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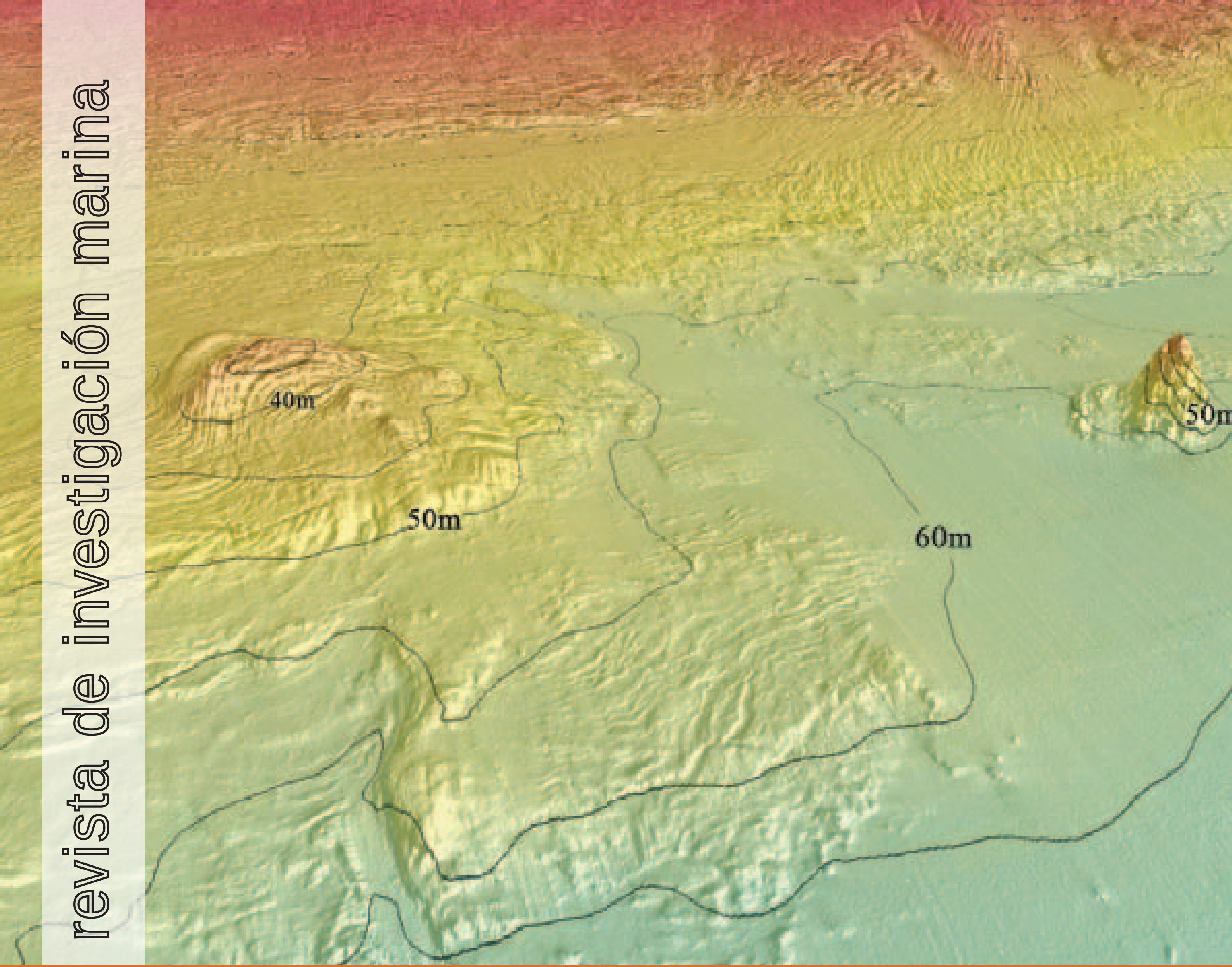

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A visual guideline for the determination of imposex in *Nassarius reticulatus* and *Nassarius nitidus*

Nagore Cuevas[1], Joana Larreta, José Germán Rodríguez & Izaskun Zorita

Abstract

Imposex assessment (imposition of male characters in female dioecious gastropods due to organotin compounds) in two gastropod species, *Nassarius reticulatus* and *Nassarius nitidus*, is detailed by a visual guideline. The information that appears in different sections is accompanied by photographs, diagrams and data summary reports with the aim of solving the doubts that can arise in the process of imposex evaluation. Additionally, this guideline also reviews alternative imposex classification indices currently in use.

Resumen

En este trabajo se detalla de manera visual el proceso de evaluación del imposex (imposición de caracteres masculinos sobre hembras de gasterópodos dioicos, debido a compuestos organoestánicos), en dos especies de gasterópodos, *Nassarius reticulatus* y *Nassarius nitidus*. Con el objetivo de resolver aquellas dudas que pueden surgir sobre el proceso de determinación del imposex, la información en este guía se divide en diferentes secciones acompañadas de fotografías, diagramas y tablas. Además, esta guía visual expone otros índices de clasificación alternativos que actualmente están en uso.

Key Words: imposex, tributyltin, *Nassarius reticulatus*, *Nassarius nitidus*, sterility, visual guideline. tributilestaño, esterilidad, guía visual.

Introduction

Antifouling paints and tributyltin (TBT).

Antifouling paints are applied to the hulls of boats, to slow down or prevent the growth of aquatic organisms that may settle there. These paints have traditionally incorporated toxic substances, including copper or Tributyltin (TBT), into a chemical matrix; this gradually leaches the biocide from the surface layer (Lewis, 1998). However, the utilization of these kinds of paints assumed great economic benefit for this industrial activity, because: (i) fuel saving (due to less frictional drag); (ii) more spacing on entrances in the dry dock; and (iii) more availability of vessels, due to shorter periods in dry dock (Champ M.A., 2000; Dafforn *et al*. 2011)

Besides, antifouling paints are used on marine infrastructures (electric power stations and fuel extraction systems), as well as on oceanographic instrumentation. Furthermore, during the 1960's, one of the most used antifouling paint was TBT. Organotin compounds were applied also in other sectors such as wood preservation, agriculture, cooling systems, paper mills, textile materials, food and control and diseases eradications (Bennett, 1996).

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TBT in the marine environment.

In the marine environment, the main input of TBT is caused by antifouling paints. Thus, areas with high shipping traffic, or ports, are those where concentrations of this compound would be higher. TBT consists of three butyl groups, joined to a tin atom (IV) by covalent bonds (De Mora, 1996) (Figure 1).

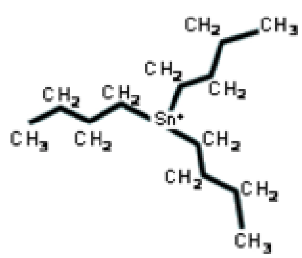

Figure 1. TBT structure.

TBT can be degraded by a wide range of organisms, mainly by a biotic factor (bacteria), following a sequential debutilation to its products: dibutyltin (DBT) and monobutyltin (MBT) process (TBT→DBT→MBT→Sn (IV)) (Valkirs *et al.,* 1991) (Figure 2). The principal product of this degradation is DBT, forming a lesser volumen of MBT. These latter products are less toxic than TBT. Aditionally, TBT degradation can also occur in response to photolysis.

TBT half-life in water, *i,e.*, the time that is required to reduce its concentration by half, is from few days to several

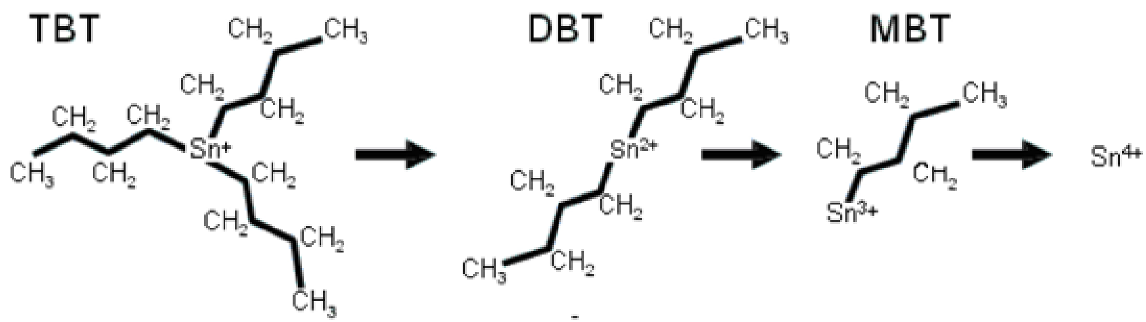

Figure 2. TBT sequential debutilation process.

weeks, depending upon the prevailing environmental conditions (Watanabe *et al.,* 1992). After TBT is freed into water, it tends to be adsorbed by suspended particles, due to its hydrophobic character. Hence, in sediments this lifetime would be longer and, under anoxic conditions, TBT's lifetime may even reach decades (Sarradin *et al*., 1991; Watanabe *et al.*, 1995). TBT concentration depends upon several factors (the quantity of suspended particles, organic matter, salinity and pH); because of this, port areas near estuaries with an high input of suspended particles in conducive hydrodynamic conditions (for example, without stratification) are the most responsive sites to accumulate TBT in sediments.

TBT toxicity in the marine environment: Imposex.

TBT's toxic effects are shown in a wide range of organisms, detecting sublethal and lethal damages in microorganisms, invertebrates and vertebrates (Crothers, 1989; Austen & McEvoy, 1997; and Martinez-Llado *et al*., 2007). These damages include acute toxicity, RNA alterations, neurotoxicity, teratogenicity (alteration of physic, embryonic or larval phases) and inmunotoxicity (immunosuppression). Therefore, TBT is classified as one of the most toxic xenobiotic that is introduced by humans, into marine waters (Goldberg, 1986).

Within macroinvertebrates, possibly molluscs have been studied the most, in relation to TBT. In gastropods (especially neogastropods) and bivalves, this compound behaves as an endocrine disruptor in many species. These chemical compounds could be active at very low concentrations, causing adverse effects in organism or progeny health (Oehlmann & Schulte-Oehlmann, 2003; Oetken *et al.*, 2004; Grun & Blumberg, 2006; and Oehlmann *et al.*, 2007).

