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Baseline

Extended imposex monitoring in N Atlantic Spain confirms punctual attainment of European environmental objectives for TBT

José M. Ruiz[a,*], Belén Carro[a], Naiara Albaina[a], Rodolfo Barreiro[a], Diego Rial[b], Juan Bellas[b]

[a] Universidade da Coruña, Grupo BIOCOST, Centro de Investigacións Científicas Avanzadas (CICA), Depto. BA, BV y Ecología, Facultade de Ciencias, Campus da Zapateira, 15071 Coruña, Spain

[b] Instituto Español de Oceanografía, Apdo. 1552, 36280 Vigo, Spain

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ABSTRACT

Legislation in the European Union (EU) aimed at reaching by 2015 a Good Ecological Status in regard to tributyltin (TBT, the biocide used in traditional antifouling paints). With a view to check such an achievement in N Atlantic Spain, baseline monitoring of gastropod imposex (the recommended assessment tool) was extended up to that date. In Galicia (the Western part of the study area) the use of the rock snail *Nucella lapillus* since 1996 had shown this environmental objective to be met as soon as 2009, but new surveys reveal no further improvement thereafter. As for the Eastern Cantabrian coast, imposex levels in the mud snail *Nassarius reticulatus* progressively declined from 2006 to 2015, when records finally complied with expectations. Both data sets are confronted and discussed in relation to the diverse environmental factors that may be determining the distribution of gastropods in these regions.

The organometallic compound tributyltin (TBT) has been worldwide used as a biocide in ship antifouling paints over the last half century, from its first marketing around 1960 (Clark et al., 1988) to its 2008 global ban by the International Maritime Organization (http://www.imo.org). TBT pollution had been shown to cause deleterious effects on marine life and, among them, imposex [i.e. the superimposition of male sexual characteristics (penis included) onto female gastropods (Smith, 1971)] proved of paramount relevance to this long story. Imposex in field populations has been reported for hundreds of species, and in several cases it has been specifically linked to TBT. In Atlantic Europe these include *Nucella lapillus* (L.), a rocky shore carnivore (Bryan et al., 1986), and *Nassarius reticulatus* (L.), a soft-bottom carrion feeder (Stroben et al., 1992).

This unequivocal cause-effect relationship led OSPAR (the Convention for the Protection of the Marine Environment of the NE Atlantic) to include the recording of imposex within its mandatory monitoring program (OSPAR, 2004). In addition, an Ecological Quality Objective (EcoQO) was formulated so that levels of imposex should agree with exposure to water annual average (AA) concentrations below the Environmental Quality Standard (EQS) set for TBT at 0.2 ng TBT/L (OSPAR, 2011). As a member of the OSPAR Convention, the European Union (EU) has also assumed this EQS through the Water Framework Directive (WFD, (EC, 2008)); this ambitious legislation set out an overall timetable according to which 2015 was the first deadline for achieving a Good Ecological Status in regard to priority hazardous substances such as TBT (http://ec.europa.eu/environment/water/waterframework/info/timetable_en.htm).

The imposex EcoQO, initially devised for the intertidal rock snail *Nucella lapillus*, is based on the development onto females of a male genital structure, the *vas deferens*: the values of the *vas deferens* sequence index [VDSI, first proposed by (Gibbs et al., 1987)] in samples define the Assessment Classes, and their lowest two indicate that the EcoQO has been met. Later, the lack of *N. lapillus* in some areas led to apply this classification to other species (such as the mud snail *Nassarius reticulatus*) for which the pertinent equivalences had been established [(OSPAR, 2004), see Table 1]. The present work reports on the evolution of imposex in N Atlantic Spain, from a baseline established before the IMO's ban to the primary date targeted to meet EU environmental objectives for TBT (i.e. 2105).

