Universidade de Aveiro Departamento de Biologia
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Ruy Miguel Alfama Lopes dos Santos

Imposex em *Cantharus viverratus* na Ilha de São Vicente (Cabo Verde)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Carlos Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e coorientação da Doutora Susana Galante-Oliveira, Investigadora em Pós-Doutoramento do Centro de Estudos do Ambiente e do Mar (CESAM) e da Doutora Corrine Almeida, Professora do Departamento de Engenharias e Ciências do Mar da Universidade de Cabo Verde.

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“Education is the most powerful weapon which you can use to change the world”

- Nelson "Madiba" Mandela

palavras-chave

Tributilestanho, TBT, Poluição por TBT, *imposex*, bioindicador, *Cantharus viverratus*, Ilha de São Vicente, Cabo Verde, Costa Ocidental Africana

resumo

Os compostos de tributilestanho (TBT) foram agentes biocidas amplamente utilizados em sistemas antivegetativos desde os anos 60. Contudo, a sua toxicidade em organismos não alvo conduziu à progressiva restrição da sua utilização, culminando na sua proibição global a partir do ano de 2008. No entanto, a utilização extensiva destes compostos nos anos anteriores a essa proibição levou à sua acumulação nos sedimentos de zonas costeiras por todo o mundo. Tal facto, juntamente com a forte possibilidade da sua utilização não ter cessado em países onde não existe a fiscalização da sua regulamentação, justifica a necessidade de monitorização regular da poluição por TBT.

O *imposex* (superimposição de caracteres sexuais masculinos em fêmeas de gastrópodes prosobrânquios), fenómeno causado pela exposição ao TBT, é um biomarcador efetivo deste tipo de poluição e, por isso mesmo, amplamente utilizado em programas de monitorização ambiental. O *imposex* já foi observado em mais de 260 espécies de gastrópodes por todo o mundo, no entanto existe uma extrema falta de informação sobre a ocorrência do fenómeno em espécies da costa ocidental Africana. Essa quase total ausência de informação acerca da ocorrência de *imposex* e a inexistência de espécies bioindicadoras na costa ocidental Africana, constituem um impedimento não apenas a uma correta gestão ambiental e conservação dos recursos marinhos dos países nessa região, mas também ao esforço global de monitorização e redução da poluição por TBT à escala mundial.

Por esse motivo, o presente trabalho pretende propor, pela primeira vez, uma espécie bioindicadora que pode ser usada para monitorizar os efeitos da poluição por TBT na costa ocidental Africana e aplicá-la na primeira avaliação dos níveis de poluição por TBT na ilha de São Vicente, onde se localiza o principal porto da República de Cabo Verde.

Devido à sua ampla distribuição geográfica, desde as Ilhas Canárias e o arquipélago de Cabo Verde até Angola, a sua abundância e aparente sensibilidade ao TBT, a espécie *Cantharus viverratus* (Kiener, 1834) foi seleccionada como potencial candidata para monitorizar os efeitos biológicos da poluição por TBT pela resposta *imposex*. Procedeu-se à colheita de animais adultos de *C. viverratus* na ilha de São Vicente entre Agosto e Outubro de 2012 e foi analisada a expressão morfológica do *imposex* nesta espécie, desenvolvendo-se um esquema de VDS (sequência do vaso deferente) com base nos que já existem para outros bioindicadores.

resumo (cont.)

Obteve-se um esquema de VDS atualizado que descreve fielmente a expressão morfológica do *imposex* nesta espécie, incluindo sete estádios de masculinização das fêmeas (de estádio 0 a 6), seguindo pelo menos três vias de evolução diferentes (a,b e c).

Após ter sido definido o novo esquema de VDS, foram avaliados os níveis de *imposex* ao longo da costa de São Vicente através do cálculo da percentagem de fêmeas afetadas pelo fenómeno (%I), do comprimento médio do pénis das fêmeas (FPL),do comprimento relativo do pénis (RPLI), do índice da sequência do vaso deferente (VDSI) e da percentagem de fêmeas estéreis (%S). Foi ainda determinado o conteúdo em compostos de butilestanho nos tecidos das fêmeas em seis estações de amostragem, que se revelou positivamente relacionado com os níveis de *imposex* registados.

Foram encontradas fêmeas com *imposex* apenas nas estações sob influência do Porto de São Vicente, exibindo valores tão elevados como VDSI≥5 em locais no interior deste porto.

Os resultados revelaram que a baía onde se localiza o Porto de São Vicente constitui a principal fonte de poluição por TBT na ilha, devido à ocorrência de elevado tráfego naval e à atividade dos estaleiros, e possivelmente devido à libertação de TBT dos sedimentos como consequência da contaminação histórica por estes compostos.

keywords

Tributiltyn, TBT, TBT pollution, imposex, bioindicator, *Cantharus viverratus*, São Vicente Island, Cabo Verde, West African Coast

abstract

Tributyltin (TBT) compounds were biocide agents widely used in antifouling systems since the 60s. However, their high toxicity over non-target organisms led to a progressive restriction of their use, culminating into their global ban by 2008. The extensive use of these compounds in years prior to the ban led to their build up in the sediment compartment of coastal areas around the world. This fact, together with their possible continued use in countries without regulatory supervision, justifies the need to perform regular TBT pollution monitoring.