For example, TBT causes oyster shell thickening, produced by the alteration of calcium fixation metabolism; it induces also reproductive failures (De Mora, 1996). These changes have accounted for many economic losses in French mollusc farming. In other cases, TBT modifies the morphology of sexual characters in neogastropods. The circumstance most studied is "imposex", which refers to the imposition of sexual characters; this is caused by exposure to sub-lethal concentrations of organotins compounds (Bettin *et al.*, 1996; Gibbs & Bryan, 1996; Tester *et al*., 1996; and Gibbs *et al*., 1997). This alteration generates the presence of male sexual characters, in the females of some dioic species (Gibbs & Bryan, 1996). Furthermore, due to the correlation between dose and TBT bioavaiability, imposex in some species is proposed by international organisations (OSPAR-JAMP 2004, ICES 2000) to evaluate the presence of this organotin compound in the marine environment.

On the other hand, in neogastropod species, there exists also another phenomenon known as "intersex", which consist of modification or supplanting female sexual characteristics, into male ones (Casey *et al.*, 1998; Barroso *et al.*, 2000; De Wolf *et al.*, 2001; Birchenough *et al*., 2002; De Wolf *et al.*, 2004; and Sloan & Gagnon, 2004).

Such environmental problems have introduced early response monitoring, with the objective of determining the TBT pollution in marine waters. Within this context, many countries accomplish routine controls such as: the United Kingdom (Dowson *et al*., 1992; Franklin & Jones, 1994; Law *et al*., 1994; Thomas *et al*., 2000; and Thomas *et al*., 2001); the United States (Lauenstein, 1995; O'Connor & Lauenstein, 2006); or France (Mauvais & Alzieu, 1991; Michel & Averty, 1999; and Michel *et al*., 2001). Additionaly, other countries have carried out specific investigations: Portugal (Barroso *et al*., 2000; Barroso & Moreira, 2002; Barroso *et al*., 2005; and Santos *et al*., 2004); Brazil (Fernandez *et al*., 2002; Fernandez *et al*., 2005); Italy (Bortoli *et al*., 2003; Pavoni *et al*., 2007); Spain (Barreiro *et al.*, 2001; Ruiz *et al*., 2005; Rodríguez *et al*., 2007; Rodríguez *et al*., 2009; and Rodríguez *et al*., 2009b); China (Shi *et al*., 2005); Denmark (Strand *et al*., 2003); and Marocco (Lemghich & Benajiba, 2007).

In order to be able to assess the biological effects of TBT in gastropods, different parameters are used systematically to characterize imposex (see below):

- Percentage of affected females
- VDSI (Vas Deferens Sequence Index)
- RPLI (Relative Penis Length Index)
- AOS (Average of Oviduct Stage)
- Percentage of females with aborted egg capsule

These indicators are explained more specifically below.

Restrictions of the use of TBT in antifouling paints.

Since 1982, legislation introduced by many countries has forbidden, or restricted, the use of TBT in antifouling paints. The reasons for this restriction are due mainly to the toxicity effect on various species, such as oysters (Alzieu *et al.,* 2000).

In 1990, the International Maritime Organization (IMO) passed a resolution which recommended that governments should take steps to eliminate antifouling paints containing TBT on vessels <

25m in length, stationary marine constructions and fishing gear. In 1999, the IMO appealed for a global ban against the application of organotin compounds as biocides, in vessel antifouling systems, for the 1st January 2003; likewise, a total ban opposed completely to the presence of the aforementioned compounds in every ship for the 1st January 2008.

In 2001, after the appeal of a global ban, the IMO considered an international agreement on vessel antifouling systems damage control (Anti-Fouling System agreement, AFS); apart from performance of TBT ban, it would create a mechanism that would avoid other harmful substances being used in antifouling paints. In Europe, the Regulation (EC) 782/2003 of Parliament and of the Council of 14th April 2003, which is related to the organotin compounds ban in shipping, confirms the AFS agreement. According to this regulation, after the 1st January 2008, commercial vessels with an active covering of TBT on hulls would not be accepted in Community ports.

These measures have involved several studies promoting the effect on the recuperation of the quality of marine environment (with TBT reduction), of the above mentioned bans imposed (Quintela *et al.*, 2006; Sousa *et al.*, 2007; Wirzinger *et al.*, 2007; and Smith *et al.*, 2008).

The aim of the present study is to lead the assessment of TBT effects. Consequentely, the development of an useful and conprenhensive guideline, for those inexperienced in this area of research who want to monitor imposex levels in gastropods, is necessary. This guideline is focused on two species of gastropods, *Nassarius reticulatus* and *Nassarius nitidus*; these are good indicators to evaluate imposex, due to their wide spatial distribution and sensitivity.

Methodology

Sediment sampling.

As contaminants tend to accumulate in sediments over a longer time period than in the water column, the collection of sediments close to a sampling station could be an interesting complementary dataset to characterise the TBT level of the substratum. These chemical results use to be coherent with the imposex assessment outcomes in gastropods.

Sediment sampling is carried out in different ways, depending upon the tidal characteristics. In subtidal areas, cores of superficial sediment can be collected. The use of these samples permits the maintenance of the sediment structure (Figure 3). Sediments are sampled also by diving diving, and thus, in some port places often result unviable due to high maritime traffic or low visibility. In this case, sampling can be carried out using Van Veen or Shipek grabs (Figure 4).

Figure 3. Cylinder with piston used for superficial subtidal sediment sampling.

Figure 4. Van veen (left) and Shipek (right) dredge.

Species included in the guideline.

Two species of gastropods are included in this Guideline: *Nassarius reticulatus* (Linnaeus, 1758) and *Nassarius nitidus* (Jeffreys, 1867). The first is distributed usually in outer estuarine or coastal zones, where the salinity tends to be higher than inner parts of an estuary, whereas the second inhabits in inner estuarine areas. Hence, *N. reticulatus* has been utilised previously for the determination of TBT pollution, in many Atlantic European coastal studies (Stroben *et al*., 1992a; Stroben *et al*., 1992b; Bryan *et al*., 1993; Barreiro *et al*., 2001; Barroso *et al*., 2002a; Barroso *et al*., 2002b; Barroso *et al*., 2005; Sousa *et al*., 2005; Rato *et al*., 2006; Wirzinger *et al*., 2007; Rodríguez *et al*,. 2009; and Rodríguez *et al*,. 2009b). In comparison, *N. nitidus* has been used in only a few studies, located mainly along the Venetian and Swedish coasts (Magnusson *et al*., 2005; Pavoni *et al*., 2007; Rodríguez *et al*. 2007, Rodríguez *et al*., 2009, Rodríguez *et al*., 2009b; and Lahbib *et al*., 2011). Therefore, these two species are good candidates for TBT monitoring, within their wide area of distribution.

Species identification.

Nassarius reticulatus and *Nassarius nitidus* are considered as sympatric species (Rolán & Luque, 1995) with many similarities in external morphology (Figures 5, 6, 7 and 8). Consequently, the differentiation betweewn both species should be undertaken, taking into account the contrasting characteristics (Table 1). If any specimen appears with intermediate characteristics, of both *Nassarius* species, it would be excluded from the study.

Figure 5. General view of an adult specimen of *Nassarius nitidus* (Jeffreys, 1867)

Figure 6. Obtuse siphonal view of *Nassarius nitidus*.

Figure 7. General view of an adult of *Nassarius reticulatus* (Linaeus, 1758)

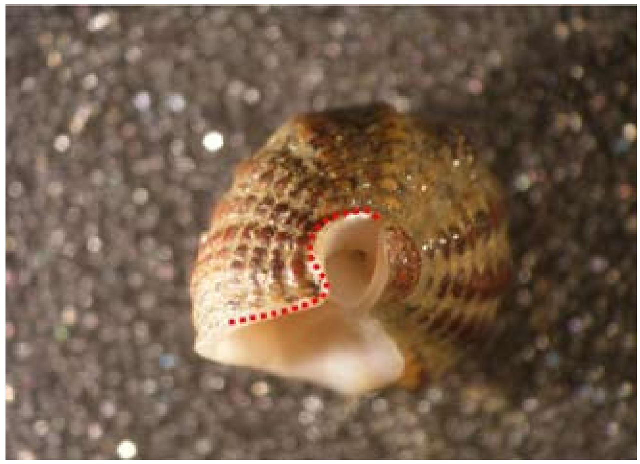

Figure 8. Acute siphonal view of *Nassarius reticulatus* (Linaeus, 1758)

Table 1. The different characteristics of both species: Nassarius reticulatus (Linnaeus, 1758) and Nassarius nitidus (Jeffreys, 1867) based on Rolán & Luque (1995).

Character	**Nassarius nitidus (Figure 8 and 9)**	**Nassarius reticulatus** (Figure 10 and 11)
Shell external coloration	Dark brown, violaceous	Light brown, yellow, cream
Coloration of inner part of shell aperture	Violaceous	White
Columellar callus (adults)	Translucent	White
Number of groove/ stretch in siphonal canal	3	4-5
Profile shape	Stepped	Gently marked
Angle of aperture with siphonal canal	Obtuse	Right or acute

Gastropod sampling and aquaria maintenance.

Sampling of gastropods from intertidal sites can be carried out during low tide (generally on spring tide). In some cases, this requires the use of fish, as bait, to facilitate the capture. In subtidal areas, the sampling does not depend upon the tidal cycle and usually it is necessary to use baited hoop nets and /or fishing cages. These are left from between 1 and 48 hours (Figure 9 and

10). Further, depending upon the sampling site, thus could be achieved directly by diving.

The transport of gastropods to the laboratory should be undertaken in plastic containers, with water collected from the sampling site, in order to facilitate the survival of the majority of the specimens.

Before imposex evaluation, gastropods are maintained in artificial marine seawater, for at least 24 hours without feeding; this is to depurate the digestive tract of the gastropods and to avoid interferences in TBT bioaccumulation analysis. Seawater can be prepared at the same salinity of that at the sampling site of origin, with artificial commercially available marine salt.

The maintenance of gastropods in aquaria has to be with aeration, mechanical and biological filtration and protein separation (by use of the skimmer). Also, the temperature of the aquaria should be similar to that at the origin.

Figure 9. Baited fishing cages and hoop nets used for subtidal gastropods capture.

Figure 10. Installation of pots in a port.

Imposex assessment.

Adult selection.

