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Imposex levels and concentrations of organotin compounds (TBT and its metabolites) in *Nassarius nitidus* from the Lagoon of Venice

Bruno Pavoni [a,*], Elena Centanni [a], Sara Valcanover [a], Marco Fasolato [a], Silvia Ceccato [a], Davide Tagliapietra [b]

[a] *Dipartimento Scienze Ambientali, Università di Venezia, Calle Larga S. Marta 2137, 30123 Venezia, Italy*
[b] *CNR Istituto di Scienze Marine, Riva sette martiri 1364/a, 30122 Venezia, Italy*

Abstract

Specimens of *Nassarius nitidus* were collected in seven stations of the Venice Lagoon to assess the levels of tributyltin (TBT) and its metabolites monobutyltin and dibutyltin in the tissues and monitor their effect on organisms, in particular the phenomenon of imposex (superimposition of male sexual characteristics on females). The following values of population indices were found: vas deferens sequence: 1.2 ± 0.7–4.0 ± 0.5; relative penis length: 6–47%. The least impacted station was situated in the northern part of the Lagoon, where females without imposex were found and Butyltin (BuTs) concentrations in the organisms (average sum of BuTs = 43 ± 14 ng Sn g^{-1} w.) were significantly lower than in the other stations (range of average sum of BuTs: 101 ± 22–217 ± 27 ng Sn g^{-1} d.w.). Population indices were found to be related to the TBT content in the tissues. In particular VDSI had a significant logarithmic correlation: $r = 0.95$, $n = 8$, $p < 0.05$.

Keywords: *Nassarius nitidus*; Imposex; TBT; Lagoon of Venice

1. Introduction

Imposex, consisting of the superimposition of male sexual characters, such as penis and vas deferens, on female gastropods, is an example of widespread endocrine disruption, rather common also in the Mediterranean Sea. It has been observed in *Hexaplex trunculus* (Linnaeus, 1758) along the Italian coasts (Terlizzi et al., 1998, 1999, 2004; Chiavarini et al., 2003; Pellizzato et al., 2004; Garaventa et al., 2006a, 2007) and in Malta (Axiak et al., 1995); *Nassarius reticulatus* (Linnaeus, 1758) (syn. *Hinia reticulata*) in Spain (Barreiro et al., 2001) and Portugal (Barroso et al., 2000, 2002); *Bolinus brandaris* (Linnaeus, 1758) along the Catalan (Ramón and Amor, 2001; Solé et al., 1998) and Italian coasts (Chiavarini et al., 2003); and *Nucella lapillus* (Linnaeus, 1758) in Portugal (Barroso et al., 2000).

Imposex was firstly reported by Blaber, 1970. Afterwards other scientists identified the organotin compounds (OTs), mainly tributyltin derivatives (TBT) from antifouling paints, as the main responsible chemicals (Smith, 1981a,b; Bryan et al., 1986; Gibbs and Bryan, 1986). The toxicity of OTs has been recognized by the European community who limited their use with the Regulation 782/2003 of the Parliament and Council of 14 April 2003. The application of organotin based anti-fouling systems is prohibited from 1st July 2003; from 1st January 2008 also previously applied paints will have to be removed or covered in order to avoid the release of organotin compounds in the environment. However the amounts stored in the sediments, those from early applications and a possible illegal use render these compounds still available to organisms.

* Corresponding author. Tel.: +39 041 2348522; fax: +39 041 2348582.
E-mail address: brown@unive.it (B. Pavoni).

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Even though organotin compounds, were considered the main cause of imposex, recently, hypotheses about the involvement of other factors have been suggested (Garaventa et al., 2006b); Nias et al. (1993) observed that copper and environmental stress can cause imposex in *Lepsiella vinosa* (Lamarck, 1822); Evans et al. (2000) showed that nonylphenol promotes the phenomenon in *N. lapillus*. Recently an epidemiologic study conducted by Maran et al. (2006) suggested that organochlorine pesticides, polychlorinated biphenyls and, at a lower extent, polycyclic aromatic hydrocarbons can have a role in the imposex development. However the induction of imposex by other pollutants deserves more investigation.