The imposex (superimposition of male characters onto female of prosobranch gastropods), which is caused by TBT exposure, is an effective biomarker to assess environmental TBT pollution levels. Imposex is already reported for more than 260 species of gastropods around the world, but there is an astonishing lack of information regarding the occurrence of this phenomenon in the west coast of Africa. There is also no information about suitable bioindicator species for this region, which constitutes a serious setback, not only for the countries in the region, as for the global effort to implement regulatory policies and monitor their effectiveness.

Therefore, the present work aimed to propose for the first time a suitable bioindicator species that can be used for monitoring biological effects of TBT pollution in the western African coast and use it to make the first assessment of TBT pollution in the São Vicente island, where is located the main harbour of the Republic of Cabo Verde.

Because of its widespread distribution area, from the Canary islands and the Cabo Verde Archipelago to Angola, its abundance and apparent TBT sensibility, the species *Cantharus viverratus* (Kiener, 1834) was selected as a potential candidate to monitor the biological effects of TBT pollution through the imposex response.

Adult *C. viverratus* were collected between August and October 2012 along the coast of São Vicente island and the morphological expression of imposex was assessed and compared to general VDS (vas deferens sequence) schemes available for other bioindicators. An updated VDS scheme that faithfully describe the morphological expression of imposex in *C. viverratus* was therefore determined, including seven levels of female virilization (from stage 0 to 6), following at least three different evolution pathways (a, b and c).

abstract (cont.)

After the new VDS scheme was established, the assessment of imposex levels was performed through the estimation of the percentage of females affected with imposex (%I),the mean female penis length (FPL), the relative penis length index (RPLI), the vas deferens sequence index (VDSI) and the percentage of sterilized females (%S). The organotin body burden was also determined at 6 sampling stations, revealing a positive relation with the imposex levels registered. Females affected with imposex were only found at sites under the São Vicente's harbour influence, exhibiting values as high as VDSI≥5 at hotspots inside this harbour.

The results revealed that the São Vicente harbour is the focus of TBT pollution in the island due to the occurrence of high naval traffic and shipyard activities, and possibly to the TBT release from sediments as a consequence of historical contamination by this compound.

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Chapter 1

General Introduction

1.1. General Introduction

1.1.1.Tributyltin-based antifouling paints and imposex: Background History

Aquatic biological fouling, also known as biofouling or bioincrustation, consists in the undesirable settling and growth of organisms on artificial surfaces immersed in water (Omae 2003). This phenomenon is especially detrimental to vessels as it causes higher fuel consumption, increase in the frequency of dry-docking operations and deterioration/corrosion of hulls (Omae 2003, Yebra et al. 2004). Consequently, preventing bioincrustation has always been a priority for the shipping industry and antifouling (AF) paints have been the first choice for protection from biofouling since late 19th century, when they first appear (Omae 2003).

These paints are composed by a matrix – the paint background – plus biocides and pigments (Omae 2003, Yebra et al. 2004). When applied on ship hulls, they release low quantities of biocidal chemicals forming a toxic layer that prevents the undesirable fouling (Hoch 2001). Initially, copper salts, DDT, organomercurials and arsenic compounds were among the main biocidal agents added to AF paints (Bennett 1996) but in mid 1960s tributyltin (TBT) was introduced for the first time as a biocide and proved to be very successful (WHO 1990, Omae 2003). TBT was, in fact, very effective in keeping the vessels hull clean while it was considered to be less harmful to non-target organisms than other biocides used at that time, like DDT and arsenic (WHO 1990, Dowson et al. 1996, De Mora and Pelletier 1997). In the beginning, during the 1960's and 1970's, TBT was considered ideal because although the bonds between Sn and the organic substituents are stable in the presence of water, atmospheric oxygen and heat, they are rapidly cleaved when exposed to UV radiation and decomposed by microorganisms, originating less toxic degradation products (figure 1.1) (WHO 1990, Dowson et al. 1996, Hoch 2001).

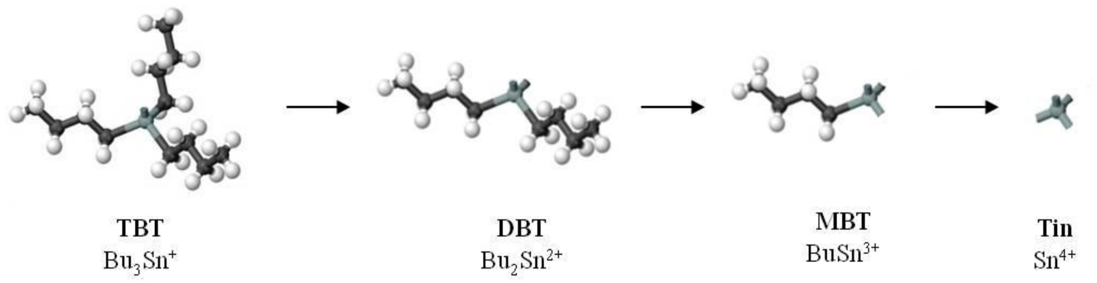

Figure 1.1.TBT moiety and its degradation products. TBT degradation occurs by the sequential loss of n-butyl chains (Bu), originating the less toxic products dibutyltin (DBT), monobutyltin (MBT) and lastly, inorganic tin (Sn). Adapted from Domingues (2012).

