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Critical analysis of the relationship between imposex and butyltin body burden in *Nassarius reticulatus* and *Nucella lapillus*☆

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ABSTRACT

Imposex is a disorder caused by organotins, mainly tributyltin, which results in the appearance of male sexual characteristics in females of gastropod mollusks. The main objective of this work was to make a critical analysis of the relationship between imposex and butyltin body burdens in *Nucella lapillus* and *Nassarius reticulatus*. Specifically, this study evaluates possible additive effects among butyltins, proposes scales of effects based on robust statistical criteria as alternatives to existing ones and defines the body burdens of TBT in *N. lapillus* and *N. reticulatus* corresponding to the assessment classes (ACs) of the Vas Deferens Sequence Index (VDSI) established by OSPAR. Data of organotin body burdens and biological effects was retrieved from the ICES Dataset and from scientific literature. All responses, except the percentage of females displaying Imposex (IMPF) in *Nucella lapillus*, showed a sigmoidal profile regarding to the body burden of mono- (MBT), di- (DBT) and tributyltin and sum of butyltins (SumBTs). TBT and the SumBTs were better indicators of the VDSI or Relative Penis Size Index/Relative Penis Length Index (RPSI/RPLI) responses than MBT or DBT in most cases. From a statistical point of view, RPSI/RPLI and VDSI were better indicators of contamination by TBT than IMPF, although both RPSI and RPLI showed lower sensitivity than VDSI. The model used for describing the joint effect of butyltins provided a statistically significant fitting to the data assuming a null effect for both MBT and DBT for *N. lapillus*, and a lower toxic contribution of MBT and DBT with respect to TBT for *N. reticulatus*. RPSI or RPLI values, equivalent to the ACs for VDSI, were proposed as alternative criteria when measuring moderate to high levels of imposex. TBT concentrations in *N. reticulatus* and *N. lapillus* tissues, corresponding to the ACs were calculated and provided valuable information for cross-species comparisons.

1. Introduction

Tributyltin (TBT) had a widespread use since the late 1960s, because of its exceptional properties as antifouling biocide (Santillo et al., 2002). The great toxicity of this compound to marine life and its environmental stability led, in the 1980s, to the limitation of its use on certain vessels in some European countries (France, Great Britain), and to Resolution A.895 (21) of the International Maritime Organization that involved the phasing out of organotin paints in 2003 and its global ban in 2008 (Santillo et al., 2002). TBT accumulated in sediments can be released in episodes of sediment disturbance and, although there is currently a recovery of the ecosystems most affected by TBT, in some cases their effects still persist or even increase (Stewart, 1996; Ruiz et al., 2017).

Imposex is a disorder caused by organotins, mainly TBT, which results in the appearance of male sexual characteristics (penis and vas deferens) in females of gastropod mollusks. The measurement of imposex in gastropods is an appropriate indicator to monitor the effects of TBT in the environment because of its high specificity and its sensitivity in relation to other species and endpoints (Alzieu, 1996), which is comparable to that of analytical chemistry techniques (Stebbing, 1996). Imposex is currently a mandatory technique for assessing TBT contamination in the OSPAR Convention and has also been proposed for implementation within the Water Framework Directive (WFD) and the Marine Strategy Framework Directive (MSFD) (Lyons et al., 2010; Laranjeiro et al., 2015a). Dog-whelk (*Nucella lapillus*) and netted-whelk (*Nassarius reticulatus*) are

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the most commonly used species for the study of imposex on the European Atlantic coast within the framework of these regulations. Both species provide complementary information as *N. lapillus* might reflect better the contamination in the water column and *N. reticulatus* that from sediments (Laranjeiro et al., 2017).

The determination of the potential negative effects of a toxicant on the biota involves establishing a quantitative relationship, usually in the laboratory, between exposure and some type of response that expresses damage to the organism or group of organisms under study. The interpretation of dose-response relationships is based on assuming that: a) the response is proportional to the concentration at the site of action in the organism; b) the concentration at the site of action is dose-related; and c) the response is causally-related to the administered compound (Timbrell, 1995).

Exposure-response relationships for a pollutant can also be derived from field data. Suter (2007) has pointed out the causal evidences that can be established when examining field data: exposure-response relationships from other field studies or laboratories, exposure-response relationships from ecological simulation models, modification of exposures from a sample at the laboratory to simulate the biological effects, possible mechanistic causes and the existence of agents analogous to the causal agent. TBT is a paradigmatic toxicant since it presents an acceptable definition of all of this causal evidences. Its action as an endocrine disruptor presents a monotonic response, well defined thresholds of effects and sensitive ecotoxicological tests with predictive capacity of effects in other generations are also available (Matthiessen et al., 2017).