Pellizzato et al. (2004) monitored the extent of imposex in *H. trunculus* in the Lagoon of Venice during the period 2001–2002. This species lives at a depth between 1 and 100 m on rocky and muddy substrates, has a direct development without larval stage and therefore a limited spreading capacity. In the Lagoon of Venice it is found on the seaward side of the barrier islands and near the ports, where the influence of the sea is significant.

Here we extended the study of organotin contamination in the inner parts of the Lagoon to the gastropod *Nassarius nitidus* (Jeffreys, 1867).

This species has been generally described as *N. reticulatus mamillatus* (Risso, 1826); the Mediterranean subspecies of *N. reticulatus*. Rolán and Luque (1994) reported that *N. reticulatus* is an Atlantic species entering the Mediterranean Sea along Spanish coasts and it is found only in the western part of this Sea. Conversely, *N. nitidus* is widespread in the Mediterranean. As mentioned by Rolán and Luque (1994) many authors with the exception of Jeffreys (1867) consider the distinction between the two species still not clear. Sanjuan et al. (1997), contributed to give *N. nitidus* the taxonomic status of species through allozyme analysis in sympatric populations of *Nassarius* spp. At present *N. nitidus* is considered as a separate species both by the European Register of Marine Species (Costello et al., 2004) and Checklist of the Species of the Italian Fauna (Minelli et al., 1993). References to literature quoted in the present paper refer, therefore, to *N. reticulatus sensu latu*, without distinction between the two species.

N. reticulatus is usually considered an intertidal species living down to a depth of 15 m on sandy and rocky shores with patches of soft materials into which it burrows (Stroben et al., 1992 and references therein); however it was also found in off-shore areas (Santos et al., 2004). It can live in areas where the salinity ranges between 35 and 20 psu or less. Throughout Europe *N. reticulatus* starts to spawn in mid-winter or spring and finishes in summer and its development is through a free swimming veliger larva, which drifts as plankton until it settles (Barroso and Moreira, 1998 and references therein).

Comparing *N. reticulatus* with *H. trunculus*, Axiak et al. (1995 and references therein) reported that the latter is more sensitive to organotin contamination: imposex starts at a concentration of TBT < 1 ng Sn g^{-1} d.w. for *H. trunculus* and at 25 ng Sn g^{-1} d.w. for the other species.

The above mentioned characteristics provide *N. nitidus* with a higher spreading capacity and explain the presence of this organism in the inner parts of the Lagoon.

The present work aims at assessing the possibility of using *N. nitidus* as a bioindicator of organotin contamination in the inner part of the Lagoon of Venice; measuring the extent of imposex in *N. nitidus*; quantifying a possible correlation between imposex and organotin concentrations in tissues of organisms; starting a database to monitor the OTs contamination trend in the Lagoon after the limitations.

2. Methods and materials

2.1. Sampling

A number of organisms ranging between 50 and 100 were sampled using traps and dead flesh for bait in 7 stations of the Lagoon of Venice (Fig. 1), during the period October 2004–July 2005.

The station "S. Nicolò del Lido" is situated next to the port of Lido, which connects the Lagoon to the Adriatic Sea; it's characterized by the transit of passenger ships and pleasure crafts. In this station, sampling was repeated in October 2004 and in June 2005.

"S. Erasmo" is located near S. Nicolò del Lido, in front of the island of S. Erasmo, where agriculture is the predominant activity.

"Carbonera" and "Murano" are in the inner part of the Lagoon north of the city of Venice; these areas are characterized by pollution coming from little shipyards and the glass manufacture.

"S. Maria del Mare" is located near the port of Malamocco; through this entrance, oil tankers enter the Lagoon and reach the industrial district of Porto Marghera.

"Ex Poveglia" is near the homonymous island in the central part of the Lagoon south of the city of Venice.