However the TBT persistence in the deeper and anoxic natural sediments was being seriously underestimated. The TBT degradation process in deep sediments occurs in a much slower rate than in the water column, resulting in TBT accumulation in this compartment (Dowson et al. 1996, Radke et al. 2013). This fact, together with the worldwide high input of TBT into coastal waters, made the sediment compartment a dangerous reservoir of this biocide, slowly remobilizing TBT to the water column or contaminating benthic organisms trough particles ingestion (Burton et al. 2006, Lee et al. 2006, Galante-Oliveira et al. 2010). Moreover, the high rate of bioacumulation and the extreme toxicity to non-target organisms turned this compound into a nightmare pollutant for the following decades.

The first report of TBT toxicity over non-target organisms arose in the late 1970's when reproductive failure and shell malformations of commercial Pacific oysters (*Crassostrea gigas*), at Arcachon Bay in France, provoked major stock losses and a decline of their market value (Alzieu 2000). Concurrently, at the coasts of USA and Europe, females of prosobranch species presenting male sexual characters began to appear (Blaber 1970, Smith 1971). Smith (1971) coined this phenomenon as “imposex”, a term globally adopted by the scientific community since then. In later works (Smith 1981a, b) the author related the imposex with TBT leaching from boats. The relationship between imposex and TBT contamination was later established for many other species around the world.

Since then imposex has been reported for over 260 species of gastropods worldwide (Titley-O'Neal et al. 2011) and in many of them it ultimately causes female sterilisation that may lead to population decline or extinction (Gibbs et al. 1987, Stroben et al. 1992, Gibbs 1996, Oehlmann et al. 1996, Matthiessen and Gibbs 1998, Barreiro et al. 2001). Consequently, and despite some initial resistance, several measures to control and regulate the use of TBT based AF paints started to be implemented in many countries, culminating in a global ban in 2008 (see below). Thereafter, many alternative biocides became more used as boosters (e.g., Seanine, Irgarol, Diuron, and many others) together with the classical AF-metal biocides (such as copper and zinc oxides), but their AF efficacy seems to have no parallel comparing to TBT (Thomas 2001).

1.1.2. International regulations on the Use of TBT-based AF paints

The first legal restrictions on the use of TBT-based AF paints were issued by the French government, in 1982, as a measure to prevent the detrimental effect of these substances on the oyster farming (Hoch 2001). The use of these paints was prohibited in all vessels with less than 25m in length, at first in areas of intense oyster production and later extended to the entire coastline. Analogous measures started to be adopted in other countries like United Kingdom, USA and Australia (WHO 1990, Hoch 2001, Sonak et al. 2009). In 1989, the European Commission implemented the council directive (89/677/EEC) that prohibited the use of organotin-based AF paints in vessels smaller than 25m and equipment used in fish or shellfish farming (European-Commission 1989). The rise of the global awareness on the ecological risks of TBT compounds led the International Maritime Organization (IMO) to implement the “International Convention on the Control of Harmful Antifouling Systems on Ships”, known as the AFS Convention, that banned the application of organotin paints in all kind of vessels since September 2008 (IMO 2001).

Despite the success of the AFS Convention in regulating the use of TBT-based AF paints at a global scale, many countries did not ratify the convention. Consequentially, in these countries, the possibility of a continued use of these AF-paints still remains. According to the IMO conventions status of December 2013, the Republic of Cabo Verde is yet to officially ratify the AFS Convention (IMO 2013).

1.1.3. Imposex as a Biomarker of TBT Pollution

Imposex is, undoubtedly, one of the most notable adverse effects of TBT pollution and one of the best-studied examples of endocrine disruption in wildlife. The acquired knowledge about this phenomenon has allowed the use of imposex as a biomarker of environmental TBT pollution (Gibbs et al. 1987, WHO 1990, Matthiessen and Gibbs 1998, Titley-O'Neal et al. 2011, Sousa et al. In Press). Its strong specificity, sensitivity and dose-dependent relationship to TBT environmental levels has provided a low cost and less time-consuming method to monitor TBT pollution. Triphenyltin (TPT) is an environmental contaminant that may also induce imposex, but the very low values of TPT in marine environment exclude it from being a probable imposex indutor in natural populations (Barroso et al. 2002).