Imposex is assessed preferably in adult individuals. To differentiate these specimens from juveniles the following characteristics of the shell should be considered (Figure 11 and 12): (i) absence of developed teeth in inner face of shell aperture's lip, (ii) thin and/or sharp lip and (iii) in adult specimens of *Nassarius reticulatus* the columellar wall is white opaque, whereas in juveniles it is not well developed.

Figure 11. Juvenile and adult specimens of *Nassarius reticulatus (*Linnaeus, 1758)

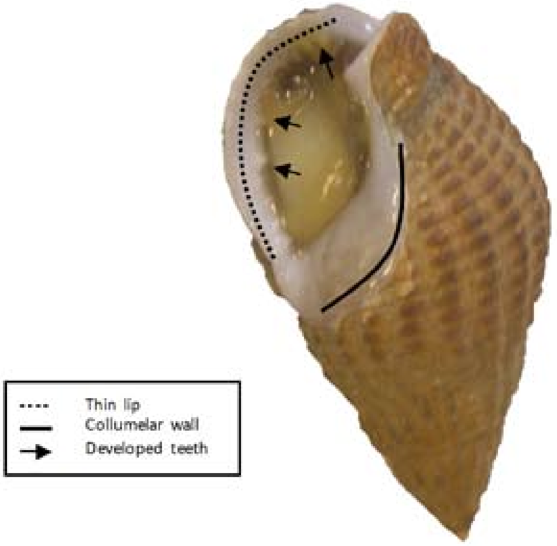

Figure 12. An adult specimen of *Nassarius reticulatus.*

Necessary intrumentation for analysis.

- Dissecting microscpe
- Tweezer
- Scalpel
- Dissecting spring scissors
- Dissecting needle
- Mechanic vice
- Digital caliper
- Petri dish
- Beaker
- Numbered container
- Stirring rod
- Filter paper

Narcotization.

Before shell removal, gastropods were narcotized in a two-step process; firstly, they were submerged in a mixture of 1:1 (v/v) of artificial seawater (salinity of 33 PSU) and $MgCl_2$ (7% w/v) aerated solution for, at least, an hour. Secondly, individuals were transferred to a $MgCl_2$ (7% w/v) solution for, at least, 30 minutes (Fernández *et al*., 2007).

Shell length measurement.

The height and the aperture of the shell was measured in each individual using a digital caliper (Figure 13 and 14). All data are annotated in a summary data report (Annex I).

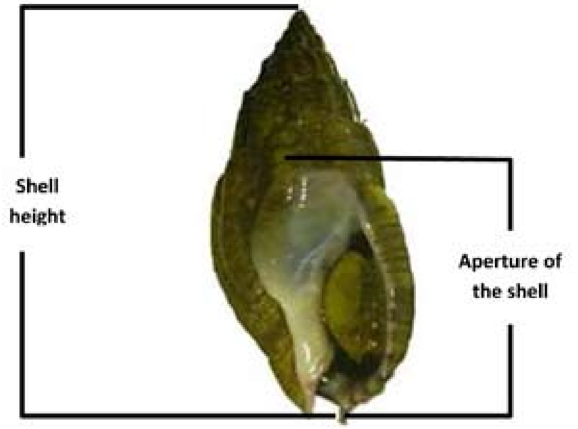

Figure 13. Measures of length (SL) and aperture (SA) of the shell in *Nassarius nitidus.*

Figure 14. Measures of height and aperture of the shell by millimetric caliper.

Shell removal.

To determine the sexual structure of gastropods it is necessary to remove the shell completely; for this, a mechanical vice was used to crack the shell, using pressure (Figures 15 and 16). Afterwards, the columellar muscle, which makes the union of the shell and soft body possible, was cut and the opercule was removed using a binocular dissection microscope (Figures 17,18 and 19).

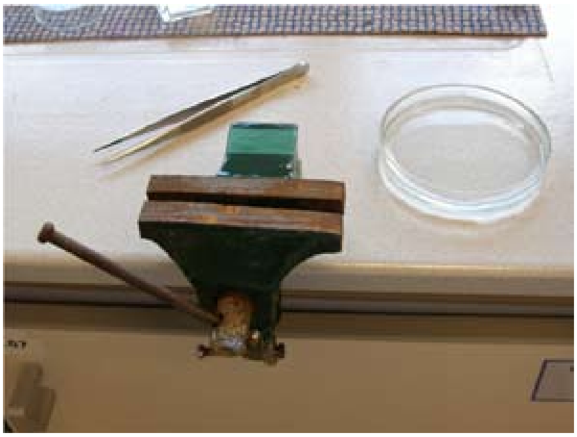

Figure 15. Material for shell removal.

Figure 16. Shell removal technique and numbered container with specimens without shell ready for analysis.

Figure 17. Equipment required for imposex assessment.

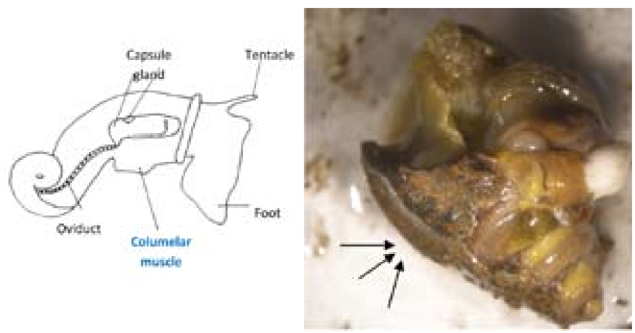

Figure 18. The position of collumellar muscle in a gastropod diagram (left) and after cracking the specimen (right)(This diagram was adapted by Invertebrate Anatomy online by Richard Fox, Lander University).

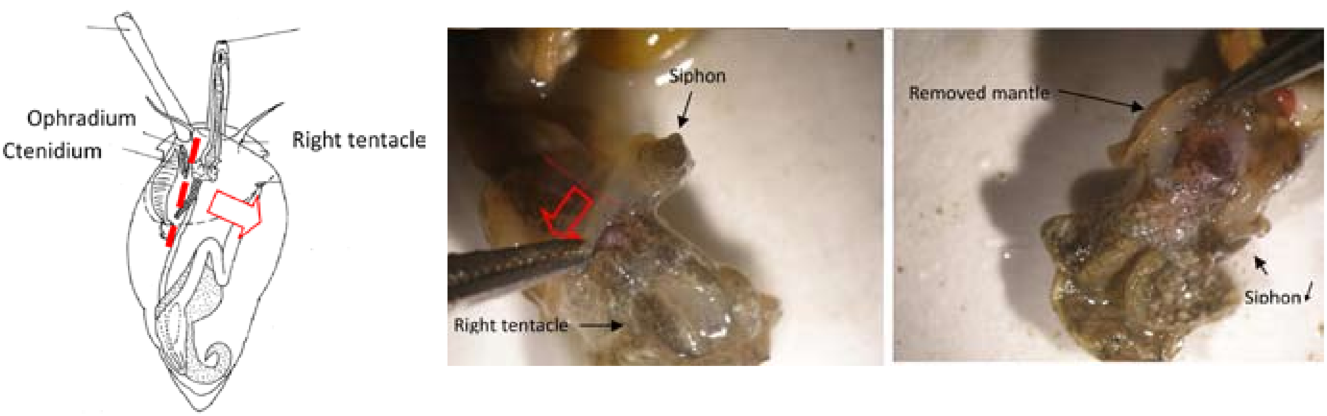

Figure 19. Process of removing the mantle of a *Nassarius reticulatus*.

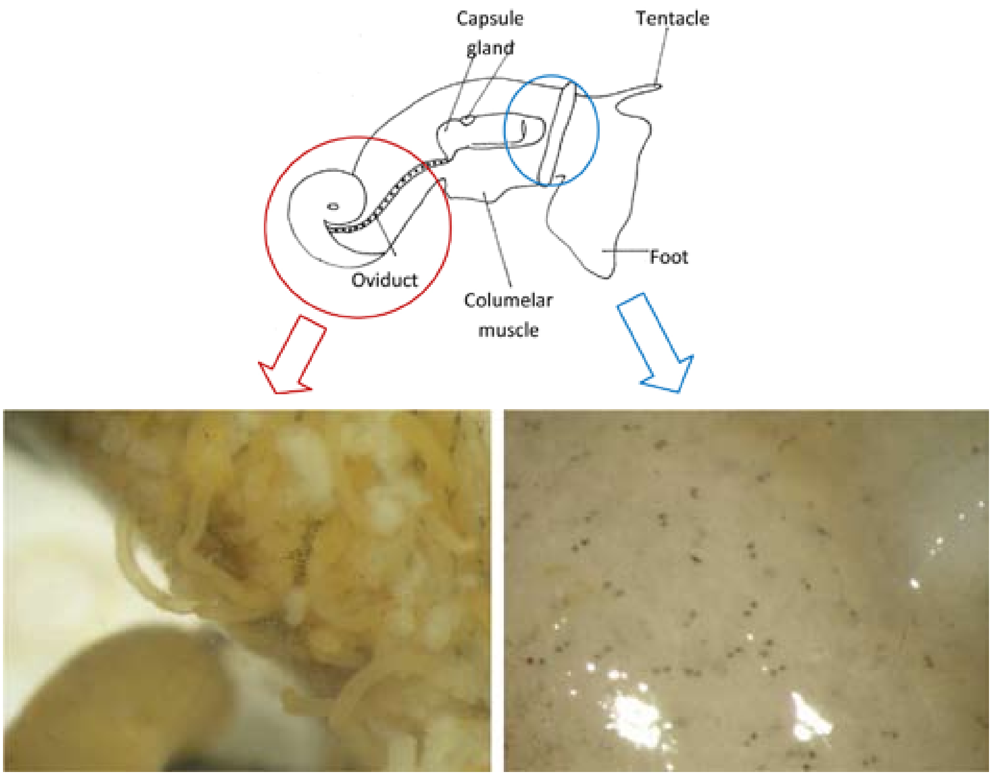

Figure 20. Presence of parasites in gastropod (left: gonad-digestive complex, backside; right: rectum surface) (This diagram was adapted by Invertebrate Anatomy online by Richard Fox, Lander University).