"Canale Rigà" is situated in the northern basin of the Lagoon and it was chosen as a remote site far from input sources.

After sampling, organisms were frozen to −20 °C until biological analysis.

2.2. Biological analysis

After thawing, length and width of each shell were measured with a calliper, and then the shell was cracked in a vice. Organism was extracted and mantle was cut to expose pallial cavity; penis length was measured; sex and imposex degree, in females, were determined referring to Stroben et al. (1992). The authors described imposex development with 4 stages; every stage, except number 2, is characterized by two different types: 1a, 1b, 3a, 3b, 4, 4+. In type a (1a and 3a), the first male accessory sexual organ developing

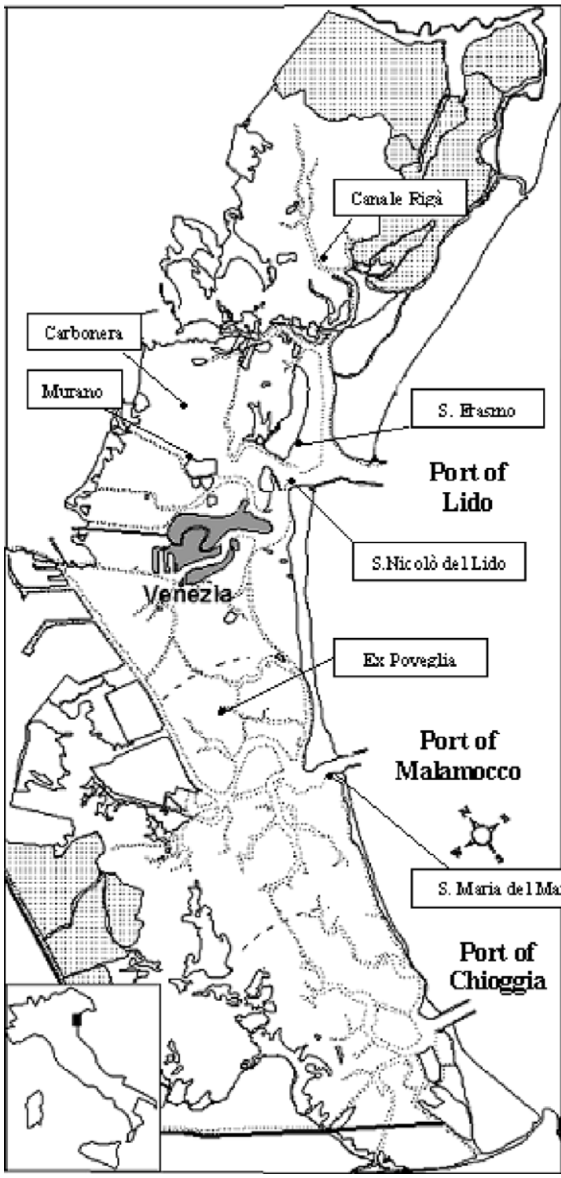

Fig. 1. Map of the Lagoon of Venice and sampling stations.

is the penis, while in type b (1b and 3b) no penis is observed, but only a vas deferens.

In order to compare different populations, vas deferens sequence index (VDSI = mean of imposex degrees in each population) and relative penis length index (RPLI = female penis length/male penis length × 100) were calculated. According to Bryan et al. (1993), since the penes of *N. reticulatus* are very elongated, RPLI is preferable to relative penis size index (RPSI = (female penis length)^3/(male penis length)$^3 \times 100$), which is generally used with other species. For calculation of VDSI, a value of 4.5 is associated with stage 4+ according to Barreiro et al. (2001).

After biological analysis, specimens of the same sex and the same stage of imposex (or close to) were pooled, put in a glass container and frozen to −20 °C. Before chemical analysis, tissues were freeze-dried and homogenized, then they were kept in the fridge.

2.3. Chemical analysis

Chemical analysis and instrumental characteristics were reported in details elsewhere (Pellizzato et al., 2004).