The relationship between TBT concentration in water and imposex in marine gastropods has been the subject of extensive study (Huet et al., 1995; Stroben et al., 1995). However, the relationship between the toxic response measured as imposex and TBT dissolved in water is not straightforward, since the concentration in the diet and in the sediment can significantly affect the concentration reached in the tissues (Meador, 2011). The body burden of a pollutant reflects the accumulation of the bioavailable fraction present in the environment, and provides a more integrated information than the concentration dissolved in water, which may reflect intermittent or episodic exposures (Landrum et al., 2012). The lack of data of TBT body burdens for marine snails suffering imposex is rather shocking (OSPAR, 2011), but probably stems from TBT environmental history: early concerns in the 1970s led to developing analytical methods to determine organotin species first in the abiotic matrices traditionally used in marine monitoring, considering only afterwards some organism such as those included in ‘Mussel Watch’ programmes. Later on, following the mid 1980s studies linking TBT, imposex and population decline (Bryan et al., 1986; Gibbs and Bryan, 1986), many researchers have tried and analysed organotin body burden in whelks worldwide. As a result, a number of surveys on the European gastropods have concurrently determined both the chemical tissue residues and imposex to illustrate this cause-effect relationship. However, these have been somewhat isolated and/or sporadic attempts, and so far no sound figures have been proposed to be incorporated to the OSPAR scheme for TBT (OSPAR, 2004), which is based on imposex and includes tissue concentrations only for mussels (OSPAR, 2011).

The objectives of this work are to: a) evaluate the relationship between the body burden of butyltins and the degree of imposex, in two species of marine gastropods, *Nucella lapillus* and *Nassarius reticulatus*; b) compare the different indices used to evaluate the imposex response of gastropod populations; c) evaluate possible additive effects among butyltins to promote imposex; d) propose scales of effects based on robust statistical criteria as alternatives to existing ones and; e) define the body burdens of TBT in *N. lapillus* and *N. reticulatus* corresponding to the assessment classes established by OSPAR.

2. Material and methods

2.1. Data collection

Organotin concentrations as Monobutyltin (MBT), Dibutyltin (DBT), Tributyltin (TBT) and Sum of Butyltins (SumBTs) in whole soft-body of *Nucella lapillus* and *Nassarius reticulatus* and biological effects as Percentage of Females displaying Imposex (IMPF) and Vas Deferens Sequence Index (VDSI), for both species, and Relative Penis Size Index (RPSI) specifically for *N. lapillus* and Relative Penis Length Index (RPLI) for *N. reticulatus* were retrieved from: a) the ICES Dataset “Contaminants and biological effects” (ICES, 2017); b) tables shown in the literature (see Table 1); and c) digitized through ‘WebPlotDigitizer’ version 3.11 (Rohatgi, 2017) from figures of Bryan et al. (1986); Gibbs and Bryan (1996); Oehlmann et al. (1998); Laranjeiro (2016). While triphenyltin (TPT) may contribute to imposex in these two species, the literature available on this compound and other organotins is rare and localised mostly to Portugal (e. g. Sousa et al. (2009) even analysed octyltins). Thus, in order not to add further heterogeneity to the dataset, no organotin other than the three butyltins above have been taken into account in this study. Databases for both *N. lapillus* and *N. reticulatus* were made up by interpreting, to the best of our knowledge, all available references but excluding repetitions (Table 1). Heterogeneity between studies in sample collection, treatment and analyses is large, but in some occasions, differences have been surmounted. For instance, a couple of surveys in Portugal (1997–98 and 2003) only reported VDSI in a non-standard system (i.e. VDSI5), but original data have been converted to the usual VDSI4 scale by using the equivalences provided by the same group for other surveys (2005 and 2008, see Table 1 for *N. reticulatus*). However, some other works could not be considered because serious inconsistencies, either in the methodology used or in the time of sampling (e. g. animals for imposex and for chemical analyses were collected one year apart, see Guðmundsdóttir et al. (2011)).

Data of butyltins concentration and effects measured in *N. lapillus* and *N. reticulatus* was downloaded as an Excel file from the ICES Dataset, imported in Microsoft Access, linked according to the sampling station and date, and saved as Excel files. For some data a wet weight to dry weight transformation was performed according to the values reported in the ICES Dataset (33.9 ± 3.9% and 30.3 ± 3.5% dry weight for *N. lapillus* and *N. reticulatus*, respectively).

2.2. Statistical methods

The dose-response relationship for each combination of BT and type of response (IMPF, VDSI or RPSI/RPLI) was obtained using the ‘drc’ package in open-source environment R (Ritz et al., 2015; R Core Team, 2016). The generalized log-logistic function was chosen because of its high capacity of describing symmetrical and asymmetrical sigmoidal profiles (Ritz et al., 2015):

$$R = \frac{d}{\left(1 + \exp(b(\ln(BT) - \ln(e)))\right)^f} \quad or \quad R = LL(BT; b, d, e, f) \qquad (1)$$

where R is the response (IMPF, VDSI or RPSI/RPLI), d is the maximum response (set as a constant and with value of 100 for IMPF, RPSI, RPLI; 4 for the VDSI of *N. reticulatus*, and 6 for the VDSI of *N. lapillus*), b and e are parameters related to the shape of the curve, BT is the concentration of the chosen butyltin or sum of butyltins, and f is a parameter related to the asymmetry of the distribution.

