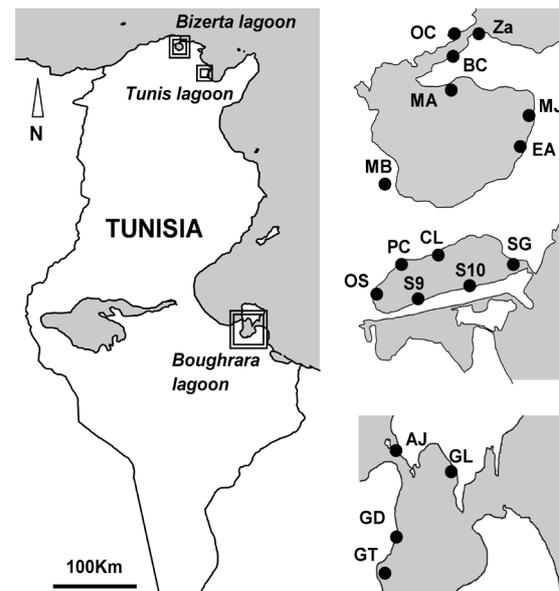


Figure 1. Sampling stations in studied lagoons from Tunisian coast. Bizerta lagoon (7): Zarzouna (Za), Cereal Office (OC), Quarry Bay (BC), Menzel Abderrahmen (MA), Menzel Jemil (MJ), Menzel Bourguiba (MB), El Azib (EA); Tunis north lagoon (6): Lake Club (CL), Small Channel (PC), Onas (OS), station 9 (S9), station 10 (S10), Steg (SG); Boughrara lagoon (5): Ajim (AJ), Gallala (GL), Gightis-Djorf (GD), Gightis (GD), Ain Maider (AM).

boats: liners, oil tankers, car ferries, gas boat, containers). This traffic is mainly concentrated in the Bizerta canal (stations of OC, Za and BC: ~ 500 fishing boats and ~ 550 commercial boats), followed by MA (~ 150 inshore fishing boats) and MB, MJ, EA (~ 30 inshore fishing boats each). We noted that the MB showed a boat repair station. In the northern Tunis lagoon, no boats were found at S9 and OS, 5 inshore boats at S10 and PC, and 165 rowing and canoeing kayaks at CL. In Boughrara lagoon, most boating traffic was concentrated at AJ (300 inshore fishing boats and 4 Car Ferry) and GT (50 inshore fishing boats). At GD, AM and GL, 6 to 20 inshore boats were recorded. All the sampled specimens were used to analyse the relationship between mollusk size and penis, which in turn affect the RPLI calculation. Thereafter, only animals in the most common size range (50-60 mm) were selected for studying imposex. The specimen numbers varied between 7 and 31 for males and between 7 and 93 for females.

#### *Analysis of imposex*

In the laboratory, snails were sacrificed by freezing. After thawing, the shell was measured to the nearest 0.01 mm and broken with a bench vice. The soft tissues were carefully removed and the mantle was longitudinally cut to analyse the pallial oviduct in females. Sex was determined from the presence or absence of the capsule gland, vagina and penis.

Imposex was quantified using the following indices: i) the imposex incidence or frequency ( $I\% = \text{percentage of imposex-affected females compared to the total number of females in the sample}$ ), ii) the vas deferens sequence index (Lahbib et al., 2007) updated for *H. trunculus*, where  $VDSI = (\text{sum of imposex stage values of all females}) / (\text{total number of females})$ , iii) the average vas deferens length for each female  $AVDL = \text{female vas deferens length} / \text{total number of females}$ , and iv) the

Relative Penis Length Index ( $RPLI = \text{female average penis length} / \text{male average penis length} \times 100$ ). The effect of reproductive activity on RPLI and the temporal trend of imposex indices were investigated using data from previous studies on reproductive cycle (Lahbib et al., 2009a) and imposex (Lahbib et al., 2008). All imposex indices were recalculated for the class-size 50-60mm from previous study (Lahbib et al., 2008), which was released in July 2004, to make reliable comparison with the present investigation. Data on monthly variations of RPLI, PL and GAI (gonad area index) were retaken again from Lahbib et al. 2009a in order to discuss the effect of reproductive cycle.