Briefly, tissues were extracted twice with a solution of tropolone in methanol (0.03%). After pre-concentration in a separatory funnel with CH_2Cl_2 and a solvent exchange from CH_2Cl_2 to isooctane, a derivatization reaction with 1 mL of pentylmagnesium bromide 2 M in diethylether was carried out. After addition of tripropyltin as internal standard, the excess of Grignard reagent was hydrolyzed with 2 mL of deionized water. The pentilated compounds were twice extracted with *n*-hexane. The organic phase was reduced to 1 mL under nitrogen and the extract was purified with a column containing 3 g of florisil and Na_2SO_4 conditioned with *n*-hexane–toluene 1:1.

The extracts were injected into a gas chromatograph with a high-resolution capillary column (HRGC model 5890 series II), coupled to a low-resolution mass spectrometer (Hewlett-Packard 5970 B). Analyses were replicated and data are reported as mean value ± (variation range)/2.

Recovery yields of the analytical procedure (80% for TBT, 74% for DBT and 84% for MBT) were evaluated by analysing the certified reference material (CRM 477, mussel tissues) and by spiking experiments.

3. Results and discussion

In Table 1 sampling stations, date of sampling, number of sampled organisms, male and female ratio, percentage of imposex affected female, average shell length and width, average female and male penis length, RPLI and VDSI values are reported. It can be observed that the percentage of imposex affected females is 100% in every station except in canale Rigà where a number of normal females with no penis and the lowest VDSI value (VDSI = 1.2 ± 0.7) were found.

The phenomenon of imposex can develop, as mentioned above, starting from the growth of the penis or the vas deferens; in this work, percentage of imposex affected females with no penis, but with a well developed vas deferens, essentially at stage 1 type b, was 0.98%; Stroben et al. (1992) found a percentage of 1.26% in Brittany and Normandy.

Three females from S. Nicolò del Lido showed a free solid cylindrical excrescence that externally obstructed the vulva causing a very probable sterility. As reported by Barroso et al. (2002), who studied organisms along the Portuguese coast, although female sterility can be in relationship with organotin contamination, there are very few experimental data confirming this observation.

The highest RPLI values were found in Murano (RPLI = 47) (ANOVA, $p < 0.05$) followed by S. Nicolò del Lido (RPLI = 37 and 32). Surprisingly, the RPLI value in S. Erasmo was only 17, significantly lower (ANOVA, $p < 0.05$) than in S. Nicolò del Lido, which is situated in front of it. A possible explanation is that the S. Erasmo

Table 1
Biological data of populations of *Nassarius nitidus* sampled in the Lagoon of Venice, Italy

Sampling stations	Date of sampling	Number of organisms	M/F	Percentage of imposex affected female	Average shell length (mm)	Average shell width (mm)	Average female penis length (mm)	Average male penis length (mm)	RPLI	VDSI
S. Nicolò del Lido 1	10/25/2004	89	33/56	100	22 ± 3	9 ± 1	4 ± 3	13 ± 3	31.6 ± 0.4	4.0 ± 0.5
S. Erasmo	11/25/2004	74	32/42	100	25 ± 2	10.4 ± 0.7	3 ± 2	16 ± 3	16.7 ± 0.3	3.5 ± 0.9
S. Maria del Mare	12/2/2004	52	22/30	100	22 ± 4	9 ± 2	3 ± 2	12 ± 2	28.2 ± 0.4	3.2 ± 0.9
Ex Poveglia	12/2/2004	100	29/71	100	24 ± 2	10 ± 1	3 ± 2	16 ± 2	19.8 ± 0.5	3 ± 1
Murano	4/28/2005	68	30/38	100	27 ± 2	12 ± 1	9 ± 3	20 ± 3	46.7 ± 0.3	3.7 ± 0.7
Carbonera	5/13/2005	64	33/31	100	27 ± 2	12 ± 1	6 ± 3	19 ± 3	32.6 ± 0.3	3.3 ± 0.9
S. Nicolò del Lido 2	6/26/2005	62	29/33	100	24 ± 2	11 ± 1	5 ± 3	13 ± 2	36.6 ± 0.4	3.5 ± 0.8
Canale Rigà	7/5/2005	100	42/58	88	27 ± 2	13 ± 1	0.8 ± 0.8	13 ± 2	6.1 ± 0.4	1.2 ± 0.7

M/F: male/female; RPLI: relative penis length index; VDSI: vas deferens sequence index.

station, which is separated by a deep canal from San Nicolò, is not influenced by waters coming from the City of Venice and the close Arsenale shipyard.