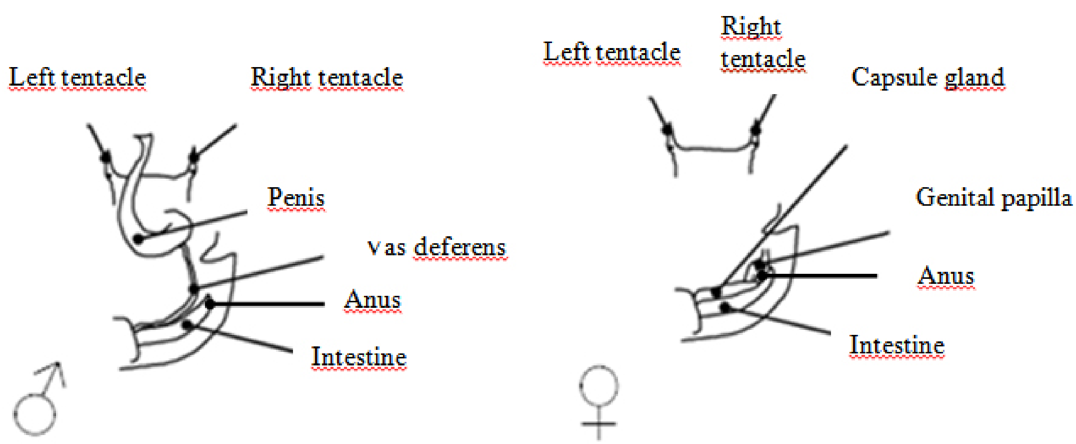

Figure 21. External morphology of male and female reproductive structures of *Nassarius reticulatus* (without imposex) after the mantle over the ophradium is cut (Gibbs & Bryan, 1996).

Determination of parasitism signs.

The presence of parasites in gastropods can alter the morphology of sexual characters (Morley, 2006). Therefore, in imposex studies, parasitized specimens are excluded. However, in some cases it is difficult to achieve an adequate number of non-parasitized organisms sin the sample; therefore, imposex needs to be assessed also in parasitized organisms. Two examples of parasitism are shown in Figure 20.

Mantle treatment.

In order to determine some of the morphological characteristics, it is necessary to remove the mantle in a precise manner; this is to open the mantle cavity, in order to observe the situation of the penis and vas deferens. For that, the mantle should be cut longitudinally through the hypobranchial gland (between rectum and ctenidium). The capsule gland is rotated to show the medial surface, as is illustrated in Figure 21.

Sex determination.

Nassarius reticulatus and *Nassarius nitidus* are dioic species, which means that males and females have distinct sexual characters (Figure 19). Taking into account that females affected by imposex can present a penis, the sex determination should be based upon other characters such as: males: the presence of seminal vesicle (Figure 22); females: presence of a capsule gland (Figures 23 and 24) and vulva (Figure 25).

In some specimens, the sex cannot be determined; this is usually because sexual structures are hidden by parasites. In such cases, individuals are not evaluated.

Figure 22. External appearance of seminal vesicle of a male individual of *Nassarius reticulatus*.

Figure 23. External appearance of capsule gland of a *Nassarius reticulatus* female specimen.

Figure 24. Thorough view of capsule gland of a *Nassarius reticulatus* female specimen.

Figure 25. External appearance of vulva of a gastropod female individual.

Determination of Vas Deferens Sequence Index (VDSI).

The imposex phenomenon is characterized by the imposition of male sexual characteristics in females. Thus, in some species of prosobranch gastropods, the imposex is described by an evolutive system of various stages, with variants. This is based upon the development of sexual characteristics (Gibbs & Bryan, 1996) (Figure 26).

Figure 26. Appearance of vas deferens in a female *Nassarius reticulatus*.

In the case of *Nassarius reticulatus* and *Nassarius nitidus* females, imposex develops through different vas deferens stages (development levels of sperm transporting duct), as shown in Fig. 27. The main scheme of vas deferens stages has been described described by *Stroben et al. 1992b*, although it has been modified slightly by other authors (Barreiro et al 2001, Pessoa eta al. 2001 ;and Barroso et al. 2002a).

Once the vas deferens stages are determined, the estimation of VDSI (Table 2) is calculated from the mean of the assigned values, which varies depending upon the scheme on which a study is based. This index is a measure of the extent of imposex, for the purpose of assessing the biological effects of TBT in gastropods.

Table 2. An example of RPLI (Relative Penis lenght), VDSI (Vas Deferens Sequence Index) and AOS (Average Oviduct Stage) data/results (PL:penis lenght,VDS: vas deferens sequence and OS: oviduct stage).

Nº specimen	Sex	PL (mm)	VDS	VDS value	OS
1	Hembra	6,51	4	4	0
2	Macho	10,82	x	x	x
3	Hembra	4,55	3c	3	0
4	Hembra	4,15	3a	3	0
5	Hembra	5,21	4	4	0
6	Hembra	1,5	2a	2	0
7	Hembra	6,01	4	4	0
8	Macho	8,58	x	x	x
9	Hembra	5,56	3a	3	0
10	Hembra	2,47	3a	3	0
11	Hembra	5,51	3a	3	0
12	Hembra	6,75	4	4	0
13	Hembra	6,49	4	4	0
14	Hembra	7,24	4	4	2
15	Hembra	5,78	4	4	0
16	Hembra	3,24	3c	3	0
17	Hembra	5,34	4	4	0
18	Macho	6,55	x	x	x
19	Macho	7	x	x	x
20	Macho	8,64	x	x	x
21	Hembra	2,78	2a	2	0
22	Macho	8,49	x	x	x
23	Hembra	3,25	4	4	0
24	Hembra	3,85	4	4	1
25	Hembra	5,89	4	4	0

RPLI	58,06
RPLI=(PL mean females/ PL mean males)*100	
VDSI	3,47
VDSI=∑VDS value/ Nº specimens	
AOS	0,16
AOS=∑OS/ Nº specimens	

A – VDSI** sensu **Stroben** et al., **1992b.

Each female should be assessed to characterize its stage of vas deferens sequence: the following descriptions are based upon Stroben *et al*., (1992b).

Stage 0 of the Vas Deferens Sequence (VDS 0): Without any male characteristics, neither the presence of a penis nor vas deferens.

Stage 1a of the Vas Deferens Sequence (VDS 1a): Tiny penis, without a penis duct and vas deferens, behind the right ocular tentacle.

Stage 1b of the Vas Deferens Sequence (VDS 1b): No penis, but a short and distal vas deferens tract behind the right ocular tentacle.

Stage 2a of the Vas Deferens Sequence (VDS 2a): Short penis with a closing or closed penis duct behind the right ocular tentacle (Figure 28).

Stage 3a of the Vas Deferens Sequence (VDS 3a): Penis, with penis duct continuing in an incomplete distal tract of the vas deferens that is growing out successively towards the vaginal opening.

Stage 3b of the Vas Deferens Sequence (VDS 3b): Penis lacking; vas deferens running continuously from the right ocular tentacle, over the bottom of the mantle cavity, up to the vulva (Figure 29).

Stage 4 of the Vas Deferens Sequence (VDS 4): Penis, with a penis duct and a continuous vas deferens from the penis up to the vulva; exceptionally the vas deferens can touch the vulva.

Stage 4+ of the Vas Deferens Sequence (VDS 4+): Penis with duct and vas deferens, that passes the vaginal opening and runs into the ventral channel of the capsule gland (Figures 30 and 31) .

In the case of *Nassarius nitidus*, the vas deferens normally finishes on the front side of the capsule gland.

For the estimation of the Vas Deferens Squence Index (VDSI) in all of the females recorded, for each sampling site, the mean of VDSI stages has been calculated (Table 2). In the case of 4+, the value assigned is 4.

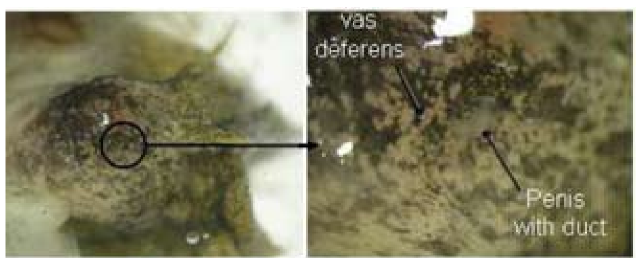

Figure 28. Stage VDS 2a in *Nassarius nitidus*.

Figure 29. Distal and proximal part of vas deferens in stage VDS 3b of *Nassarius reticulatus*.