Table 1
List of references where the level of imposex in *Nucella lapillus* and Nassarius reticulatus is related to the butyltin body burden. Mention is made of the country and year where the samples were collected, as well as the imposex indices and the butyltins reported (see text for full explanation of acronyms). Some more data for these and other countries were retrieved from ICES and included in the analyses. 3BTs: all 3 butyltins were analysed; unpubl: unpublished own results.

Species	Country	Year partial ban	Year sam-pling	Imposex indices	BTs analysed	Publication
Nucella lapillus	France	1982	1988–96	VDSI	TBT	Oehlmann et al. (1998)
	Ireland	1987	1988–96	VDSI	TBT	Oehlmann et al. (1998)
	UK	1987	1984–86	RPSI, VDSI	TBT	Gibbs and Bryan (1996)
			1998–00	IMPF, RPSI	3BTs	Birchenough et al. (2002)
			1991	RPSI	TBT	Douglas et al. (1993)
			2006	IMPF, RPSI, VDSI	TBT, DBT	Oliveira et al. (2009)
	Norway	1989	1993–95	RPSI, VDSI	3BTs	Følsvik et al. (1999)
			1998–00	IMPF, RPSI	3BTs	Birchenough et al. (2002)
	Spain	1990	1996	IMPF, RPSI, VDSI	3BTs	Ruiz et al. (1998)
			2003	IMPF, RPSI, VDSI	3BTs	Ruiz et al. (2008); Ruiz et al. (2017); unpubl.
			2006	IMPF, RPSI, VDSI	3BTs	Ruiz et al. (2010); Ruiz et al. (2017); unpubl.
			2009	IMPF, RPSI, VDSI	3BTs	Ruiz et al. (2015); Ruiz et al. (2017); unpubl.
	Portugal	1993	2000	IMPF, RPSI, VDSI	3BTs	Barroso and Moreira (2002)
			2003	IMPF, RPSI, VDSI	TBT, DBT	Galante-Oliveira et al. (2006)
			2006	IMPF, RPSI, VDSI	TBT, DBT	Galante-Oliveira et al. (2009)
			2011	IMPF, RPSI, VDSI	3BTs	Oliveira (2017)
			2012	IMPF, RPSI, VDSI	3BTs	Laranjeiro (2016)
			2014	IMPF, RPSI, VDSI	3BTs	Laranjeiro (2016)
Nassarius reticulatus	UK	1987	1985–93	RPLI	TBT, DBT	Bryan et al. (1993)
	Spain	1990	1998–99	RPLI, VDSI	3BTs	Barreiro et al. (2001)
			2000	IMPF, RPLI, VDSI	3BTs	Ruiz et al. (2005); unpubl.
			2005	IMPF, RPLI, VDSI	3BTs	Ruiz et al. (2008); Ruiz et al. (2017); unpublished
			2006	IMPF, RPLI, VDSI	3BTs	Couceiro et al. (2009)
			2007	IMPF, RPLI, VDSI	3BTs	Rodríguez et al. (2009)
			2008	IMPF, RPLI, VDSI	3BTs	Ruiz et al. (2010); Ruiz et al. (2010); unpubl.
			2011	IMPF, RPLI, VDSI	3BTs	Cuevas et al. (2014)
			2011	IMPF, RPLI, VDSI	3BTs	Ruiz et al. (2015); Ruiz et al. (2017); unpubl.
	Portugal	1993	1997–98	IMPF, RPLI, VDSI5	TBT, DBT	Barroso et al. (2005)
			2000	IMPF, RPLI, VDSI	3BTs	Barroso et al. (2002)
			2003	IMPF, RPLI, VDSI5	3BTs	Sousa et al. (2005)
			2005	IMPF, RPLI, VDSI	3BTs	Sousa et al. (2007)
			2008	IMPF, RPLI, VDSI	3BTs	Sousa et al. (2009)

Robust non-linear regression was applied to obtain parameter estimates of eq. (1). The adjusted R^2 was computed taking into account the observed and predicted response, the number of cases and the parameters of the model. The effective concentrations of TBT corresponding to the VDSI values proposed in the assessment criteria for imposex (OSPAR, 2004) were calculated using the 'delta' method in ED function of the 'drc' package (Ritz et al., 2015).

An additive model was used to estimate the toxic contribution of MBT, DBT and TBT to imposex responses in *N. reticulatus* and *N. lapillus*:

$$R = LL(u_1 MBT + u_2 DBT + TBT; b, e, f) \tag{2}$$

where *MBT, DBT* and *TBT* are the measured concentrations of butyltins and u_1 and u_2 the factors which show the toxic potency of MBT and DBT, respectively, regarding to TBT. The same parameters (*b*, *e* and *f*) are assumed for all butyltins.