The imposex intensity observed in adult gastropods represents a dose-dependent and irreversible response of the total TBT integrated throughout its life, especially during the juvenile stage when the genital tract is in formation (Gibbs and Bryan 1994). It is commonly measured by different parameters that are computed as average values for a given population. Among many others (see Titley-O'Neal et al. 2011) the most popular are the vas deferens sequence index (VDSI), the female penis length (FPL), the relative female penis length (RPLI), the incidence or percentage of affected females (%I) and percentage of sterile females (%S). These parameters are explained below in more detail.

1.1.3.1. Vas deferens sequence index (VDSI)

A scheme for imposex classification has been used for numerous species of prosobranchs, based on the sequence of the vas deferens and penis development, as well as the degree of modification of the oviduct that may lead to sterility. This scheme was originally based on a three-stage scale (Gibbs and Bryan 1986), which was later extended to six stages (Gibbs et al. 1987) categorising the development of the vas deferens and associated organs. This classification scheme is commonly known as the "Vas Deferens Sequence" (VDS) scheme. Fioroni et al. (1991) adapted this VDS scheme in order to represent the morphological expression of imposex in 69 species of prosobranchs. Their classification system has been modified continuously (Oehlmann et al. 1991, 1992, Stroben et al. 1992, Oehlmann et al. 1996) in order to accommodate as many species as possible.

According to this VDS scheme, imposex expression is classified into seven stages, with three types (a, b and c) in the first three stages and stage 5, and with two types in stages 4 and 6 (figure 1.2). Stage 0 refers to a normal female without any male characters. Stage 1 is characterized by a small penis without a penis duct behind the right tentacle (type a), a short distal vas deferens section also behind the right tentacle (type b), or a short proximal vas deferens section beginning at the vaginal opening (type c). The vas deferens grows progressively (either forming the penis duct or growing in extension over the mantle floor) in stages 2 and 3. In stage 4 the vas deferens completes its development from the base of the penis up to the vaginal opening, or it passes by the vaginal opening and runs into the ventral channel of the capsule gland (type 4*). In stage 5, the vagina is replaced by a small prostate gland (type a), the vaginal opening is occluded by proliferating vas deferens tissue (type b), or the ontogenetic closure of the pallial oviduct is incomplete (type c). From this stage onwards, imposex development leads to reproductive failure and to sterility, because egg capsules cannot be released (types a, b) or produced (type c). In stage 6, the lumen of the capsule gland and its vestibulum are filled with aborted egg capsules (Fioroni et al. 1991, Oehlmann et al. 1991, 1992, Stroben et al. 1992, Oehlmann et al. 1996).

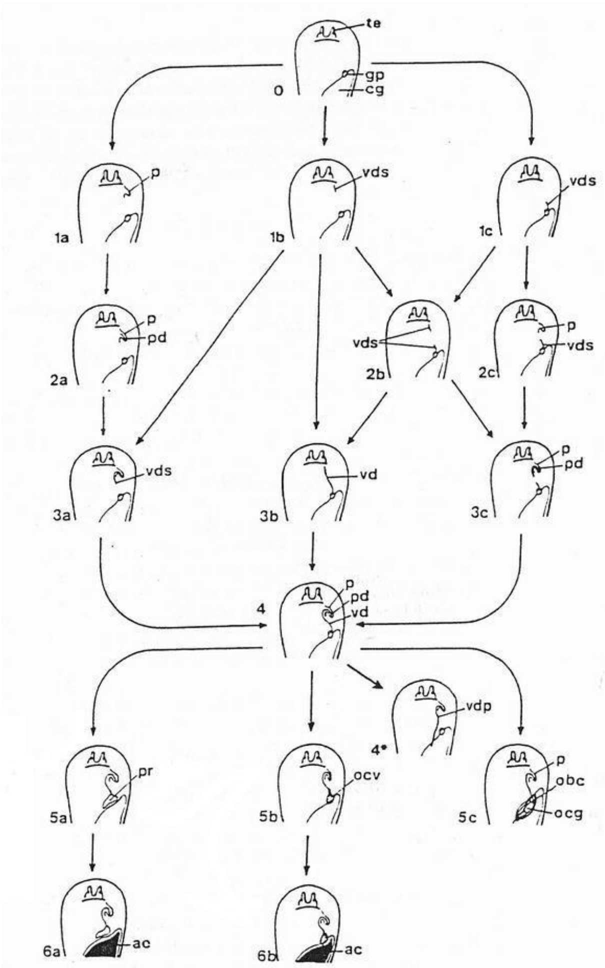

Figure 1.2. General scheme of imposex evolution in prosobranchs. Adapted from Stroben et al. (1992). (*ac*, aborted capules; cg, capsule gland; *gp*, genital papilla; *obc*, open bursa copulatrix; *ocg*, open capsule gland; *ocv*, occlusion of the vulva; *p*, penis; *pd*, penis duct; *pr*, prostate; *te*, tentacle; *vd*, vas deferens; *vds*, vas deferens section; *vdp*, vas deferens section passage by the vaginal opening).