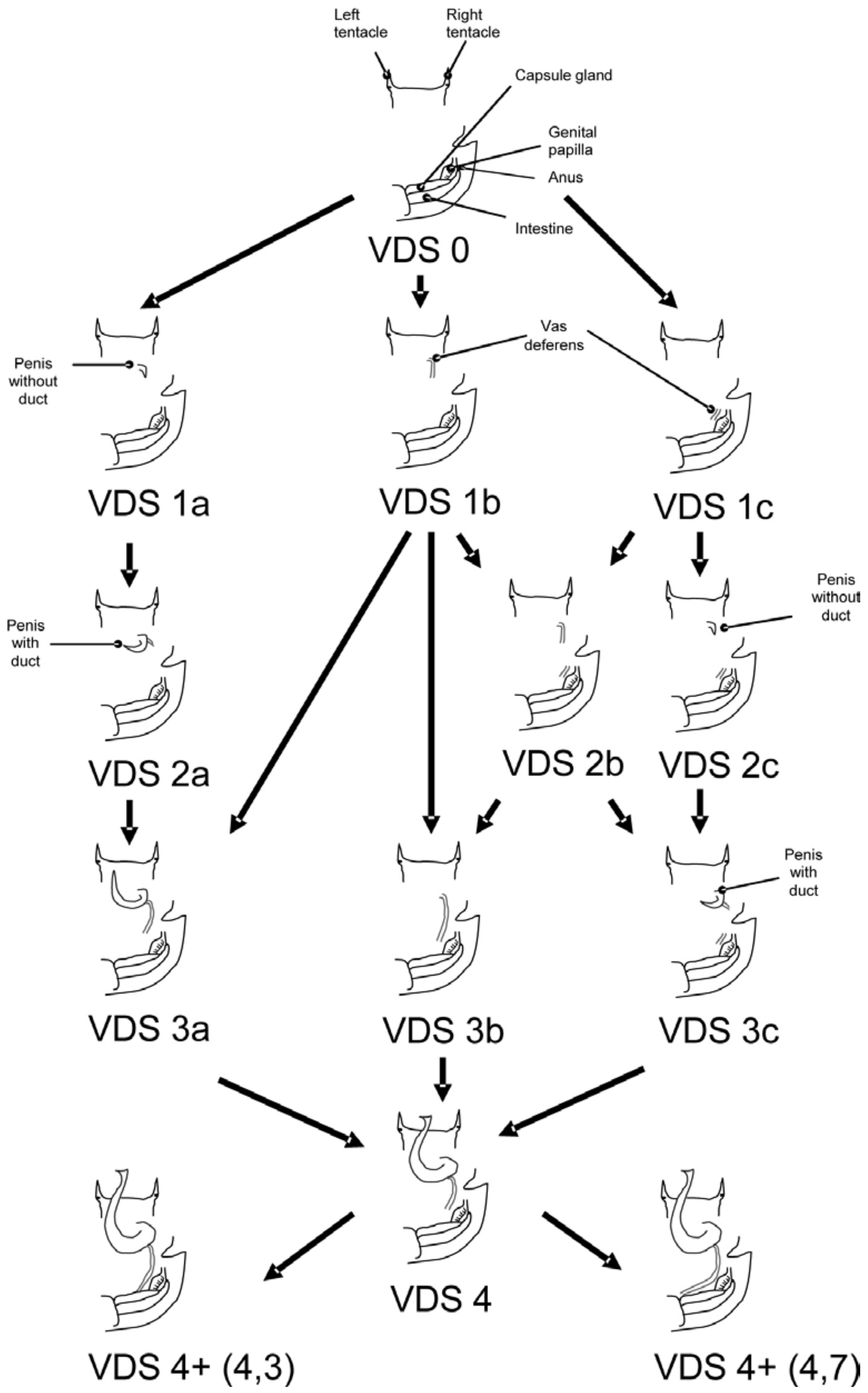

Figure 27. General diagram of Nassarius reticulatus and *Nassarius nitidus* imposex evolution, based on Stroben et al., 1992b. The cases of VDS4+ (4,3) and VDS 4+ (4,7) are according to VDS 4,3 and VDS 4,7 in the description of Pessoa et al., 2001. Some times in stages 3b, 4, 4+ (4,3) and 4+ (4,7) the vas deferens can pass by vaginal opening.

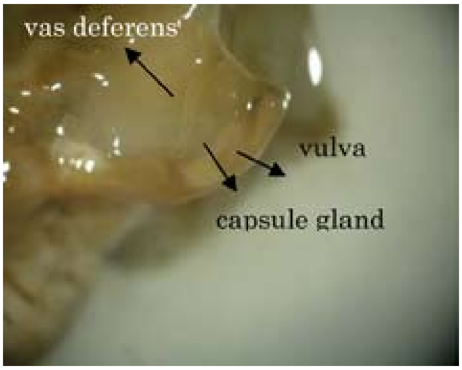

Figure 30. Distal part of vas deferens in Stage 4+ of *Nassarius nitidus* (the vas deferens penetrates through the capsule gland).

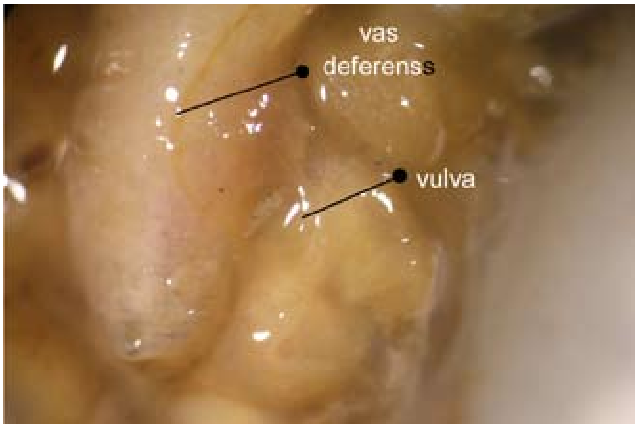

Figure 31. Distal part of VDS 4+ of *Nassarius nitidus*.

B – VDSI** sensu **Barreiro** et al., **2001.

In this case, for the estimation of Vas Deferens Sequence Index (VDSI) there are five stages: 1, 2, 3, 4, 4+; this is in agreement with Stroben *et al.,* 1992b, but with slight modifications.

Stage 0 of the Vas Deferens Sequence (VDS 0): Without any male characteristics, neither the presence of penis nor vas deferens.

Stage 1 of the Vas Deferens Sequence (VDS 1): Tiny penis, without a penis duct and a very short vas deferens, behind the right ocular tentacle.

Stage 2 of the Vas Deferens Sequence (VDS 2): a penis together, with a distal vas deferens of which the length was less than one half of the distance between the base of the penis and the vulva.

Stage 3 of the Vas Deferens Sequence (VDS 3): Penis, with penis duct continuing in an incomplete distal tract of the vas deferens that is growing out successively towards the vaginal opening; its measure is half of the distance between the penis and vulva.

Stage 4 of the Vas Deferens Sequence (VDS 4): Penis, with a penis duct and a continuous vas deferens from the penis up to the vulva; exceptionally the vas deferens can touch the vulva.

Stage 4+ of the Vas Deferens Sequence (VDS 4+): Penis with duct and vas deferens that passes the vaginal opening and runs into the ventral channel of the capsule gland. However, in some specimens the VD follows a course parallel to the oviduct beyond the vulva, ending in the posterior section of the pallial cavity, instead of contacting the oviduct. For the stage 4+ the value assigned is 4,5.

C – VDSI** sensu **Pessoa** et al., **2001.

The stages are the same as those proposed by Stroben *et al.,* 1992b , but the calculation of VDSI is made also on the basis of the mean of stages. However, for 4+ (4,3) the assigned value is 4,3 and for 4+ (4,7) is 4,7.

Stage 0 of the Vas Deferens Sequence (VDS 0): Female, without any male characteristics.

Stage 1 of the Vas Deferens Sequence (VDS 1): Tiny penis, without a penis duct behind the right ocular tentacle.

Stage 2 of the Vas Deferens Sequence (VDS 2): Penis with a closing or closed penis duct, behind the right ocular tentacle.

Stage 3 of the Vas Deferens Sequence (VDS 3): Penis, with a penis duct continuing in an incomplete distal tract of the vas deferens that is growing out successively towards the vaginal opening.

Stage 4 of the Vas Deferens Sequence (VDS 4): Penis, with a penis duct and a continuous vas deferens from the penis up to the vulva.

Stage 4+ of the Vas Deferens Sequence (VDS 4,3): Penis, with a penis duct and a continuous vas deferens, which passes slightly the vaginal opening.

Stage 4+ of the Vas Deferens Sequence (VDS 4,7): The vas deferens passes the vaginal opening and runs into the ventral channel of the capsule gland (Figure 32).

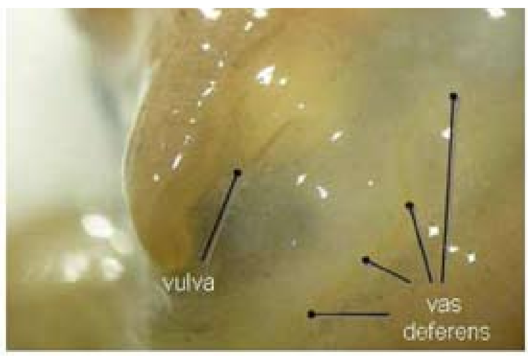

Figure 32. Distal part of vas deferens in stage 4+ (4,7) of *Nassarius reticulatus (*the vas deferens does not penetrate in capsule gland, running parallel to this).

D – VDSI sensu Barroso** et al**., 2002a.

The scored system to assess VDSI is the same as that proposed by Stroben *et al.,* 1992b, except that these authors express their results of VDSI in two forms: either considering VDS 4+ = 4 (as in the pioneering work of Stroben *et al*., (1992b) or VDS 4+ = 5 (as in the Barroso *et al*,. (2002a) study).

E – Other stages described.

Apart from the descriptions of VDS that are explained previously, there are other stages detailed in these two species of *Nassarius* (see below).

Stage 1c of the Vas Deferens Sequence (VDS 1c): No penis, but a tiny vas deferens tract near the vulva.

Stage 2b of the Vas Deferens Sequence (VDS 2b): No penis, the presence of a vas deferens spreads in a discontinuous form into the vicinity of the vulva.

Stage 2c of the Vas Deferens Sequence (VDS 2c): Tiny penis, without tract and associated vas deferens nearby to the right ocular tentacle; presence of a tiny vas deferens next to the vulva.

Stage 3c of the Vas Deferens Sequence (VDS 3c): Presence of a small penis, with a penis duct and a vas deferens that spreads discountinuously until the vicinity of the vulva.

In these cases, the numerical value for the VDSI calculation is given by the Stage number (for example, 3c = value3).

Determination of relative penis length (RPLI).

Other indicator of imposex intensity is the Relative Penis Length Index, which correlate the female and male penis length.

Length of the penis in males and females is measured with a digital caliper, which measures hundredth of millimetre, using a binocular magnifying glass (Figures 33).

Relative Penis Length Index (RPLI) is calculated (Table 2), by dividing the mean of the female penis lenght by the mean of the male penis length, multiplied by 100 (e.g. Barreiro *et al*., 2001).

In the case of specimens with two penis, or with a diverged penis (Figure 34), the length of the longest penis is used for the RPLI estimation (Table 2).

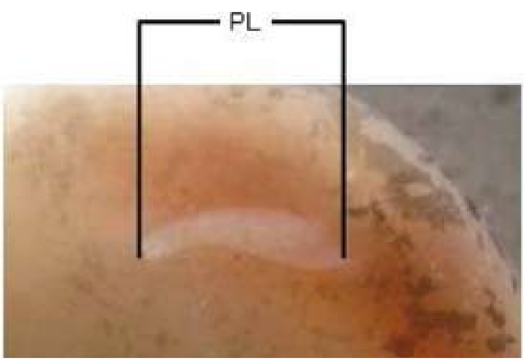

Figure 33. *Measure of Nassarius* reticulatus penis length (PL).

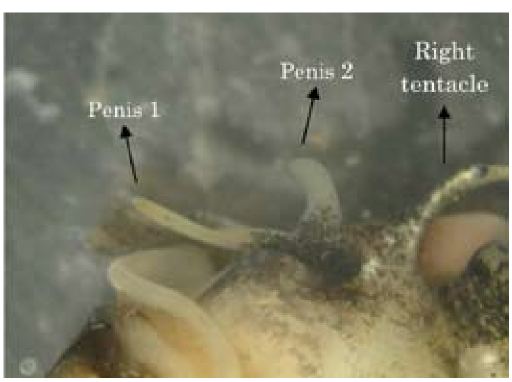

Figure 34. *Nassarius nitidus* female with diverged penis.