The Chi-square and Mann Whitney rank sum tests were used respectively for comparison of I (%) and VDSI. However, the paired sample t-test was used to compare differences in AVDL, APL and RPLI. The degree of correlation between specimen size and male penis length was tested using Fisher test. Statistical tests were performed in 2004 and 2007 data using the software Sigmastat® 3.5 for Windows.

## **Results**

### *Imposex analysis*

Imposex was detected in 17 stations out of 18, in both campaigns, while no imposex was observed at AM station in Boughrara lagoon in 2004, and at PC in Northern Tunis lagoon in 2007. The level and intensity of imposex were higher in Bizerta lagoon, than in the other two lagoons. At this site, imposexed females ranged from 60% to 100% in 2004; and from 28% to 100% in 2007. Females were found all abnormal in five stations out of 7 in 2004 and three in 2007. From 2004 till 2007, the VDSI remained above 3.3 in four stations (OC, Za, BC and MA) and decreased in the others (Fig. 2). Overall, the most affected station was OC, where sterile females were observed (21.4% in 2004 and 5% in 2007,

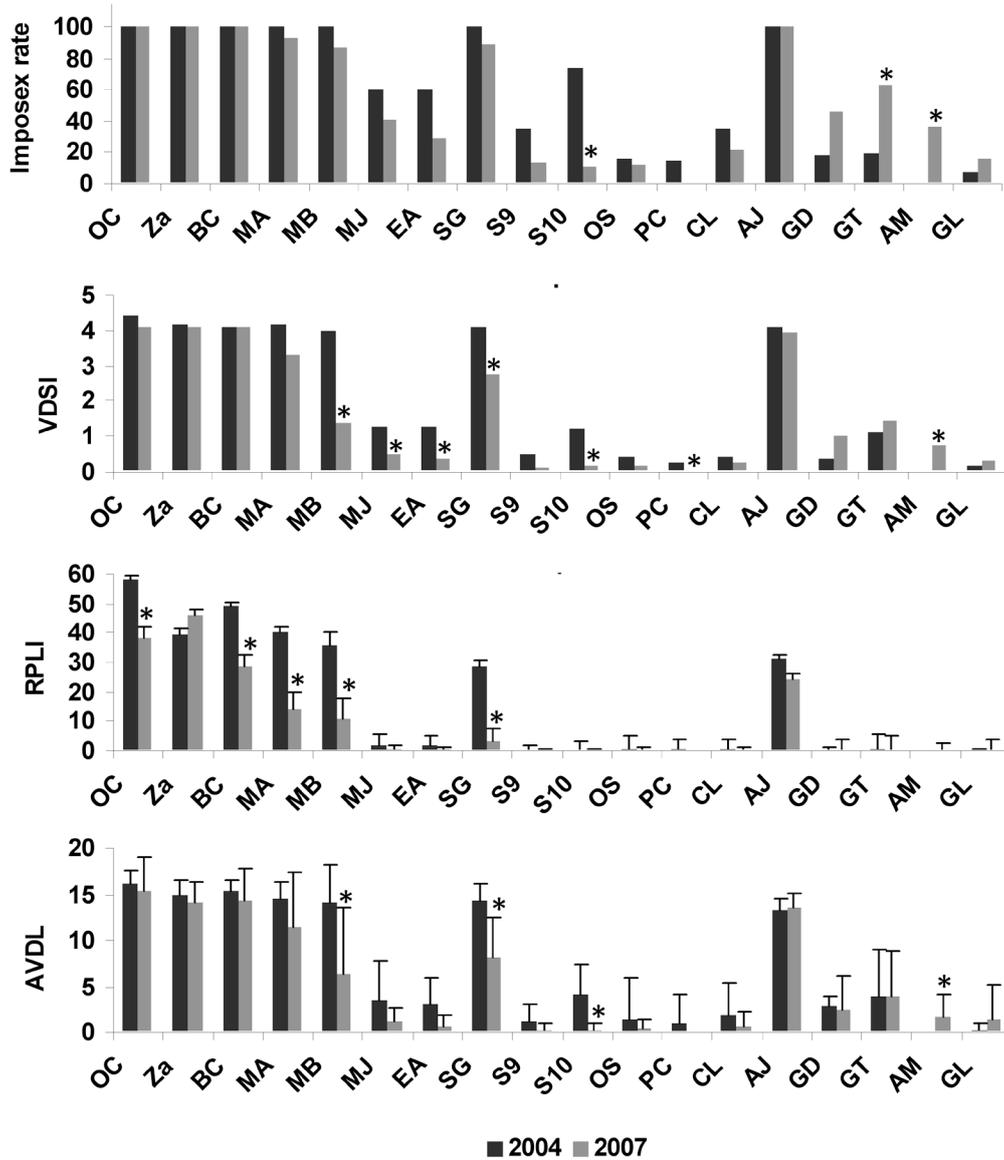


Figure 2. Comparison of imposex indices in *Hexaplex trunculus* from the studied lagoons between 2004 and 2007. (\*) denotes significant differences at  $p < 0.05$ .