As it can be observed in Fig. 2, the average concentration of the sum of butyltin compounds in Murano and S. Nicolò del Lido was the highest (concentration = 168 ± 31–217 ± 27 ng^{-1} w.), in Canale Rigà the lowest (concentration = 43 ± 14 ng^{-1} d.w.) (ANOVA, $p < 0.05$) as expected. Intermediate values were found in the other stations: this trend of organotin concentration parallels that of RPLI values as shown in Fig. 3.

In Table 2, sampling stations and date, pool separated on the basis of sex and imposex degree, and TBT, DBT and MBT concentration with their associated error are reported. Analyses of triphenyltin (TPhT) and its derivatives were conducted too, but monophenyltin (MPhT) and diphenyltin (DPhT) concentrations were always below limit of detection of the analytical method (LOD_{MPhT} = 0.4 ng g^{-1} d.w. LOD_{DPhT} = 0.4 ng g^{-1} d.w.) and TPhT concentrations ranged between <LOD = 0.1 ng g^{-1} d.w. and 8 ng g^{-1} d.w. from the Murano station.

In the two samplings of S. Nicolò del Lido, chemical and biological data are not significantly different (ANOVA, $p > 0.05$). The range of time of sampling is relatively brief to expect a variation of the organotin levels.

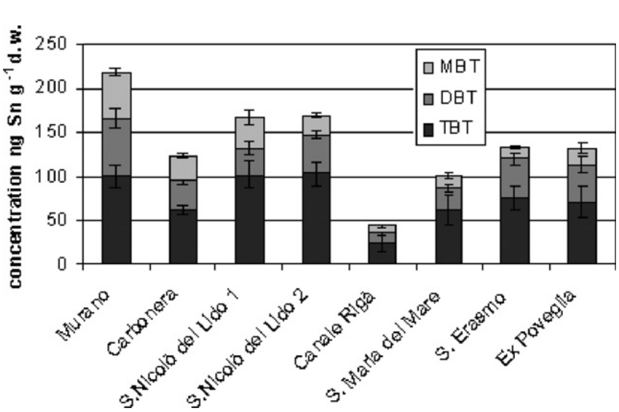

Fig. 2. Average concentrations of TBT, DBT, and MBT, expressed as ng Sn g^{-1} d.w., in every station.

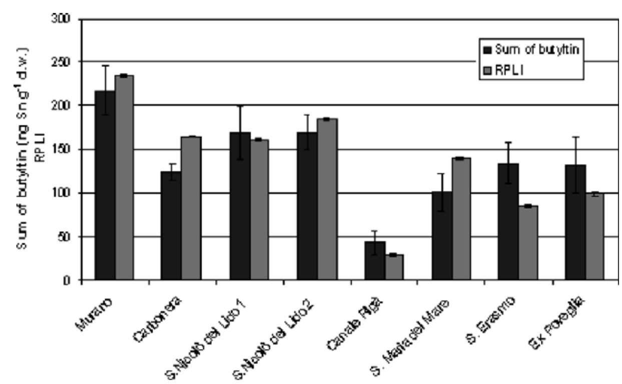

Fig. 3. Comparison between average TBT concentration (ng Sn g^{-1} d.w.) and RPLI (×5).