The calculation of the average stage of the VDS in a sample of females provides the VDS index (VDSI) by which is possible to compare the intensity of imposex in different populations and correlate to TBT levels in the environment and tissues (Gibbs and Bryan 1994).VDSI is considered the most complete index for imposex studies because (i) it allows the comparison of TBT sensitivity and levels between different species, (ii) there are no seasonal changes associated with VDSI and (iii) is the only index (together with %S) that demonstrates ecological significance. Generally, VDSI values >4 identify the proportion of sterilised females in a population (Galante-Oliveira et al. 2010b, Gibbs et al. 1987, Titley-O'Neal et al. 2011).

1.1.3.2. Female penis length (FPL) and relative female penis length (RPLI)

The FPL corresponds to the average length of the penis in the females' population whereas RPLI indicates the FPL as a percentage of the mean length of the male's penises within the population (MPL). It is calculated by the formula $\frac{FPL}{MPL} \times 100$. Thus, this index allows the comparison between FPL values from different sampling stations independently of the size of the animals and of the males' penises for each population. There are two potential drawbacks on its use: (i) seasonal changes in male penis length that are common to occur and (ii) the existence of imposex affected females without a penis, which can result in an underestimation of imposex severity. Nevertheless, the use of RPLI can still be useful in providing supplementary data especially when used together with the VDSI (Galante-Oliveira et al. 2010b, Gibbs et al. 1987,Gibbs 1993, Titley-O'Neal et al. 2011).

1.1.3.3. Percentage of females affected by imposex (%I)

The parameter (%I) indicates the prevalence of the imposex within the population. Despite giving useful information regarding the extension of the phenomenon, this measure alone fails to differentiate between highly TBT polluted sites (Gibbs et al. 1987, Titley-O'Neal et al. 2011).

1.1.3.4. Percentage of sterile females (%S)

The percentage of sterile females (%S), which becomes extremely useful in highly TBT polluted sites, allows the analysis of the impairment of female's reproductive capacity and the possibility of the population decline (Gibbs et al. 1987, Titley-O'Neal et al. 2011).

1.1.4. Gastropods as Bioindicators of TBT Pollution

The correct classification of the morphological expression of imposex in analysed females, into the adequate VDS stage, is of crucial matter in any study assessing TBT contamination. The scheme presented in figure 1.2, although generally valid for imposex descriptions in most prosobranchs, will probably fail to cover all morphological variations, given that the characters of imposex demonstrate widely inter-specific variability (Fioroni et al. 1991, Stroben et al. 1992). To lessen this problem, several schemes have been proposed for different species, faithfully describing the morphological expression of imposex in the given species, validating its use as a bioindicator, for example *Nucella lapillus* (Gibbs et al. 1987*), Nassarius reticulatus* (Stroben et al. 1992, Barroso and Moreira 1998), *Hydrobia ulvae* (Schulte-Oehlmann et al. 1997, Galante-Oliveira et al. 2010a), among many others.

However, since different oceanic, climatic and biotic conditions pertain to different world regions, it is relevant to find a broader base of validated indicator organisms which should be indigenous within areas under investigation. Despite imposex had already been reported for 260 species of gastropods around the world (Titley-O'Neal et al. 2011), there is an astonish asymmetry between the studied geographic regions.

The main gap of information occurs undoubtedly in the African Continent where, to date, imposex have only been reported for species collected in Tunisia (Abidli et al. 2009, Abidli et al. 2012, Abidli et al. 2013, Lahbib et al. 2013), the Mediterranean coast of Morocco (Lemghich and Benajiba 2007), Ghana (Nyarko and Evans 1997) and the eastern seaboard of South Africa (Marshall and Rajkumar 2003) (figure 1.3). Hence, in addition to the almost total lack of information regarding the occurrence of imposex for the West coast of Africa, no prosobranch species have been proposed as a validated TBT bioindicator for the region.

Figure 1.3. Map of the African continent highlighting in grey the countries where relevant investigations on imposex have been undertaken.

This fact constitutes a serious setback both for the countries, hindering TBT biomonitoring studies and ecological risk assessments at the regional scale, and for the global effort to implement regulatory policies and monitor their effectiveness, e.g. the AFS Convention.

1.1.5. Study Area

The Republic of Cabo Verde (figure 1.4) is constituted by an archipelago of 10 volcanic islands and 8 islets located in the eastern Atlantic Ocean at 600 Km off the Coast of Senegal, between latitude 14º 48’ and 17º 12’ N and longitude 22º 40’ and 25º 22’ W. It belongs to the biogeographic region of Macaronesia, which also includes Madeira, Azores and the Canary Islands. With only 4033 km^2 of land and 1020 km of coastline, the country possesses an exclusive economic zone of 734,265 km^2, what clearly denotes the importance of maritime resources for country's progress (Almada 1993, Medina et al. 2007, Lopes 2009).