Table 1). The BC station also showed up to 6.7% of sterile females in 2007, reflecting an increase in imposex degree (Table 1). The less affected populations showing weak values of VDSI were found at MJ and EA stations. RPLI and AVDL underwent a wide variability, from 1.7 to 57.9% and 3.0 to 16.0 mm in 2004, showing a decrease in all stations, except for RPLI at station Za (Fig. 2).

In the Northern Tunis lagoon, imposex was detected in all the stations in 2004 and has significantly decreased after three years namely in PC where a total recovery occurred (93 analyzed females) (Table 1). The most affected station in both campaigns was SG (Fig. 2). In the other stations, the phenomenon was less developed with VDS0 being the dominant stage (Table 1).

Table 1 - Percentage (%) of imposex stages (VDSrange) in the studied stations in 2004 (value above) and 2007 (value below). N: female number, SL: shell length. VDS: vas deference sequence (imposex stage). Bizerta lagoo: Za (Zarzouna), OC (Cereal Office), BC (Quarry Bay), MA (Menzel Abderrahmen), MJ (Menzel Jemil), MB (Menzel Bourguiba) and EA (El Azib); Tunis north lagoon: CL (Lake Club), PC (Small Channel), OS (Onas), S9 (station 9), S10 (station 10) and SG (Steg); Boughrara lagoon: AJ (Ajim), GL (Gallala), GD (Gightis-Djorf), GD (Gightis) and AM (Ain Maider).