This study deals with the Spanish littoral between the French and the Portuguese border, a continuum separated by an oceanographic boundary into a neatly Atlantic stretch to the West and one another characterised by warmer waters to the East (e.g. (Prego et al., 2012)). Both are roughly coincident with the traditional distinction between the Galician and the Cantabrian coasts, respectively, and we have thus compartmentalized our work. While the preferred gastropod for imposex watch in Europe (i.e. *Nucella lapillus*) is well distributed in the former area (http://www.marinespecies.org), populations further East

* Corresponding author.
E-mail addresses: jmruiz@udc.es (J.M. Ruiz), b.carro@udc.es (B. Carro), rodolfo.barreiro@udc.es (R. Barreiro), diego.rial@vi.ieo.es (D. Rial), juan.bellas@vi.ieo.es (J. Bellas).

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Table 1
Imposex monitoring in N Atlantic Spain. OSPAR scheme for TBT-specific biological effects, where the values of the *vas deferens* sequence index (VDSI) in the rock snail *Nucella lapillus* are the criteria originally defining the Assessment Classes and those in sympatric populations of the mud snail *Nassarius reticulatus* are considered equivalent. The green colours in A and B mean that the Ecological Quality Objective is met (OSPAR, 2004).

Assessment Class	*Nucella* VDSI	*Nassarius* VDSI
A	< 0.3	
B	0.3 – < 2.0	< 0.3
C	2.0 – < 4.0	0.3 – < 2.0
D	4.0 – 5.0	2.0 – 3.5
E	> 5.0	> 3.5

are too rare and small for sufficient and recurrent sampling ((Peña and Kendall, 1990), and personal observations). Thus, for the biomonitoring of the latter coastal section we had to resort to the alternative species in Table 1 (i.e. *Nassarius reticulatus*).

The baseline survey with the rock snail *Nucella lapillus* in the Galician coast was conducted in the summer (July–September) of 1996 and, for the present work, sampled 9 populations; their location is detailed in (Ruiz et al., 1998) and in the map in supplementary data below. All of them were revisited in 2003 and every 3 years thereafter up to 2015. The collection, laboratory treatment and imposex assessment of animals is also described there, and summarised in (Ruiz et al., 2017). The VDS in each female runs from 0 (unaffected animal) to 4 (individual with penis and full *vas deferens*) and further to 6 (top value with aborted egg capsules inside the sterilized female), and so does the sample mean value or *vas deferens* sequence index (VDSI). On the other hand, the first study in the Cantabrian coast with the mud snail *Nassarius reticulatus* was carried out in August 2006 and included a dozen populations (see (Couceiro et al., 2009)). However, due to operational reasons the subsequent surveys only revisited 9 (2008 and 2012) and 6 (2015) of them during the spring months; their position is detailed there and in the map in supplementary data below. Gastropods were dealt with as described in (Couceiro et al., 2009), who followed the OSPAR *vas deferens* sequence (VDS) (OSPAR, 2003) established for females after (Stroben et al., 1992): it spans from 0 (no trace of imposex) to 4 (animal showing both penis and full *vas deferens*), and so does the sample mean value or VDSI.

Since imposex monitoring should ideally compare adult females of the same age (or size as a surrogate), the shell height (SH) range we had previously defined for each gastropod species was used and maintained (i.e. SH from 17.5 to 32 mm and from 20 to 27 mm, respectively for *N. lapillus* and *N. reticulatus*, see (Ruiz et al., 2017)). In addition, the SH homogeneity between surveys was checked by means of pairwise nonparametric Mann-Whitney (Wilcoxon) W-tests (MWW). For *N. lapillus* these compared the medians of every couple of consecutive campaigns in Galicia, and found no statistically significant SH differences ($p > 0.05$) in 4 out of 5 contrasts. Specimens' size was then considered homogeneous within these 4 pairs so that regional VDSI changes could be confidently assessed. This was done with contrasts considering a number of populations equal to the lowest involved, either a parametric *t*-test when VDSI data sets were normally distributed (2009 vs. 2012 –$n = 9$– and 2012 vs. 2015 –$n = 8$–) or a MWW test when sets were not so (2003 vs. 2006 and 2006 vs. 2009, $n = 9$ in both cases). The survey conducted in 1996 collected gastropods whose SH resulted heterogeneous when compared with that of 2003, and was therefore not subject to statistical imposex tests. The pertinent pairwise contrasts between groups of *N. reticulatus* sampled in the Cantabrian coast were also made, testing for SH homogeneity between every couple of consecutive surveys (i.e. 2006 vs. 2008, 2008 vs. 2012, and 2012 vs. 2015) plus an extra pair formed by 2006 and 2012. The corresponding MWW tests proved there were no statistically significant differences between median SH in any contrast ($p > 0.05$ in all 4 cases); regional changes in VDSI could thus be assessed by means of either a parametric *t*-test (2006 vs. 2008) or MWW tests (the other 3 pairs). The number of populations computed was 9 for all pairs but for 2012 vs. 2015, where it was only 6. The significance levels are depicted below as NS, * and **, respectively for $p > 0.05$, $p < 0.05$ and $p < 0.01$.