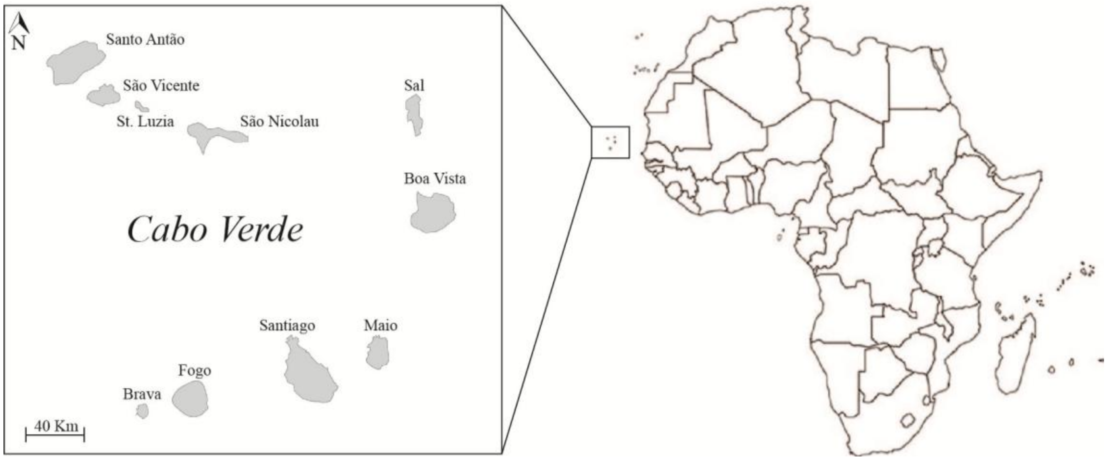

Figure 1.4. Geographic location of the archipelago of Cabo Verde.

Because of its strategic position, Cabo Verde has always been within important shipping routes between the African, European and American continents (Silva 2005, Medina et al. 2007, Enapor 2013). Cabo Verde's main harbour is located in São Vicente Island. This island, situated in the northeast of the archipelago with a total surface of 227 Km^2,is the second most populated of the country, with 76140 inhabitants (INE 2010, ONU-Habitat 2012). Its peculiar morphology, characterized by the Porto Grande Bay (figure 1.5) – a 4 Km wide, semi-circular natural embayment with depth reaching 30 meters, originated from an ancient volcanic crater – offered the right conditions to develop the countries' economy by investing in maritime infrastructures (Almeida 2004, Silva 2005).

Figure 1.5. Porto Grande Bay in São Vicente Island, Republic of Cabo Verde. Adapted from Enapor (2013).

The most important of these infrastructures is undoubtedly the Porto Grande Harbour. Built since 1962 and upgraded several times along the years, it is the main commercial harbour of the country. It possess 1.75 Km of wharfage, with depths varying from 3.5 to 12 meters and several storage facilities. This harbour has been registering a growing activity, receiving more than 2000 large-draught ships yearly (Enapor 2013). An important shipyard industry has evolved with the commercial port. Presently the shipyard of "Cabnave - Estaleiros Navais de Cabo Verde" is equipped with slipway systems of transversal elevation supporting up to six vessels of 2800 t each. This shipyard offers a great variety of services to national and international vessels, including paint stripping and antifouling coatings (Almeida 2004, ONU-Habitat 2012).

The Porto Grande Bay natural advantages also made the island a nautical tourism destination, encouraging the construction of the Mindelo Marina, which receives about 600-700 leisure boats a year.

Figure 1.6. Main harbour activities in Porto Grande Bay. **A -** Porto Grande Harbour (adapted from Enapor 2013). **B -** Cabnave shipyard. **C -**Vessel being repaired at Cabnave shipyard. **D -** Mindelo Marina.

All these infrastructures have given a major contribute in propelling both the island's and the country's economy. However, serious questions are being raised about the impact of all these anthropogenic pressures on the surrounding environment and on the population's health (Almeida 2004, Silva 2005, Abu-Raya 2009, Freitas and Silva 2009). Besides the proximity of the metropolitan area, there is a population's high dependency of the natural resources from the bay, not only for fisheries as for obtaining potable water, considering that desalination of seawater from the bay is the major water source in the island (Silva 2005, Electra 2011).

Some studies has been conducted for the assessment of the different contamination sources for the Porto Grande Bay (Almeida 2004, Silva 2005, Abu-Raya 2009, Freitas and Silva 2009). Regarding TBT pollution, some authors mentioned serious concerns on the possibility that the vessels circulating and anchored in the bay, as well as the ones being repaired in the shipyard, may have leached TBT from their AF systems to this sensible area during decades, which could have seriously affected its biota. However, until now, this situation remains to be answered.

1.1.6. Taxonomy, geographical distribution, habitat and shell of *Chantharus viverratus* (Kiener, 1834)

Cantharus viverratus is a mollusc species in class Gastropoda and subclass Caenogastropoda – although one can still find in the literature the species included in subclass Prosobranchia, even if this latter is no longer accepted as it is a paraphyletic taxon in modern malacology (WoRMS 2013). The only information available in the literature regarding this species concerns its geographical distribution, habitat and shell morphology. *C. viverratus* has been reported to occur from Canary and Cabo Verde Archipelagos and West Africa to Angola (Rolán 2005). It lives in sandy and rocky bottoms down to 10 m depth (Rolán 2005) and is common in the mid-tide zone of coastal areas (Guerreiro and Reiner 2000). The shell height can achieve a maximum of 50 mm and is fusiform, solid and heavy, with colours varying from blue to brown, with about five convex whorls, the last one being about half of its total length (Rolán 2005), (figure 1.7).