|             | N  | SL (mm)  | VDS0 | VDS1d | VDS2d | VDS2d' | VDS3a | VDS3b | VDS4 | VDS4.3 | VDS4.7 | VDS5 |
|-------------|----|----------|------|-------|-------|--------|-------|-------|------|--------|--------|------|
| <b>OC</b>   | 13 | 53.9±2.8 | 0    | 0     | 0     | 0      | 0     | 0     | 21.5 | 57.1   | 0      | 21.4 |
|             | 20 | 54.7±2.7 | 0    | 0     | 0     | 0      | 0     | 0     | 70.0 | 25.0   | 0      | 5.0  |
| <b>Za</b>   | 16 | 53.9±4.2 | 0    | 0     | 0     | 0      | 0     | 0     | 43.8 | 56.2   | 0      | 0    |
|             | 16 | 56.4±2.6 | 0    | 0     | 0     | 0      | 0     | 0     | 62.5 | 36.5   | 0      | 0    |
| <b>BC</b>   | 21 | 53.3±3.2 | 0    | 0     | 0     | 0      | 0     | 0     | 66.7 | 33.3   | 0      | 0    |
|             | 16 | 53.8±4.3 | 0    | 0     | 0     | 0      | 0     | 7     | 53.0 | 33.0   | 0      | 7.0  |
| <b>MA</b>   | 8  | 51.9±2.7 | 0    | 0     | 0     | 0      | 0     | 0     | 50.0 | 50.0   | 0      | 0    |
|             | 14 | 54.9±4.5 | 7.1  | 14.3  | 0     | 0      | 0     | 0     | 71.4 | 7.1    | 0      | 0    |
| <b>MB</b>   | 19 | 56.1±2.5 | 0    | 0     | 5.3   | 0      | 10.5  | 0     | 15.8 | 63.1   | 5.3    | 0    |
|             | 13 | 53.9±3.9 | 41.2 | 35.3  | 0     | 11.8   | 0     | 0     | 11.8 | 0      | 0      | 0    |
| <b>MJ</b>   | 23 | 52.6±2.1 | 39.2 | 13.0  | 0     | 39.1   | 0     | 0     | 8.7  | 0      | 0      | 0    |
|             | 12 | 51.9±2.9 | 58.3 | 33.3  | 0     | 8.4    | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>EA</b>   | 10 | 52.6±3.4 | 40.0 | 0     | 0     | 50.0   | 10.0  | 0     | 0    | 0      | 0      | 0    |
|             | 14 | 54.0±3.9 | 71.4 | 21.4  | 0.0   | 7.2    | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>SG</b>   | 14 | 52.9±4.0 | 0    | 0     | 0     | 0      | 0     | 0     | 64.3 | 35.7   | 0      | 0    |
|             | 40 | 53.5±2.3 | 10   | 5     | 20    | 7.5    | 15    | 2.5   | 35   | 5      | 0      | 0    |
| <b>S 9</b>  | 31 | 51.4±2.7 | 64.3 | 21.4  | 7.1   | 7.2    | 0     | 0     | 0    | 0      | 0      | 0    |
|             | 31 | 50.5±1.9 | 87.1 | 12.9  | 0     | 0      | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>S 10</b> | 30 | 52.6±3.3 | 23.3 | 43.3  | 30.0  | 0      | 0     | 3.3   | 0    | 0      | 0      | 0    |
|             | 29 | 50.1±0.4 | 89.7 | 6.9   | 0     | 3.4    | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>OS</b>   | 33 | 54.5±2.9 | 81.8 | 12.1  | 0     | 0      | 0     | 0     | 0    | 0      | 6.1    | 0    |
|             | 42 | 51.7±2.5 | 88.1 | 7.1   | 4.8   | 0      | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>PC</b>   | 14 | 55.1±3.2 | 85.7 | 0     | 0     | 14.3   | 0     | 0     | 0    | 0      | 0      | 0    |
|             | 93 | 50.9±2.1 | 100  | 0     | 0     | 0      | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>CL</b>   | 14 | 50.9±1.4 | 64.3 | 28.6  | 0     | 7.1    | 0     | 0     | 0    | 0      | 0      | 0    |
|             | 45 | 51.0±2.4 | 80.0 | 15.6  | 4.4   | 0      | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>AJ</b>   | 31 | 54.5±3.4 | 0    | 0     | 0     | 0      | 0     | 0     | 67.7 | 29.0   | 3.3    | 0    |
|             | 31 | 54.4±3.7 | 0    | 0     | 0     | 6.5    | 0     | 0     | 64.5 | 29.0   | 0      | 0    |
| <b>GD</b>   | 28 | 53.1±3.8 | 82.1 | 3.6   | 0     | 7.1    | 3.6   | 3.6   | 0    | 0      | 0      | 0    |
|             | 43 | 50.6±1.5 | 53.4 | 0     | 39.4  | 0      | 4.6   | 2.3   | 2.3  | 0      | 0      | 0    |
| <b>GT</b>   | 14 | 51.8±3.5 | 57.1 | 7.1   | 0     | 0      | 0     | 35.8  | 0    | 0      | 0      | 0    |
|             | 43 | 51.0±1.9 | 34.9 | 0     | 55.9  | 0      | 2.3   | 2.3   | 4.6  | 0      | 0      | 0    |
| <b>AM</b>   | 7  | 50.6±0.7 | 60   | 40    | 0     | 0      | 0     | 0     | 0    | 0      | 0      | 0    |
|             | 38 | 51.0±1.9 | 63.2 | 0     | 36.8  | 0      | 0     | 0     | 0    | 0      | 0      | 0    |
| <b>GL</b>   | 13 | 52.4±2.9 | 92.3 | 0     | 0     | 7.7    | 0     | 0     | 0    | 0      | 0      | 0    |
|             | 32 | 51.0±2.3 | 84.4 | 0     | 6.3   | 9.3    | 0     | 0     | 0    | 0      | 0      | 0    |