The parametric estimations of model (2) were performed by minimisation of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel spreadsheet. Subsequently, confidence intervals from the parametric estimations (Student's *t*-test) and consistence of mathematical models (Fisher's F test) were determined using the freely available 'SolverAid' macro (http://www.bowdoin.edu/~rdelevie/excellaneous/).

3. Results

Figures 1 and 2 show the relationship between the levels of MBT, DBT and TBT and the responses expressed as IMPF, RPSI/RPLI or VDSI. The overall response profile observed for IMPF, RPSI/RPLI or VDSI was sigmoidal, except for the IMPF in *Nucella lapillus*, where the trend was not clear (Figures 1 and 2). The sensitivity of the indices was in all cases: IMPF > VDSI > RPSI/RPLI (Figures 1 and 2). The pattern for *N. lapillus* was similar to that of *N. reticulatus* with regard to the sensitivity of the indices, although differences were less marked in terms of absolute values (curves were closer) and the curves were not so clearly defined due to the lower number of cases.

The dispersion of the measured response (IMPF, VDSI or RPSI/RPLI) serves to assess its robustness as estimator of the concentration of MBT, DBT, TBT or SumBTs (Figures 1 and 2). The dispersion of the IMPF response was high for *N. lapillus* (at least 2–3 orders of magnitude) and *N. reticulatus* (1–2 orders of magnitude). The dispersion of the VDSI response was around 1–2 orders of magnitude for the BTs tissular concentrations or their sum, in both species. The lowest data dispersion occurred in the combination of TBT and RPSI/RPLI for both *N. lapillus* and *N. reticulatus*.