Average Oviduct Stage (AOS) determination.

In general, the oviduct of the *Nassarius reticulatus* and *Nassarius nitidus* females is straight, transparent and thin. However, when these species are affected by organotin compounds, the oviduct tends to be convoluted as a male seminal vesicle (Barreiro *et al*., 2001).

Generally, the Oviduct Stage is estimated in each female, following the scale proposed by Barreiro *et al*., (2001):

Stage 0 of Gonadal Oviduct: Females with normal oviduct (rectilinear, transparent and narrow (Figure 35)).

Stage 1 of Gonadal Oviduct: Females with a slightly sinuous, swelled and generally whitish oviduct (Figure 36).

Stage 2 of Gonadal Oviduct: Females with convoluted oviduct, very thick and, generally, whitish or yellowish in colour (Figure 37).

The Average Oviduct Stage (AOS) convolution is determinded for each sampling site by calculating (Table 2) the mean of the oviduct stage of females.

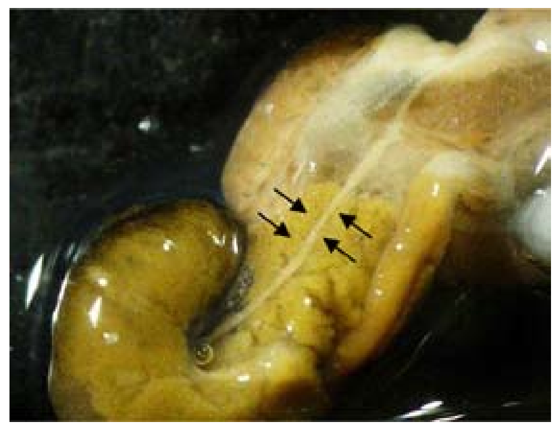

Figure 35. *Nassariusreticulatus* female with Oviduct Stage 0.

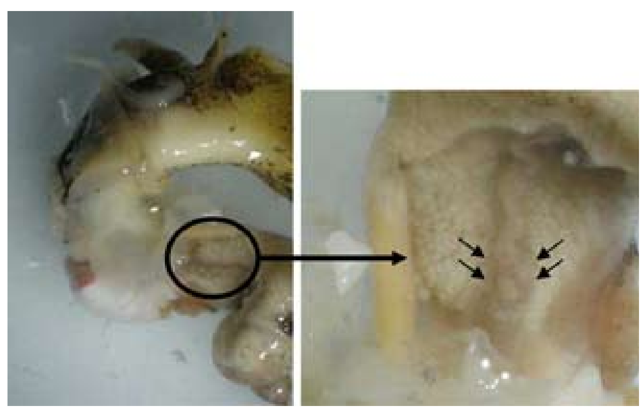

Figure 36. *Nassariu reticulatus* female with Oviduct Stage 1.

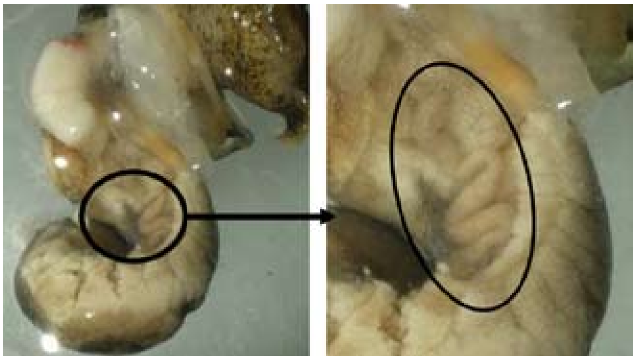

Figure 37. *Nassarius reticulatus* female with Oviduct Stage 2.

Aborted egg capsules assessment.

In cases where imposex is so advanced, several species could present aborted egg capsules. In *Nassarius reticulatus* and *Nassarius nitidus*, the presence of aborted egg capsules can bedescribed. However, although the presence of aborted egg capsules is consistent with advanced imposex stages, it is not known whether TBT is responsible for sterilization of these species.

To assess the presence of aborted egg capsules, the content of the capsule gland of all females is examined. Usually, females with aborted egg capsules present a dark capsule gland. In other cases, aborted egg capsules are located in the back or the front side of the capsule gland, deforming its appearance. In Figure 38, normal capsule is shown; in Figures 39 and 40a, deformed capsule gland (caused by the presence of aborted egg capsule masses) is observed.

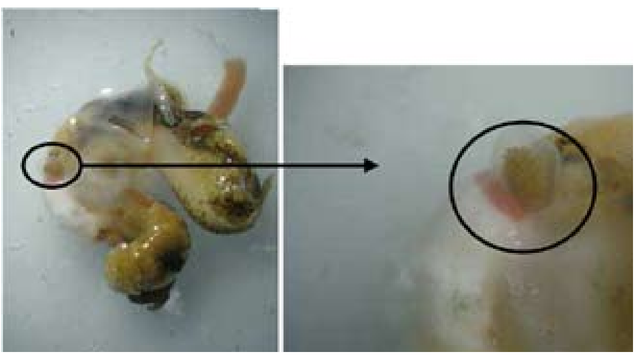

Figure 38. *Nassarius reticulatus* female with normal egg capsules.

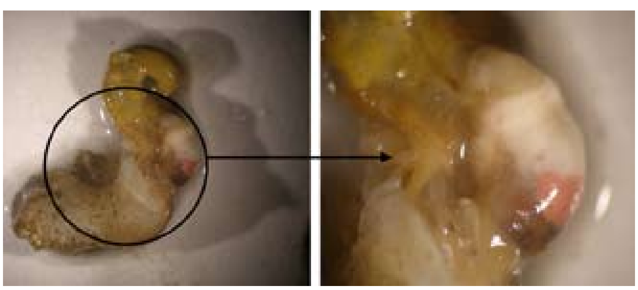

Figure 39. *Nassarius reticulatus* female with aborted egg capsules.

Figure 40. *Nassarius reticulatus* female with aborted egg capsules mass.

There are four types of capsules identified (Rodríguez *et al*. 2009b), as described below.

<u>Aborted Egg Capsules, Type I.</u> These are soft capsules (*i.e*, can be deformed by forceps) and inside they have structures containing recognizable walls of egg capsules. Commonly, they are a reddish or translucent in colour (Figure 41a).

<u>Aborted Egg Capsules, Type II.</u> There are soft capsules (they can be deformed by forceps) and they contain a dark brown amorphous mass (Figure 42a).

<u>Aborted Egg Capsules, Type III.</u> There are capsules recovered with a hard surface but, once broken, the inside is soft (Figure 41b).

<u>Aborted Egg Capsules, Type IV.</u> They are hard capsules, practically imposible to break by forceps. Normally, they are smaller than Type III (Figures 41c and 42b).

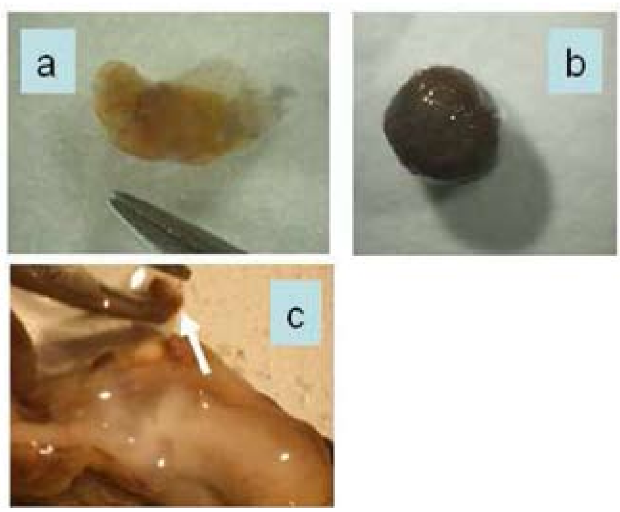

Figure 41. Aborted egg capsules mass. a: Aborted egg capsule type I; b: aborted egg capsule type III; c: aborted egg capsule type IV.

Figure 42. *Nassarius reticulatus* female with aborted egg capsule. a: type II; b: type IV

Assessment of Morphological Anomalies.

Apart from imposex, TBT could cause other adverse effects in gastropods (Hagger *et al*., 2006). However, unlike imposex, these disturbances could be produced also by other compounds or parasitization, which means that these changes could not be attributed only to the presence of TBT in the environment. Nevertheless, the evaluation of the presence of different anomalies can be useful for the general assessment of the population status; thus, some alterations are shown in Figures 43 and 44. It is worth notting that organisms showing adverse effects should be removed (for example, dumpton syndrome) from imposex assessment.

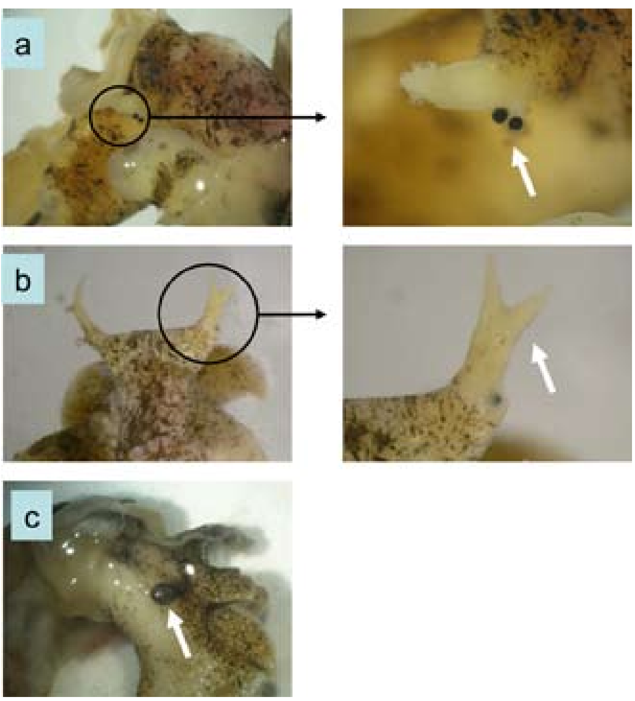

Figure 43. Examples of morphological anomalies in *Nassarius reticulatus* a: specimen with a right tentacle without eye and the left one with two eyes, b: specimen with both tentacles diverged, c: specimen with amorphous penis.