In the lagoon of Boughrara, imposex was reported in 4 stations out of 5 in 2004. Except AJ, the sole station with high imposex level and degree (I: 100%, VDSI >4, AVDL > 13mm and RPLI > 30%), the deformity was found at basic stages not exceeding the VDS3 (Fig. 2, Table 1). In 2007, the status remained at the same level

in the AJ station, while it increased in the other four stations (Fig. 2).

*Importance of male penis variation in RPLI calculation*

Penis length in males was significantly correlated with shell length ( $r = 0.731$ ,  $P < 0.001$ ). Bigger males have larger penis

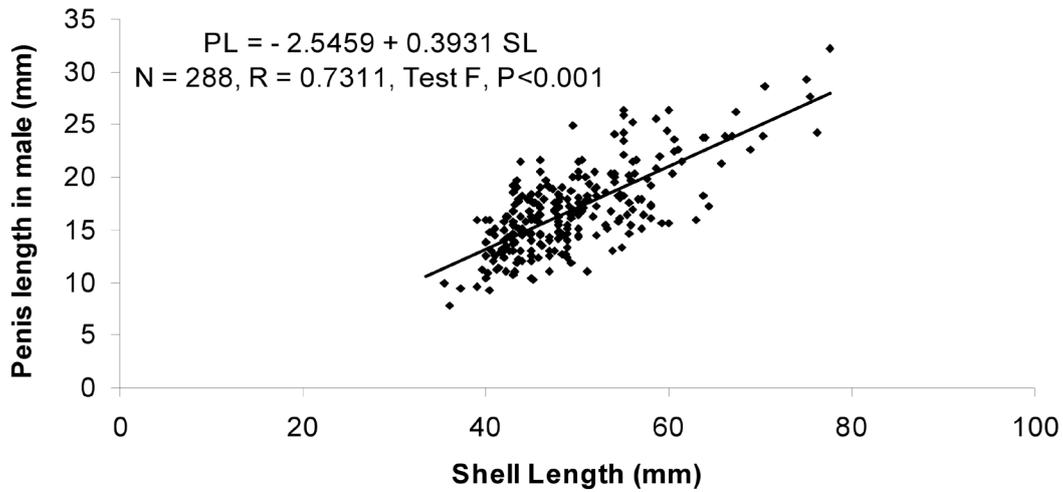


Figure 3. Linear regression established between penis length and shell length in males collected in all stations.