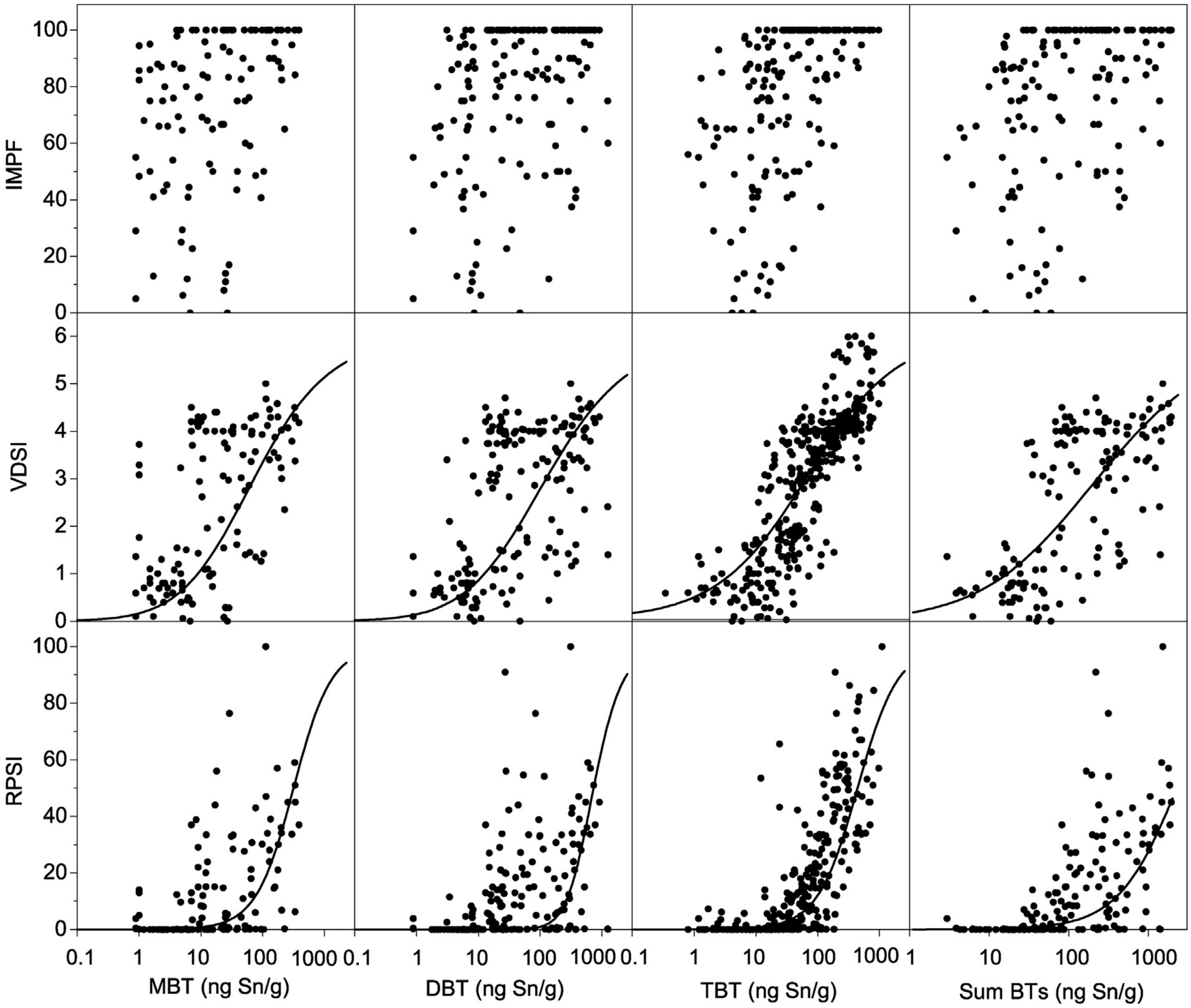

Figure 1. IMPF, VDSI and RPSI obtained for *Nucella lapillus* at the indicated concentration of MBT, DBT, TBT or Sum of BTs (ng Sn/g dry weight). See text for full explanation of acronyms.

In general, RPSI/RPLI responses were always better correlated with TBT and the SumBTs, than with MBT or DBT based on adjusted R^2 values (Table 2). Higher correlations were obtained for TBT than for SumBTs in *N. lapillus*; whereas in *N. reticulatus* the SumBTs yielded higher correlation coefficients (Table 2). For IMPF, adj. R^2 values were low for *N. reticulatus* (Table 2) and it was not possible to adjust the curve to *N. lapillus* data (Figure 1). It was not clear which estimator (MBT, DBT, TBT or SumBTs) for IMPF in *N. lapillus* is better (Figure 1). RPLI showed a higher correlation (adj. R^2) with the concentration of TBT than IMPF or VDSI in *N. reticulatus* (Table 2). In *N. lapillus* the higher value of adj. R^2 was found for the combination of VDSI and TBT (Table 2).

Table 3 shows the Assessment Classes (ACs) set by OSPAR (OSPAR, 2004) and the corresponding TBT concentrations as estimated by fitting eq. (1) to the dataset used here. The scale proposed by OSPAR for *N. lapillus* is gradual and the range of estimated TBT concentrations as body burden was 0.4–961.1 ng Sn/g dw. The ACs for *N. reticulatus* are less staggered and the range of estimated TBT concentrations is narrower than for *N. lapillus* (2.3–96.9 ng Sn/g dw). Estimated TBT values, equivalent to ACs, were lower for *N. reticulatus* than for *N. lapillus* in all cases. Table 3 also shows the RPSI/RPLI values equivalent to the ACs values established for VDSI in both species. The values calculated for class A in *N. lapillus* (<0.01% RPSI) and class B in *N. reticulatus* (<0.2% RPLI) did not allow to discriminate TBT concentrations corresponding to low effect levels (Table 3). However, calculated RPSI/RPLI values for classes C, D and E were robust estimators of the measured TBT concentrations in organisms.

The concentration addition model (eq. (2)) provided a statistically significant fitting for both RPSI and RPLI ($p < 0.001$), whereas the model was not suitable for describing VDSI data. For RPSI (*N. lapillus*) only one parameter was significant (b, -0.74 ± 0.56; e, 0.01; f, 164.14; u_{MBT}, 0; u_{DBT}, 0; adj. R^2, 0.613). The parameters of the model were significant for RPLI (*N. reticulatus*) (b, -1.39 ± 0.38; e, 228.06 ± 161.94; f, 0.95 ± 0.60; u_{DBT}, 0.39 ± 0.22; u_{MBT}, 0.50 ± 0.32; adj. R^2, 0.843). The parameter values representing the toxic potency of MBT or DBT were null for *N. lapillus* and statistically significant for *N. reticulatus*.

4. Discussion

The use of a sigmoidal dose-response model (eq. (1)) provided an adequate description of the relationships between the tissue concentrations of butyltins and the response types commonly used for monitoring the effects on *N. lapillus* and *N. reticulatus* (IMPF, VDSI, RPSI/RPLI), except for the IMPF measured in *N. lapillus*. From a