When relating imposex indices (VDSI and RPLI) with average TBT concentrations in tissues of organisms from all stations it resulted a good correlation between VDSI and TBT with a logarithmic curve ($r = 0.95$, $n = 8$, $p < 0.05$, VDSI = −4.91 + 1.81 ln TBT, Fig. 4) and a less significant one between RPLI and TBT ($r = 0.79$, $p < 0.05$). This result is in agreement with what observed by Stroben et al. (1992) who recommended the VDSI as the best index for TBT biomonitoring, since the mean male penis length of *N. reticulatus* exhibits seasonal changes, whereas the mean female penis length exhibits random changes, and RPLI, being the percent ratio between these two variables, can be affected by these variations.

Comparing the relationship found in this work with those from the literature, it can be observed that, if we calculate the VDSI by approximating stage 4+ to 4, 100 ng TBT-Sn g^{-1} d.w. correspond to a VDSI of 1.3 and a RPLI of 2% in Brittany and Normandy (Stroben et al., 1992), a VDSI of 2 and a RPLI of 10% in Portugal (Barroso et al., 2002) and a VDSI of 3.4 and a RPLI of 39% in the lagoon of Venice. However, a few limitations should

Table 2
Concentrations of TBT, DBT and MBT, expressed as ng Sn g^{-1} d.w., in every pool of *Nassarius nitidus*

Sampling station	Date of sampling	Sex and imposex degree	TBT	DBT	MBT
S.Nicolò del Lido 1	10/25/2004				
		Female 3	89 ± 18	29 ± 6	30 ± 6
		Female 4	85 ± 14	29 ± 4	28 ± 10
		Female 4+	244 ± 13	47 ± 15	82 ± 4
		Male	57 ± 18	25 ± 7	22 ± 10
S. Erasmo	11/25/2004				
		Female 2	144 ± 29	132 ± 26	26 ± 5
		Female 3	62 ± 7	46 ± 2	21 ± 1
		Female 4	42 ± 6	22.0 ± 0.2	8 ± 1
		Female 4+	66 ± 13	19 ± 2	11 ± 1
		Male	85 ± 17	46 ± 9	13 ± 3
S. Maria del Mare	12/2/2004				
		Female 2	71 ± 14	27 ± 5	25 ± 5
		Female 3	88 ± 18	45 ± 9	21 ± 4
		Female 4	60 ± 5	24 ± 2	9.9 ± 0.4
		Male	52 ± 23	17 ± 3	11 ± 3
Ex Poveglia	12/2/2004				
		Female 2	36 ± 19	28 ± 6	8 ± 3
		Female 3	59 ± 15	36 ± 13	12 ± 5
		Female 4	69 ± 23	35 ± 3	20 ± 4
		Female 4+	123 ± 25	78 ± 10	33.14 ± 0.04
		Male	79 ± 13	41 ± 13	24 ± 7
Murano	4/28/2005				
		Female 3	114 ± 18	85 ± 17	56 ± 6
		Female 4/4+	44 ± 2	48 ± 5	32 ± 4
		Male	133 ± 16	65 ± 13	63 ± 2
Carbonera	5/13/2005				
		Female 2	106 ± 7	34 ± 5	22 ± 5
		Female 3	57 ± 5	26 ± 1	20 ± 4
		Female 4/4+	64 ± 9	34 ± 4	29 ± 3
		Male	56 ± 3	31 ± 3	35 ± 1
S.Nicolò del Lido 2	6/26/2005				
		Female 2/3	80 ± 15	45 ± 8	25 ± 3
		Female 4/4+	82 ± 11	44 ± 1	16 ± 1
		Male	129 ± 15	44 ± 5	24 ± 2
Canale Rigà	7/5/2005				
		Female 0	24.8 ± 0.2	10 ± 2	10 ± 2
		Female 1	26 ± 3	12 ± 1	9 ± 1
		Female 2/3	28 ± 2	12 ± 1	8 ± 1
		Male	25 ± 20	8 ± 2	6 ± 3