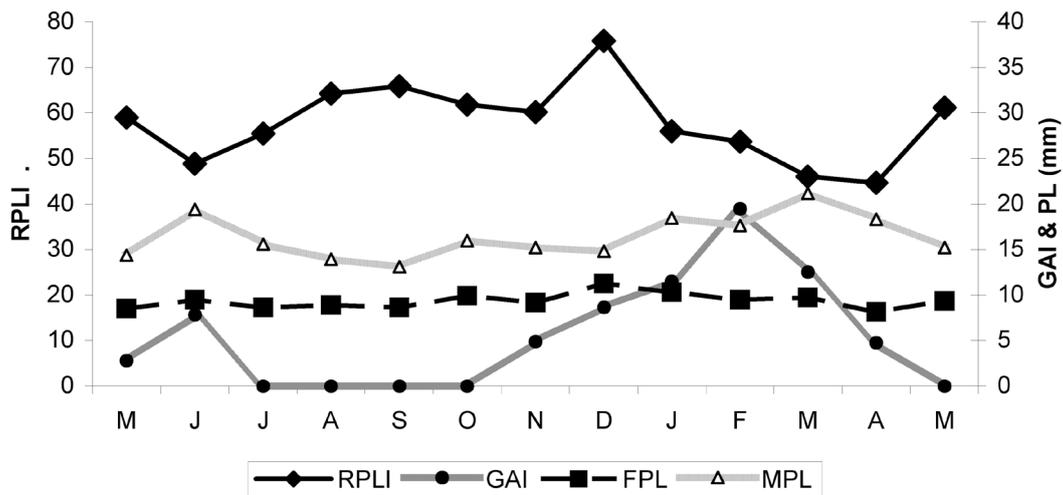


Figure 4. Monthly variations of RPLI, PL and GAI in Bizerta canal from May 2004 to May 2005 (Lahbib et al. 2009a). FPL: female penis length, MPL: male penis length, GAI: gonad area index.

(Fig.3). In both sexes, variations in PL were recorded during a one year study period in the Bizerta canal. While PL females showed a random and not significant variation throughout the year, without any discernible temporal trend, male penis presented evident seasonal variation with a continuous increase from December to March and decrease from

March to May. This trend was similar to that of GAI, penis length increased with the degree of gonadic maturation (Fig.4). However, the temporal trend of RPLI was inversely related with MPL.

**Discussion**

Among the three studied sites, the Bizerta

lagoon was the most affected by imposex. This was likely due to the high boating traffic, which generated a great TBT discharges through the channel of the commercial port of Bizerta. Imposex level and degree decreased progressively in the lagoons with less boat traffic. However, here imposex level and degree were greater only in one station in each lagoon, while in the remaining stations the deformity was weaker. The high imposex recorded in the SG station, despite there is no boating traffic, is due to the input of polluted waters from the channel of Kheiredine, which links this station to the commercial port of La Goulette (more than 1400 boats/year).

The temporal trend of imposex indices in *H. trunculus* showed some evidence of imposex recovery between 2004 and 2007 in the lagoons of Bizerta and Tunis north. This was confirmed by the decrease in TBT burden in these locations between 2004 and 2007 (Lahbib et al., 2009b), which probably resulted from banning TBT. The dredging of the Kheiredine channel and Northern Tunis lagoon in 1984 has certainly caused a decrease of TBT concentrations in this site. In the lagoon of Boughrara, the slight increase of TBT concentration in 2007 (Lahbib et al., 2009b) was sufficient to augment imposex levels, in agreement with Axiak et al. (1995) who found that imposex was triggered by TBT concentrations lower than 1 ng Sn g<sup>-1</sup> dw.

This study allowed to test that RPLI depended on specimen size and reproductive cycle (Oliveira et al., 2010). The penis length in males collected from the three lagoons was significantly correlated with the shell length, suggesting that class size should be fixed in imposex investigations to get more reliable quantification.

Data on imposex and reproductive cycle of *H. trunculus* from a previous work showed that male penis also exhibits seasonal variation, related to gonadic development. Whilst, in imposexed females the penis did not show

significant variation during the reproductive cycle (Fig.4) but was probably related with TBT concentrations in the environment.

The sampling of gastropods during July (resting period, Fig. 4), has certainly minimized the fluctuation of penis length (and consequently RPLI) caused by the reproductive activity but despite that, some variations were still present between sites, which also has implications on spatial comparison of imposex using RPLI. The spatial variation of male penis length could be related to the asynchrony in reproductive cycles between sites and to genetic variability among populations. Thus, for inter-site comparison of imposex the average female penis length (AFPL) seems to perform better than RPLI, because FPL did not show variations during reproductive cycle.

The highest degree of imposex found in the present study was less than that reported in the lagoon of Venice (Italy), but it was greater than that reported for the same species in the Ria Formosa lagoon (Portugal) (Vasconcelos et al., 2006).