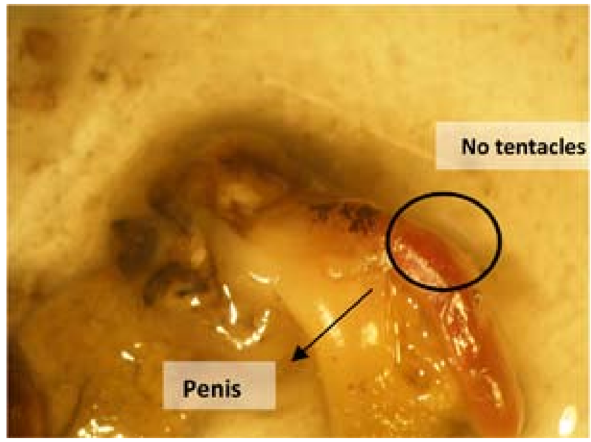

Figure 44. A specimen without tentacles (morphological anomaly).

Conclusions and recommendations

Once the imposex assessment has been completed, the different biological indices explained before (RPLI, VDSI, AOS and females percentage with aborted eggs capsule) were calculated and integrated, to achieve the conclusions of the study. Besides, the result should be an integrative response between biological and chemical outcomes; normally, this integration shows coherent conclusions and help to understand the biomonitoring results.

Furthermore, this visual guideline for an inexperienced colleague in this area of research is useful and comprenhensive; and it can solve the doubts that can arise in the process of imposex assessment.

Moreover, for imposex assessment it is recommended to read some related scientific papers such as:

- ICES 2000: http://www.ices.dk/products/CMdocs/2000/E/E0400.PDF
- JAMP guideline: http://www.ospar.org/documents/dbase/decrecs/agreements/04-15e_REVISED%20TBT%20specific%20biological%20effects%20criteria.doc
- Stroben, E., J. Oehlmann, P. Fioroni, 1992b. The morphological expression of imposex in *Hinia reticulata* (Gastropoda, Buccinidae) - a potential indicator of tributyltin pollution. *Marine Biology*, 113: 625- 636.

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References

Alzieu, C, 2000. Environmental impact of TBT: the French experience. *The Science of the Total Environment,* 258 (1-2): 99-102.

Austen, M. C., A.J. McEvoy, 1997. Experimental effects of tributyltin (TBT) contaminated sediment on a range of meiobenthic communities. *Environmental Pollution*, 96: 435-444.

Barreiro, R., R. Gonzalez, M. Quintela, J.M. Ruiz, 2001. Imposex, organotin bioaccumulation and sterility of female *Nassarius reticulatus* in polluted areas of NW Spain. *Marine Ecology-Progress Series,* 218: 203-212.

Barroso, C. M., M.H. Moreira, P.E. Gibbs, 2000. Comparison of imposex and intersex development in four prosobranch species for TBT monitoring of a southern European estuarine system (Ria de Aveiro, NW Portugal). *Marine Ecology-Progress Series,* 201: 221-232.

Barroso, C. M., M.H. Moreira, 2002. Spatial and temporal changes of TBT pollution along the Portuguese coast: inefficacy of the EEC directive 89/677. *Marine Pollution Bulletin,* 44: 480-486.

Barroso, C. M., M.H. Moreira, M.J. Bebianno, 2002a. Imposex, female sterility and organotin contamination of the prosobranch *Nassarius reticulatus* from the Portuguese coast. *Marine Ecology-Progress Series*, 230: 127-135.

Barroso, C. M., M.A. Reis-Henriques, M.S. Ferreira, M.H. Moreira, 2002b. The effectiveness of some compounds derived from antifouling paints in promoting imposex in *Nassarius reticulatus*. *Journal of the Marine Biological Association of the United Kingdom,* 82: 249-255.

Barroso, C. M., M.A. Reis-Henriques, M. Ferreira, P.E. Gibbs, M.H. Moreira, 2005. Organotin contamination, imposex and androgen/oestrogen ratios in natural populations of *Nassarius reticulatus* along a ship density gradient. *Applied Organometallic Chemistry*, 19: 1141-1148.

Bennett, R. F.,1996. Industrial manufacture and applications of tributylin compounds. In S. J. de Mora (Ed.): Tributyltin: case study of an environmental contaminant, Cambridge University Press, Cambridge, 23-61.

Bettin, C., J. Oehlmann, E. Stroben, 1996. TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. *Helgolander Meeresuntersuchungen,* 50: 299-317.

Birchenough, A. C., N. Barnes, S.M. Evans, H. Hinz, I. Kronke, C. Moss, 2002. A review and assessment of tributyltin contamination in the North Sea, based on surveys of butyltin tissue burdens and imposex/intersex in four species of neogastropods. *Marine Pollution Bulletin,* 44: 534-543.

Bortoli, A., A. Troncon, S. Dariol, F. Pellizzato, B. Pavoni, 2003. Butyltins and phenyltins in biota and sediments from the Lagoon of Venice. *Oceanologia,* 45: 7-23.

Bryan, G. W., G.R. Burt, P.E. Gibbs, P.L. Pascoe, 1993. *Nassarius reticulatus* (Nassariidae, Gastropoda) as an indicator of tributyltin pollution before and after TBT restrictions. *Journal of the Marine Biological Association of the United Kingdom,* 73: 913-929.

Casey, J. D., S. De Grave, G.M. Burnell, 1998. Intersex and *Littorina littorea* in Cork Harbour: results of a medium-term monitoring programme. *Hydrobiologia,* 378: 193-197.

Champ, M.A., 2000. A review oforganotin regulatory strategies, pending actions, related costs andbenefits. *The Science of the Total Environment*, 258: 21-71.

Crothers, J. H., 1989. Has the population decline due to TBT pollution affected shell-shape variation in the dog-whelk, *Nucella lapillus* (L.)? *Journal of Molluscan Studies,* 55: 461-467.

Dafforn, K.A., J.A. Lewis, E.L. Johnston, 2011. Antifouling strategies: History and regulation, ecological impacts and mitigation. *Marine Pollution Bulletin*, 62: 453-465.

De Mora, S. J., 1996. The tributyltin debate: ocean transportation versus seafood harvesting. In S. J. de Mora (Ed.): Tributyltin: case study of an environmental contaminant, Cambridge University Press, Cambridge, 1-20.

De Wolf, H., W. De Coen, T. Backeljau, R. Blust, 2001. Intersex and sterility in the periwinkle *Littorina littorea* (Mollusca : Gastropoda) along the Western Scheldt estuary, the Netherlands. Marine *Environmental Research,* 52: 249-255.

De Wolf, H., C. Handa, T. Backeljau, R. Blust, 2004. A baseline survey of intersex in *Littorina littorea* along the Scheldt estuary, The Netherlands. *Marine Pollution Bulletin*, 48: 592-596.

Dowson, P. H., J.M. Bubb, J.N. Lester, 1992. Organotin distribution in sediments and waters of selected east coast estuaries in the UK. *Marine Pollution Bulletin,* 24: 492-498.

Fernández, M. A, A.M. Maia, I.B. Castro, A.C. Martins, R. Wagener, 2002. Occurrence of imposex in *Thais haemastoma*: possible evidence of environmental contamination derived from organotin compounds in Rio de Janeiro and Fortaleza, Brazil. *Cadernos de Saúde Pública,* 18: 463-476.

Fernández, M. A., A.D.R. Wagener, A.M. Limaverde, A.L. Scofield, F.M. Pinheiro, E. Rodrigues, 2005. Imposex and surface sediment speciation: A combined approach to evaluate organotin contamination in Guanabara Bay, Rio de Janeiro, Brazil. *Marine Environmental Research,* 59: 435-452.

Fernández, M.A., F.M. Pinheiro, J.P. de Quadros, E. Camillo, 2007. An easy, non-destructive, probabilistic method to evaluate the imposex response of gastropod populations. Mar*ine Environment Research*, 63(1): 41–54.

Franklin, A., J. Jones, 1994. Monitoring and surveillance of non- radiactive contaminants in the aquatic environment and activities regulating the disposal of wastes at sea, 1992. *Aquatic environment monitoring report*, 40: 1-83.

Gibbs, P. E., G.W. Bryan, 1996. TBT-induced imposex in neogastropod snails: masculinization to mass extinction. In S. J. de Mora (Ed.): Tributyltin: case study of an environmental contaminant, Cambridge University Press, Cambridge, 212-236.

Gibbs, P. E., M.J.Bebianno, M.R. Coelho, 1997. Evidence of the differential sensitivity of neogastropods to tributyltin (TBT) pollution, with notes on a species (*Columbella rustica*) lacking the imposex response. *Environmental Technology,* 18: 1219-1224.

Goldberg, E. D.,1986. TBT - an environmental dilemma. *Environment,* 28: 17-44.

Grun, F., B. Blumberg, 2006. Environmental obesogens: Organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology,* 147: S50-S55.

Hagger, J. A., .M.H. Depledge, J. Oehlmann, S. Jobling, T.S. Galloway, 2006. Is there a causal association between genotoxicity and the imposex effect? *Environmental Health Perspectives*, 114: 20-26.

ICES 2000. *Working group on biological effects on contaminant*. Nantes, France, 48-59.

JAMP 2004-15. *Provisional JAMP Assesment criteria forTBT-Specific biological effects*, 48-59.

Lahbib, Y., S, Abidli, P. Rodríguez González, J.I. Gracía Alonso, N. Trigui-El Menif, 2011. Potential of Nassarius Nitidus for monitoring organotin pollution in the laggon of Bizerta (northen Tunisia). *Journal of environmental sciences,* 23 (9): 1551-1557.

Lauenstein, G. G., 1995. Comparison of organic contaminants found in mussels and oysters from a current mussel Watch Project with those from archived mollusc samples of the 1970s. *Marine Pollution Bulletin,* 30: 826-833.

Law, R. J., M.J. Waldock, C.R. Allchin, R.E. Laslett, K.J. Bailey, 1994. Contaminants in seawater around England and Wales: Results from monitoring surveys, 1990 - 1992. *Marine Pollution Bulletin,* 28: 668-675.

Lemghich, I., M.H. Benajiba, 2007. Survey of imposex in prosobranchs mollusks along the northern Mediterranean coast of Morocco. *Ecological Indicators*, 7: 209-214.