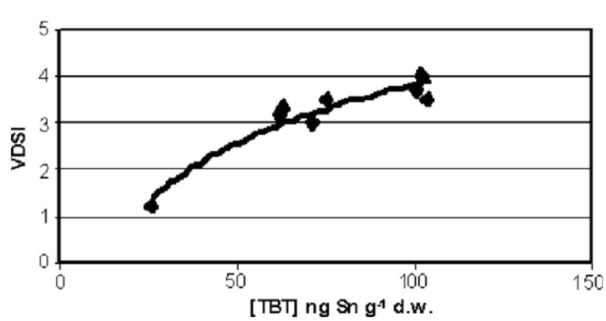

Fig. 4. Relationship between VDSI and average TBT content (ng Sn g^{-1} d.w.) in the tissue of organisms from every station ($r = 0.95$, $n = 8$, $p < 0.05$). Equation of the logarithmic curve is: $Y = -4.91 + 1.81 \ln$ TBT.

be considered for this comparison: male penis length of *N. reticulatus* exhibits changes during the reproductive period, as mentioned above; sampling periods in the different studies were not the same; in the first two works narcotised organisms were used, whereas in the present one frozen organisms were analyzed and penis length can be affected by the freezing as suggested by Minchin and Davies (1999) for *N. lapillus.* For these reasons the comparison based on the VDSI values could be the more correct one. The difference in the VDSI values in the compared studies can be due, as already suggested by Barroso et al. (2002), to a variability in the response to organotin pollution of populations from different geographic areas, or to the fact that bioaccumulation of TBT is a short-term variable, whereas imposex, being irreversible, indicates a non recent presence of organotin compounds in the environment.

To evaluate differences of sensitivity between *N. nitidus* and *H. trunculus* in the Lagoon of Venice, a comparison was made between TBT levels in tissues of the two species sampled in common stations (i.e. S. Nicolò del Lido and S. Maria del Mare) using organisms having the same VDSI. It was found that at stages of imposex equal to 3 and 4, TBT concentration in *H. trunculus* was 50 ± 9 ng Sn g^{-1} d.w. and 59 ± 16 ng Sn g^{-1} d.w. (Pellizzato et al., 2004), whereas in *N. nitidus* it was 85 ± 17 ng Sn g^{-1} d.w. and 78 ± 11 ng Sn g^{-1} d.w., respectively. It can be noticed that also in the Lagoon of Venice *H. trunculus* appears more sensitive than *N. nitidus.* However, the difference is lower than that reported in the literature and discussed in the introduction (Axiak et al., 1995). This could be due to a site-specific adaptation of the organisms. However, it should be also considered that the values reported by Axiak et al. (1995) were obtained from different laboratories and analytical methods. On the contrary, in the present case the analytical method and the laboratory are exactly the same.

This work provides preliminary data about the use of *N. nitidus* as a bioindicator of organotin contamination in the Lagoon of Venice: the collected data show that this species is suitable for the purpose and further research is in progress. In particular data from the southern part of the Lagoon, concerning both organotin contamination and imposex, are being collected to complete the study in the whole lagoon area.

4. Conclusions

In the sampled stations, *N. nitidus* showed imposex levels (VDSI) higher than three except in the station Canale Rigà situated in the north part of the Lagoon, where a number of normal females were sampled, having no penis and vas deferens and a VDSI value of 1.2 ± 0.7 was obtained.

Butyltin concentrations between 43 ng Sn g^{-1} d.w., in Canale Rigà, and 217 ng Sn g^{-1} d.w., in the most polluted station, were found.

Considering the organism sensitivity to OTC contamination, a comparison between *N. nitidus* and *H. trunculus* showed that also in the Lagoon of Venice the latter is more sensitive, although to a lesser extent than it is reported in the literature.

A correlation between average TBT content in the tissues of organisms from every station and the population index VDSI was quantified in the form of a logarithmic curve, indicating VDSI as a good index for TBT biomonitoring using *N. nitidus*.

These results can finally be a starting useful database to monitor the trend in organotin compounds contamination in the Venice Lagoon and evaluate the effects of limitations.

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