Notwithstanding the decreasing degree of imposex in most of the considered stations, we found sites with a persistent TBT contamination from sediment. The sterility significantly decreased from 21% in 2004 to 5% in 2007 and currently does not seem to affect the life cycle of Hexaplex. The RPL index is also sensitive to the gastropod size and reproductive cycle. For this reason, it should be not used in either imposex monitoring or studies.

## References

- Axiak V, Vella AJ, Micaleff D, Chircop P, Mintoff B 1995. Imposex in *Hexaplex trunculus* (Gastropoda: Muricidae): first results from biomonitoring of tributyltin contamination in the Mediterranean. *Marine Biology* 121: 685-691.
- Gibbs PE, Bryan GW, Pascoe PL, Burt GR 1987. The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *Journal of the Marine Biological Association of*

- the United Kingdom* 67: 507-523.
- Houart R 2001. *A review of the Recent Mediterranean and Northeastern Atlantic Species of Muricidae*. Evolver publisher, Rome, Italy.
- Lahbib Y, Abidli S, Le Pennec M, Flower R, El Menif NT 2007. Morphological expression and different stages of imposex in *Hexaplex trunculus* (Neogastropoda: Muricidae) from Tunisian coasts. *Cahiers de Biologie Marine* 48: 315-326.
- Lahbib Y, Abidli S, Trigui El-Menif N 2008. Imposex level and penis malformation in *Hexaplex trunculus* from the Tunisian coast. *American Malacological Bulletin* 24: 79-89.
- Lahbib Y, Abidli S, El-Menif NT 2009a. Relative growth and reproduction in Tunisian populations of *Hexaplex trunculus* with contrasting imposex levels. *Journal of Shellfish Research* 28: 891-898.
- Lahbib Y, Abidli S, Chiffolleau JF, Averty B, El Menif NT 2009b. First record of butyltin body burden and imposex status in *Hexaplex trunculus* (L.) along the Tunisian coast. *Journal of Environment Monitoring* 11: 1253-1258.
- Oliveira SG, Oliveira I, Santos JA, Pereira ML, Pacheco MP, Barroso CM 2010. Factors affecting RPSI in imposex monitoring studies using *Nucella lapillus* (L.) as bioindicator. *Journal of Environment Monitoring* 12: 1055-1063.
- Pellizzato F, Centanni E, Marin MG, Moschino V, Pavoni B 2004. Concentrations of organotin compounds and imposex in the gastropod *Hexaplex trunculus* from the lagoon of Venice. *Science of the Total Environment* 332: 89-100.
- Poppe GT, Goto Y 1991. *European Seashells, Vol. 1 (Polyplacophora, Claudofoveata, Solenogastrea, Gastropoda)*. Verlag Christa Hemmen publisher, Wiesbaden.
- Rilov G, Gasith A, Evans SM, Benayahu Y 2000. Unregulated use of TBT-based antifouling paints in Israel: high contamination and imposex levels in two species of marine gastropods. *Marine Ecology Progress Series* 192: 229-238.
- Trigui El-Menif N, Lahbib Y, Le Pennec M, Flower R, Boumaiza M 2006. Intensity of the imposex phenomenon - impact on growth and fecundity in *Hexaplex trunculus* (Mollusca: Gastropoda) collected in Bizerta lagoon and channel (Tunisia). *Cahiers de Biologie Marine* 47: 165-175.
- Terlizzi A, Geraci S, Gibbs PE 1999. Tributyltin (TBT)-induced imposex in the Neogastropod *Hexaplex trunculus* in Italian coastal waters: morphological aspects and ecological implications. *Italian Journal of Zoology* 66: 141-146.
- Vasconcelos P, Gaspar MB, Castro M 2006. Imposex in *Hexaplex (Trunculariopsis) trunculus* (Gastropoda: Muricidae) from the Ria Formosa lagoon (Algarve coast - southern Portugal). *Marine Pollution Bulletin* 52: 337-341.
- Vasconcelos P, Moura P, Gaspar MB 2010. Size matters: importance of penis length variation on reproduction studies and imposex monitoring in *Bolinus brandaris* (Gastropoda: Muricidae). *Hydrobiologia* 661: 363-375.