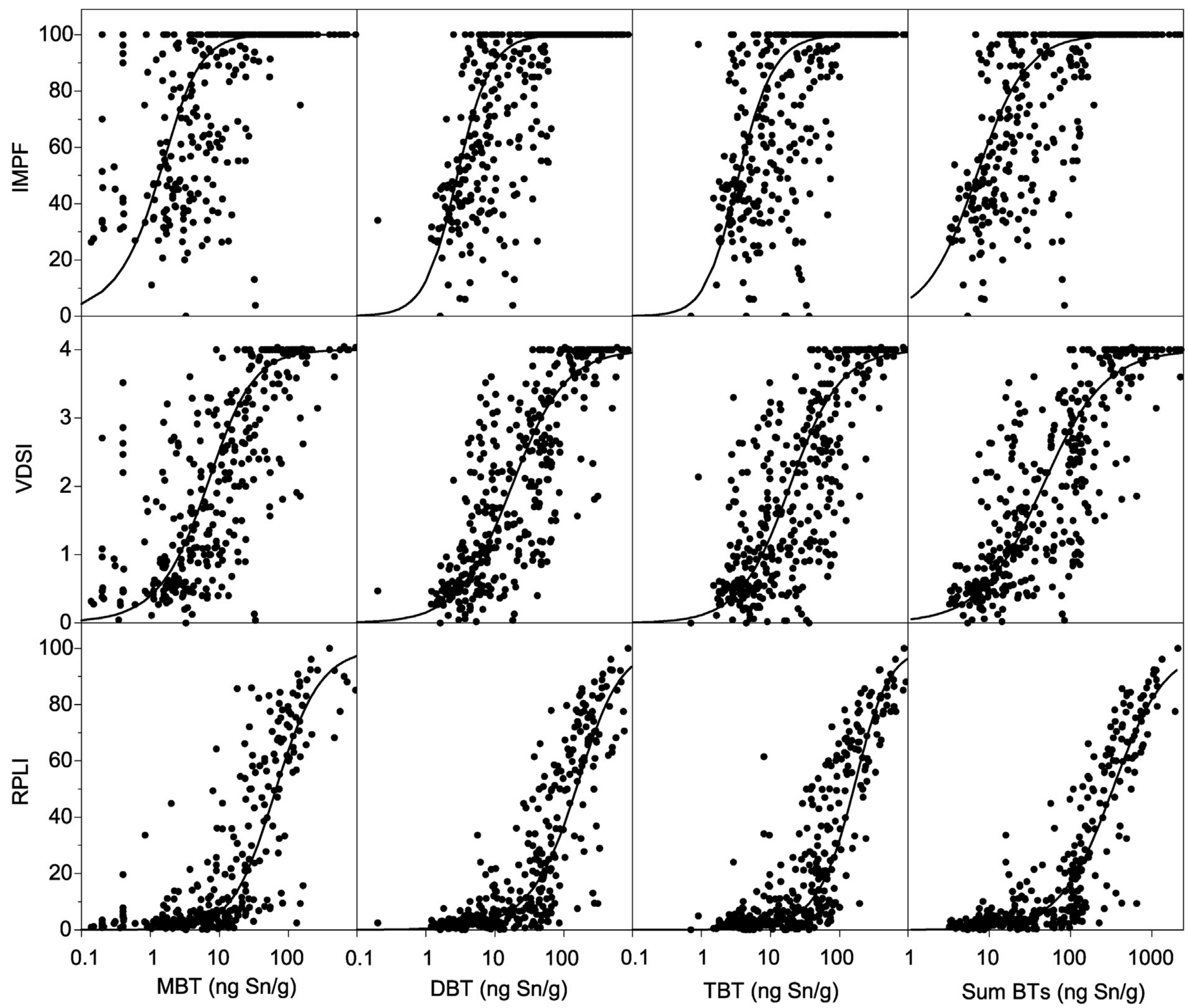

Figure 2. IMPF, VDSI and RPLI for *Nassarius reticulatus* obtained at the indicated concentration of MBT, DBT, TBT or Sum of BTs (ng Sn/g dry weight). See text for full explanation of acronyms.

statistical point of view, both RPSI and RPLI provided fair estimates of TBT concentration, though a more accurate prediction was obtained for *N. lapillus* by using VDSI values (Table 2, Figures 1 and 2). Moreover, VDSI showed higher sensitivity than RPSI or RPLI (Figure 1 and 2); which confirms its suitability for evaluating low or moderate environmental concentrations.

TBT concentrations in *N. lapillus* and *N. reticulatus* tissues, corresponding to the ACs proposed by OSPAR, provided valuable information for cross-species comparisons (Table 3). From the data exposed in Table 3 it can be argued that: a) the evaluation scales defined by OSPAR were more restrictive for *N. reticulatus* than for *N. lapillus*, regarding TBT tissue concentrations; and b) TBT concentrations corresponding to VDSI values of 0.3 and 2 (corresponding to class B in *N. lapillus* and C in *N. reticulatus*) were also smaller for *N. reticulatus* than for *N. lapillus*. The classification scheme was devised by OSPAR to link the thresholds for TBT in water, sediment and mussels with the level of imposex (OSPAR, 2011). The body burdens of TBT in mussel established by OSPAR for the assessment classes were of the same order than those calculated here for *N. lapillus* (Table 3). The RPSI/RPLI values equivalent to these ACs were also calculated (Table 3) and, given the goodness of the fit, they represent robust measures of the effects of TBT at medium and high concentrations.