Lewis, J.A., 1998. Marine biofouling and its prevention on underwartersurfaces. Mater. Forum, 22: 41-61.

Magnusson, M., A. Borgegren, A. Granmo, I. Cato, 2005. Eventuellt samband mellan halten tennföreningar i vävnaden hos nätsnäckan *Nassarius nitidus* och halten tennföreningar i sedimentet. Rapport fra *Kristinebergs Marina Forskningsstation*.

Martinez-Llado, X., O. Gibert, V. Marti, S. Diez, J. Romo, J.M. Bayona, J. de Pablo, 2007. Distribution of polycyclic aromatic hydrocarbons (PAHs) and tributyltin (TBT) in Barcelona harbour sediments and their impact on benthic communities. *Environmental Pollution*, 149: 104-113.

Mauvais, J. L., C. Alzieu, 1991. Qualité des eaux cotiéres: bases scientifiques et stratégies réglamentaires. *Equinoxe*, 36: 4-13.

Michel, P., B. Averty, 1999. Contamination of French coastal waters by organotin compounds: 1997 update. *Marine Pollution Bulletin*, 38: 268-275.

Michel, P., B. Averty, B. Andral, J.F. Chiffoleau, F. Galgani, 2001. Tributyltin along the coasts of Corsica (Western Mediterranean): a persistent problem. *Marine Pollution Bulletin*, 42: 1128-1132.

Morley, N. J., 2006. Parasitism as a source of potential distortion in studies on endocrine disrupting chemicals in molluscs. *Marine Pollution Bulletin*, 52: 1330-1332.

O'Connor, T. P., G.G. Lauenstein, 2006. Trends in chemical concentrations in mussels and oysters collected along the US coast: Update to 2003. *Marine Environmental Research*, 62: 261-285.

Oehlmann, J., U. Schulte-Oehlmann, 2003. Endocrine disruption in invertebrates. *Pure and Applied Chemistry*, 75: 2207-2218.

Oehlmann, J., P. Di Benedetto, M. Tillmann, M. Duft, M. Oetken, U. Schulte-Oehlmann, 2007. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. *Ecotoxicology*, 16: 29-43.

Oetken, M., J. Bachmann, U. Schulte-Oehlmann, J. Oehlmann, 2004. Evidence for endocrine disruption in invertebrates. *International Review of Cytology - a Survey of Cell Biology*, Vol 236: , 1-44.

Pavoni, B., E. Centanni, S. Valcanover, M. Fasolato, S. Ceccato, D. Tagliapietra, 2007. Imposex levels and concentrations of organotin compounds (TBT and its metabolites) in *Nassarius nitidus* from the Lagoon of Venice. *Marine Pollution Bulletin*, 55 :505-511.

Pessoa, M.F., A. Fernando, J.S. Oliveira, 2001. Use of imposex (pseudohermaphroditism) as indicator of the occurrence of organotin compounds in Portuguese coastal waters – Sado and Mira estuaries. *Environmental Toxicology*, 16 (3):234-241.

Quintela, M., L. Couceiro, J.M. Ruiz, R. Barreiro, 2006. Imposex in the marine gastropod *Nucella lapillus* in Galicia (NW Iberian Peninsula): An eight-year re-survey (1996-2003). *Marine Environmental Research*, 62: S236-S237.

Rato, M., A. Sousa, R. Quinta, W. Langston, C. Barroso, 2006. Assessment of inshore/offshore tributyltin pollution gradients in the northwest Portugal continental shelf using *Nassarius reticulatus* as a bioindicator. *Environmental Toxicology and Chemistry,* 25: 3213-3220.

Rolán, E., A.A. Luque, 1995. *Nassarius reticulatus* (Linnaeus, 1758) y *Nassarius nitidus* (Jeffreys, 1867) (Gastropoda, Nassaridae), dos especies válidas de los mares de Europa. *Iberus*, 12: 59-76.

Rodríguez, J.G., M.J. BelzunceA. Borja, J. Franco, J.M. Garmendia, I. González Herráiz, I. Muxika, O. Solaun, I. Tueros, V. Valencia, 2007. Organotin bioaccumulation and imposex in females *Nassarius reticulatus* and *N. nitidus* in ports and marina areas of the Basque Country. *2007 ICES Annual Science Conference.*

Rodríguez, J.G., I. Tueros, A. Borja, J.I. García Alonso, J.M. Garmendia, I. Muxika, C. Sariego, V. Valencia, 2009. Butyltin compounds, sterility and imposex assessment in *Nassarius reticulatus* (Linnaeus, 1758), prior to the 2008 European ban on TBT antifouling paints, within Basque ports and along coastal areas. *Continental Shelf Research*, 29: 1165-1173.

Rodríguez, J.G., A. Borja, J. Franco, J.I. García Alonso, J.M. Garmendia, I. Muxika, C. Sariego, V. Valencia, 2009b. Imposex and butyltin body burden in *Nassarius nitidus* (Jeffreys, 1867), in coastal waters within the Basque Country (northen Spain). *Science of the Total Environment,* 407: 4333-4339.

Ruíz, J. M., R. Barreiro, J.J Gonzalez, 2005. Biomonitoring organotin pollution with gastropods and mussels. *Marine Ecology-Progress Series*, 287: 169-176.

Santos, M. M., N. Vieira, M.A. Reis-Henriques, A.M. Santos, J.L. Gomez- Ariza, I. Giraldez, C.C. ten Hallers-Tjabbes, 2004. Imposex and butyltin contamination off the Oporto Coast (NW Portugal): a possible effect of the discharge of dredged material. *Environment International*, 30: 793-798.

Sarradin, P. M., A. Astruc, V. Desauziers, R. Pinel, M. Astruc, 1991. Butyltin pollution in surface sediments of Arcachon Bay after 10 years of restricted use of TBT based paints. *Environmental Technology*, 12: 537-543.

Shi, H. H., C.J. Huang, S.X. Zhu, X.J. Yu, W.Y. Xie, 2005. Generalized system of imposex and reproductive failure in female gastropods of coastal waters of mainland China. *Marine Ecology- Progress Series*, 304: 179-189.

Sloan, S., M.M. Gagnon, 2004. Intersex in Roe's abalone (*Haliotis roei*) in Western Australia. *Marine Pollution Bulletin*, 49: 1122-1126.

Smith, R., S.G. Bolam, H.L. Rees, C. Mason, 2008. Macrofaunalrecovery following TBT ban. *Environmental Monitoring and Assessment*, 36: 245-256

Sousa, A., S. Mendo, C. Barroso, 2005. Imposex and organotin contamination in *Nassarius reticulatus* (L.) along the Portuguese coast. *Applied Organometallic Chemistry*, 19: 315-323.

Sousa, A., C. Matsudaira, S. Takahashi, S. Tanabe, C. Barroso, 2007. Integrative assessment of organotin contamination in a southern European estuarine system (Ria de Aveiro, NW Portugal): Tracking temporal trends in order to evaluate the effectiveness of the EU ban. *Marine Pollution Bulletin*, 54: 1645-1653.

Strand, J., J.A. Jacobsen, B. Pedersen, A. Granmo, 2003. Buttyltin compounds in sediment and molluscs from the shipping strait between Denmark and Sweden. *Environmental Pollution,* 124: 7-15.

Stroben, E., J. Oehlmann, P. Fioroni, 1992a. *Hinia reticulata* and *Nucella lapillus* - Comparison of 2 gastropod tributyltin bioindicators. *Marine Biology*, 114: 289-296.

Stroben, E., J. Oehlmann, P. Fioroni, 1992b. The morphological expression of imposex in *Hinia reticulata* (Gastropoda, Buccinidae) - a potential indicator of tributyltin pollution. *Marine Biology*, 113: 625- 636.

Tester, M., D.V. Ellis, J.A.J. Thompson, 1996. Neogastropod imposex for monitoring recovery from marine TBT contamination. *Environmental Toxicology and Chemistry*, 15: 560-567.

Thomas, K. V., S.J. Blake, M.J. Waldock, 2000. Antifouling paint booster biocide contamination in UK marine sediments. *Marine Pollution Bulletin*, 40: 739-745.

Thomas, K. V., T.W. Fileman, J.W. Readman, M.J. Waldock, 2001. Antifouling paint booster biocides in the UK coastal environment and potential risks of biological effects. *Marine Pollution Bulletin*, 42: 677- 688.

Valkirs, A. O., B. Davidson, L.L. Kear, R.L. Fransham, J.G. Grovhoug, P.F. Seligman, 1991. Long-term monitoring of tributyltin in San- Diego Bay California. *Marine Environmental Research*, 32: 151-167.

Watanabe, N., S. Sakai, H. Takatsuki, 1992. Examination for degradation paths of butyltin compounds in natural waters. *Water Science and Technology*, 26: 117–124.

Watanabe, N., S. Sakai, H. Takatsuki, 1995. Release and degradation half-lives of tributyltin in sediment. *Chemosphere*, 31: 2809-2816.

Wirzinger, G., C. Vogt, J. Bachmann, M. Hasenbank, C. Liers, C. Stark, S. Ziebart, J. Oehlmann, 2007. Imposex of the netted whelk *Nassarius reticulatus* (Prosobranchia) in Brittany along a transect from a point source. *Cahiers de Biologie Marine*, 48: 85-94.

ANNEX I. Example of data summary report for imposex assessment.

Nassarius reticulatus/ nitidus

Samplig site:

Sampling date:

Imposex assessment date:

Nº of minutes in $MgCl_2$ (7% w/v) + artificial seawater:

Nº of minutes in $MgCl_2$ (7% w/v):

Nº in tray	SL (Shell length)	SA (Shell aperture)	Juvenile (shell)	Capsule gland (CG)	Seminal vesicle (SV)	Sex	% sex certainty	PL (Penis length)	VDS (Vas deferens stage)	% certainty in VDS	OS (Oviduct stage)	Oviduct wtih black balls	Dark/ degraded capsule gland	C. aborted type **I** (walls recognizable)	C. aborted type **II** (walls not recognizable)	C. aborted **type III** (hard capsule and soft inner)	C. aborted **type IV** (hard surface and hard inner)	White spot in gonadal part (a lot/little)	White spot in mantle (a lot/little)	Trematods (a lot/little)	Nematods (a lot/little)	Observations

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