Biometrical and VDSI results for the six *Nucella lapillus* surveys in Galicia are gathered in Table 2; they all refer to females of the selected size (i.e. SH from 17.5 to 32 mm). The evolution of imposex in this rock snail along these 20 years can be better appreciated in Fig. 1, where VSDI is referred to both the numerical scale defined above and also to the OSPAR scheme in Table 1. In 1996, at the beginning of this study, the mean VDSI for the 9 samples considered was 4.08 ± 0.44, and the situation showed little change by 2003 (3.81 ± 0.55). However, the 2003 EU TBT ban quickly resulted in VDSI significant decreases, first detected in 2006 (2.71 ± 1.02, MWW test, **) and further from here to 2009 (1.63 ± 0.97, MWW test, *). These results agree with our previous report on concurrent surveys that considered 4 times as many

Table 2
Imposex monitoring in N Atlantic Spain. Biometrical and *vas deferens* sequence index (VDSI) data for samples of the rock snail *Nucella lapillus* collected in Galicia along 6 surveys, including number of females selected (nF), shell height (SH, mm), and summary statistics. Asterisks denote years previously included in a more intensive study, see Ruiz et al. (2017).

		1996*			2003*			2006*			2009*			2012			2015		
Code	Site	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI
1	Ribadeo	23	22.7	3.22	21	20.8	4.00	13	19.6	3.08	17	21.0	0.65	20	21.6	0.15	–	–	–
4	Barqueiro	14	29.7	4.04	22	26.9	2.41	26	27.4	1.65	17	26.6	1.53	20	24.6	0.93	20	23.0	2.80
8	Mugardos	5	28.5	4.30	13	27.5	4.12	20	28.2	3.55	13	28.4	2.62	19	29.3	2.68	14	28.5	0.71
15	Sta. Cristina	13	26.0	4.27	21	24.7	4.07	19	25.0	3.37	18	26.5	2.14	20	27.5	2.05	25	26.7	0.80
18	Malpica	7	23.3	3.57	21	20.2	3.74	25	20.2	0.44	16	21.0	0.06	18	20.8	0.17	18	20.3	0.00
21	Corcubión	18	25.6	4.28	19	26.2	4.03	30	26.2	2.97	21	24.3	1.43	20	25.7	0.80	16	24.4	0.63
28	A Toxa	6	29.4	4.33	7	29.7	4.21	21	26.8	3.29	19	27.9	0.95	20	23.6	0.40	13	25.3	0.69
31	Marín	19	25.3	4.05	18	24.3	3.78	20	24.0	2.65	25	25.4	2.32	19	25.8	1.37	25	25.8	1.68
32	Rande	11	28.6	4.68	21	28.8	3.93	22	24.8	3.36	23	26.3	3.02	18	28.9	2.61	20	29.0	2.15
	Mean	13	26.6	4.08	18	25.5	3.81	22	24.7	2.71	19	25.3	1.63	19	25.3	1.24	19	25.4	1.18
	SD	6	2.6	0.44	5	3.3	0.55	5	3.0	1.02	4	2.7	0.97	1	3.0	1.00	5	2.9	0.93
	Max.	23	29.7	4.68	22	29.7	4.21	30	28.2	3.55	25	28.4	3.02	20	29.3	2.68	25	29.0	2.80
	Min.	5	22.7	3.22	7	20.2	2.41	13	19.6	0.44	13	21.0	0.06	18	20.8	0.15	13	20.3	0.00