Several studies indicate that *N. lapillus* is more sensitive than *N. reticulatus* (Huet et al., 1995; Laranjeiro, 2016), since the indices used to indicate the level of imposex (VDSI, RPSI or RPLI) for populations sampled in the same area were generally higher for the former. The assumption of a greater sensitivity of *N. lapillus* than *N. reticulatus* or other gastropod species apparently contrasts with the results obtained here (Stroben et al., 1992; Huet et al., 1995; Stroben et al., 1995; OSPAR, 2004) (Figure 1 and 2, Table 3). Stroben et al. (1992) conducted a comprehensive comparison of the response observed in specimens of *N. reticulatus* and *N. lapillus* exposed to TBT, and concluded that: a) the accumulation of TBT dissolved in seawater showed a similar trend in both species; b) development of the VDSI and RPSI/RPLI was more marked in *N. lapillus* than in *N. reticulatus*, and c) the bioaccumulation of TBT in *N. reticulatus* was on average 36.9% lower than that in *N. lapillus*, for populations sampled in the same area. The higher response found in *N. lapillus* in earlier works is possibly related to its greater capacity to accumulate this compound, which may be due to: a) the main prey of *N. lapillus* is the mussel, an organism that tends to bioconcentrate organic pollutants, and has been estimated that approximately 40–70% of the accumulated TBT comes from the digestive tract (Bryan et al., 1989; Davies et al., 1997); and b) *N. lapillus* would be exposed to the contaminant micro-layer, which

Table 2
Summary of the goodness of fit for each combination of imposex index and butyltin (see text for full explanation of acronyms) as adjusted R^2. *n*, number of cases.

Species	Index	Compound	n	Adj. R^2
Nucella lapillus	RPSI	MBT	129	0.231
		DBT	176	0.094
		TBT	294	0.617
		SumBTs	165	0.312
	IMPF	MBT	115	–
		DBT	158	–
		TBT	177	–
		SumBTs	147	–
	VDSI	MBT	123	0.293
		DBT	165	0.245
		TBT	372	0.705
		SumBTs	147	0.428
Nassarius reticulatus	RPLI	MBT	320	0.700
		DBT	379	0.767
		TBT	399	0.794
		SumBTs	359	0.837
	IMPF	MBT	323	0.264
		DBT	367	0.475
		TBT	387	0.339
		SumBTs	364	0.498
	VDSI	MBT	331	0.551
		DBT	375	0.636
		TBT	394	0.575
		∑BTs	372	0.640

has a higher concentration of TBT than the water column, as a result of its position in the intertidal zone (Seligman et al., 1996). From an ecotoxicological point of view *N. lapillus* is a more sensitive species than *N. reticulatus*, since it expresses a greater response to the same concentration of TBT in the environment, even though the tissue concentration at which the response is expressed is higher in *N. lapillus* than in *N. reticulatus*. Therefore, *N. lapillus* would present adequate characteristics to monitor TBT pollution, although the broad distribution of *N. reticulatus* allows its study in a wider area.

Imposex is an irreversible process whereas the TBT is dynamically accumulated, metabolized and excreted by gastropods (Stroben et al., 1992; Gibbs, 1999). This represents a clear limitation to the descriptive approach used here, i.e. to relate TBT concentrations measured in tissues with the degree of imposex, since the effect of TBT is irreversible and the maximum development of effects may be post-exposure (Bryan et al., 1988). Bryan et al. (1988) exposed females of *N. lapillus* for 14 days and assessed the degree of imposex during 200 days after the initial exposure, and confirmed that the increase of the female penis length stabilized after 69 days and there was no remission with time. Thus, it has been pointed out that the intensity of the imposex in a given gastropod population could be reflecting past TBT-exposures and that the decline of the overall imposex level is delayed with respect to the decrease in TBT concentrations (Gibbs, 1999). It is also important to note that TBT and DBT are metabolized in tissues, with reported half-times in *N. lapillus* of 90 and 98 d, respectively (Bryan et al., 1988). Thus, the measured concentrations of derivatives (DBT and MBT) are indicative of the previous exposure to the parent compound (TBT), and all 3 butyltins are usually correlated with each other. However, in the present work the relationships found between TBT and VDSI or RPSI/RPLI were sufficiently robust and clear to be considered valid.

The sum of BTs involves summarizing in a single value the concentration of a very toxic compound (TBT) and others of lower or much lower toxicity (DBT and MBT, respectively), assuming an unitary equivalence between the three compounds (Seligman et al., 1996). In eq. (2) the TBT equivalents were calculated by the toxic equivalency factors estimated for MBT (u_{MBT}) or DBT (u_{DBT}). The description obtained using eq. (2) differed markedly for the data of *N. lapillus* and *N. reticulatus*. The fitting obtained using eq. (2) (adj. R^2 0.621) was slightly higher than that obtained with eq. (1) for *N. lapillus* (adj. R^2 0.617 for TBT). Since the toxic contribution estimated for MBT and DBT was null, the observed differences are explained by the different method of calculation (robust vs least squares). This agrees with the results obtained by eq. (1) for *N. lapillus* in which TBT served to predict effects substantially better than the SumBTs (Table 2 and Figure 1). Bryan et al. (1988) found for *N. lapillus* that: a) the accumulation of dissolved DBT was limited and did not produce an increase in VDSI or RPSI; and b) DBT injected produced a slight increase in VDSI and RPSI, although differences were not statistically significant when compared to controls. For *N. reticulatus* the goodness of fit obtained using eq. (2) (adj. R^2 0.843) was slightly better than with eq. (1) (adj. R^2 0.837). According to this description, TBT would be 2 and 2.6 times more toxic than MBT and DBT respectively. However, the values of u_{DBT} and u_{MBT} obtained here for *N. reticulatus* must be taken with caution since: they were not calculated experimentally under controlled conditions, but have been deduced from field data obtained from different sources. The values of u_{DBT}/u_{MBT} determined by eq. (2) are likely to be indicative of past exposures to TBT metabolized to DBT and MBT and related to observed RPLI values.