Figure 1.7. *Cantharus viverratus.* Specimen collected at Calhau, São Vicente, Republic of Cabo Verde. Scale bar equal to 1 cm.

1.2. Objectives and Rationale of the Thesis

The present work has two main objectives. The first one, which is the focus of the **Chapter 2** of the thesis, is to fully describe the morphological expression of imposex in the caenogastropod *Cantharus viverratus*, in order to validate a new TBT pollution bioindicator for Macaronesia and the West coast of Africa. This species was selected because of its widespread distribution area in the southeast Atlantic and its apparent TBT sensitivity. Despite its potential interest as a bioindicator of TBT environmental pollution in this wide geographical region, little is known about the biology of this species and no previous studies addressed the occurrence of imposex or described the anatomy of the reproductive system of this caenogastropod, which makes this study the first contribution in the topic.

The second objective, addressed in **Chapter 3**, is to put in place and validate *C. viverratus* as a bioindicator in a field monitoring survey to assess the present status of TBT pollution in São Vicente Island, and by this way perform the first assessment of this type of pollution in the Republic of Cabo Verde.

This thesis also includes the current introductory **Chapter 1** that presents an historical overview of TBT pollution problematic and introduces the reader to the basic concepts of its use as a biomarker, the imposex phenomenon, and environmental monitoring. It also includes a brief description of the study area and of the species selected for this study, *Chantarus viverratus*.

Finally, **Chapter 4** summarizes the main conclusions of the thesis, explains how this work contributes to the scientific advances in the topic and suggests new research lines that could be explored as a continuity of this study.

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Chapter 2

The Morphological Expression and Histological Analysis of Imposex in *Cantharus viverratus* (Kiener, 1834): a New Bioindicator of Tributyltin Pollution in the western African Coast.

Lopes-dos-Santos, R.M.A., Almeida, C., Pereira, M.L., Barroso, C.M., Galante-Oliveira, S. 2013. *Marine Biology* (submitted)

2.1. Introduction

Tributyltin (TBT) compounds start to be used as biocide agents of antifouling systems in the mid-1960s and due to their unmatched effectiveness and economic advantages they soon dominated the market covering an estimated 70% of the world fleet in the early-2000s (De Mora and Pelletier 1997, Evans 1999, Yebra et al. 2004). However, the adverse effects caused by such compounds in non-target organisms led to a progressive restriction of the use of TBT as antifoulant, which culminated in a global ban of organotin-based antifouling systems by 2008 through the AFS Convention entry into force (IMO 2001).

One of the most notable effects of TBT in non-target organisms is the imposex, a condition in which female prosobranch gastropods develop male sexual characters, such as a penis and a vas deferens (Smith 1971). The specificity and strong positive relationship between imposex intensity and the level of TBT pollution, as well as the fact that imposex is triggered by very low concentrations of TBT in the environment (few nanograms per litre of seawater), has allowed the imposex phenomenon to be effectively used worldwide as a biomarker for TBT pollution (Gibbs et al. 1987, Matthiessen and Gibbs 1998, Titley-O'Neal et al. 2011). The imposex intensity is usually measured through a score system where the sequence of vas deferens and penis formation is classified into progressive stages, generally ranked from 0 (normal female) to 6 (female that become sterile due to high levels of imposex) (Gibbs et al. 1987). This classification system, usually called vas deferens sequence (or VDS scheme), is suited for each species but an extended version with multiple pathways was proposed by Fioroni et al. (1991) to fit more species, which was thereafter refined by Stroben et al. (1992) to represent the morphological expression of imposex in a wide diversity of prosobranchs.

Imposex is already known to occur in 260 species of gastropods around the world (Titley-O'Neal et al. 2011). Although this number elucidates the global scale of this phenomenon, there is an astonish asymmetry between the geographic regions for which imposex has been reported. The main gap of information occurs undoubtedly in Africa where, to date, imposex have been reported only for species collected in Tunisia (Abidli et al. 2013), the Mediterranean coast of Morocco (Lemghich and Benajiba 2007) , Ghana (Nyarko and Evans 1997) and the eastern seaboard of South Africa (Marshall and

Rajkumar 2003). The total lack of information regarding the occurrence of imposex for the almost entire West coast of Africa constitutes a serious setback, not only for the countries in the region, hindering any TBT biomonitoring studies and ecological risk assessments, as for the global effort to implement regulatory policies and monitor their effectiveness. This is of great concern since although the western African coast is a region with significant importance for shipping trade routes, several countries in the region is yet to officially ratify the AFS Convention (IMO 2013).