The dose-response relationships obtained for TBT and VDSI in both species allowed an accurate estimate of the TBT concentrations corresponding to the established levels of effect (Figures 1 and 2, Table 3). Some authors like Laranjeiro et al. (2015b) have proposed new VDSI scales, as alternatives to the values proposed by OSPAR (OSPAR, 2004), to define ecological status in the context of the Water Framework Directive for *N. reticulatus*: ‘At least good’ <0.28, ‘Good’ 0.28–0.8, ‘Moderate’ 0.8–2.4, ‘At Best Poor’ 2.4–4; and for *N. lapillus*: ‘High’ <0.3, ‘Good’ 0.3–3, ‘Moderate’ 3–4.5, ‘Poor’ 3–4.5 ,‘Bad’ 4.5–6. As an alternative, concentrations corresponding to 5, 20 and 50% of effect with respect to the maximum value of VDSI in each species (6 for *N. lapillus* and 4 for *N. reticulatus*) are used in this work to propose 4 categories of ‘ecological status’ *sensu* WFD: ‘Good’ (<5%), ‘Moderate’ (5–20%),

Table 3
OSPAR integrated assessment scheme for TBT, where the *Vas Deferens* Sequence Index (VDSI) define the Assessment Classes (ACs) and were linked to TBT concentrations in mussel by OSPAR (2011). The concentrations of TBT and the penis-based indices (RPSI/RPLI, see text for full explanation of acronyms) values obtained for both gastropods in this work are proposed to be also related to ACs. dw, dry weight.

ACs	*Nucella lapillus*			*Nassarius reticulatus*			Mussel
	VDSI	TBT (ng Sn/g dw)	RPSI	VDSI	TBT (ng Sn/g dw)	RPLI	TBT (ng Sn/g dw)
A	<0.3	<0.4 ± 0.0	<0.01				<3
B	0.3–2.0	0.4 ± 0.0–20.8 ± 0.3	0.01–1.7	<0.3	<2.3 ± 0.0	<0.2	3–30
C	2.0–4.0	20.8 ± 0.3–211.8 ± 2.3	1.7–27.1	0.3–2.0	2.3 ± 0.0–19.0 ± 0.2	0.2–4.0	30–600
D	4.0–5.0	211.8 ± 2.3–961.1 ± 17.7	27.1–73.9	2.0–3.5	19.0 ± 0.2–96.9 ± 1.1	4.0–31.0	600–900
E	>5.0	>961.1 ± 17.7	>73.9	>3.5	>96.9 ± 1.1	>31.0	900–4200
F	–			–			>4200

'Poor' (20–50%) and 'Bad' (>50%). The threshold between 'Good' and 'Moderate' can be used to define the 'Good Environmental Status' of MSFD's descriptor 8. These categories can be associated with the OSPAR Assessment Classes (Table 3); for *N. lapillus*, TBT concentrations corresponding to VDSI values of 0.3 (equivalent to 5% of effect), 1.2 (20%) and 3 (50%) are 0.4 (0.4–0.5) (Mean and 95% confidence interval), 6.4 (6.3–6.6), and 66.8 (66.2–67.5) ng Sn/g dw respectively. For *N. reticulatus*, the TBT concentrations corresponding to the VDSI values of 0.2 (5% of effect), 0.8 (20%) and 2 (50%) are 1.6 (1.6–1.6), 5.9 (5.8–6.0), and 19.0 (18.8–19.2) ng Sn/g dw respectively.

5. Conclusions

A sigmoidal dose-response relationship was observed among tissue butyltins and the indices describing the level of imposex for both *N. lapillus* and *N. reticulatus*, except for the combination of IMPF in *N. lapillus*. From a statistical point of view RPSI/RPLI and VDSI were better indicators of contamination by TBT than IMPF. The model used for describing the joint effect of butyltins provided a statistically significant fitting to the data assuming a null effect for both MBT and DBT for *N. lapillus* and a lower toxic contribution of MBT and DBT respect to TBT for *N. reticulatus*. TBT concentrations in *N. lapillus* and *N. reticulatus* tissues, corresponding to the assessment classes proposed by OSPAR, were calculated and valuable information for VDSI cross-species comparisons is provided. The assessment classes for RPSI or RPLI represent alternatives to those proposed by OSPAR for VDSI when measuring moderate to high levels of imposex.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at 10.1016/j.envpol.2018.02.090.

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