The present work describes the morphological expression of imposex in the caenogastropod *Cantharus viverratus* (Kiener, 1834), in order to make available the first TBT pollution bioindicator in the region. This species occurs from the Canary and Cabo Verde Archipelagos to Angola, living in sandy and rocky bottoms to 10 m depth (Rolán 2005). Little is known about this species biology and no previous study addressed the occurrence of imposex or described the anatomy of the reproductive system for this prosobranch, for which this study makes the first contribute.

2.2. Materials & Methods

Adult *Cantharus viverratus* were collected between August and October 2012 in the coast of São Vicente Island, Republic of Cabo Verde. These gastropods were collected by hand in the intertidal rocky shore of pristine sites in the northwest and West coast of the island and also at sites distributed along a gradient of naval traffic around the Porto Grande Bay, the major harbour in the country. In the laboratory, the specimens were narcotized during 60 min using $MgCl_2$ 7% in distilled water. The shells were cracked open with a bench vice and the reproductive systems of males and females examined using a stereo-microscope.

The observed morphological expression of imposex in *C. viverratus* was compared to the general VDS schemes available for prosobranchs (Stroben et al. 1992) and also to the VDS scheme available for an Asian tropical species belonging to the same genus, *C. cecillei* (Shi et al. 2005).

For a more detailed inspection of the anatomical changes caused by imposex and evaluate their implications for reproduction, histological studies were performed in normal individuals of both genders and also in imposex-affected females. Hence, after narcotisation and anatomical examination under a stereo-microscope, the animals were fixed in Bouin's solution for 24h and preserved in 70% ethanol afterwards. Complete series of histological sections embedded in paraffin were made (5-7 μm), stained with haematoxylin-eosin and mounted in DPX resin for light microscopy observation.

2.3. Results

2.3.1. Cantharus viverratus reproductive systems

There is a clear distinction between the male and female reproductive systems of *Cantharus viverratus*. Genders can be easily identified if animals are slightly pulled out of the shell as a conspicuous penis appears behind the right ocular tentacle in males. Further differences are finally observed when the shell is removed and the mantle cavity is exposed.

Females present a conspicuous capsule gland on the right side of the mantle cavity, beneath the rectum, leading forward to a vaginal opening (vulva) located on the right anterior region of the mantle cavity, in a ventral and anterior position in relation to the anus (figure 2.1A). Histological sections reveal that the capsule gland leads to a muscular vestibule and then to the vagina and the vulva. Between the vulva and the capsule gland (in the direction and just below the anus) the gonoduct diverges ventrally into a long oval pouch – the bursa copulatrix– that extends beneath the capsule gland for about half of its length (figure 2.1B). Histological sections frequently showed the bursa filled with spermatozoa, which denotes that, during copulation, the male inserts the penis into the vulva and discharges sperm and prostatic fluid into the bursa (figure 2.1B5). From here, the sperm is conducted posteriorly through the ventral channel for fertilization somewhere close to the posterior end of the capsule gland, in the receptaculum seminis. The albumen gland lies in the posterior end of the duct behind the capsule gland and between the two glands it is visible a shallow darker ingesting gland. The oviduct continues posteriorly in direction to the ovary, which lies over the digestive gland in the visceral mass.

In turn, males present a conspicuous and very large penis behind the right ocular tentacle that may reach, at rest, as much as 1/3 of the full length of the animal (figure 2.1C). The penis is composed of an extensible basal portion and a tip that is enclosed inside a sheath. Inside the whole organ runs a channel – penis duct – through which the sperm is discharged into the female (figure 2.1D7). This duct leaves the penis and continues posteriorly as a vas deferens, at first straight, but then becomes highly convoluted to form a distal prostate (figure 2.1D8). This prostate, located behind the penis, when fully developed, enlarges and elevates smoothly above the mantle floor. From here the vas deferens resumes its linear configuration and runs backward along the right side of the mantle floor in the direction of the rectum. When the vas deferens passes ventrally close to the rectum it starts to convolute again and, clothed by an epithelium, produces a voluminous and conspicuous second prostate (the "proximal prostate") that accompanies ventrally the intestine along the pallial cavity; the convoluted coils of the vas deferens can be seen through transparency inside the epithelium (figure 2.1D9). Hereafter, the duct runs posteriorly in the direction of the testis that lies close to digestive gland in the visceral mass. In ripe males, below the surface of the visceral mass, the vas deferens is greatly coiled and distended with a white sperm mass forming a seminal vesicle.

2.3.2. Imposex development

The female reproductive system described above is valid for animals collected out of Porto Grande Bay and is referred as the "normal condition". However, females caught at sites entering this Bay exhibited different degrees of abnormal virilisation or imposex.

Females collected at the innermost side of the Porto Grande Bay were the most affected, showing a complete penis with a sheath and a tip inside it (a character that is no longer valid to distinguish genders in these locations) and a vas deferens running through the mantle floor up to the vulva (12% of the cases) or passing laterally close to the vulva (88% of the cases), (figure 2.1E). Some females with advanced stages of imposex developed a distal and proximal prostates, resembling the males (figure 2.1E). Some of these females also presented an enlarged capsule gland, tissue excrescencies near the vulva and one female presented aborted egg capsule inside the capsule gland.

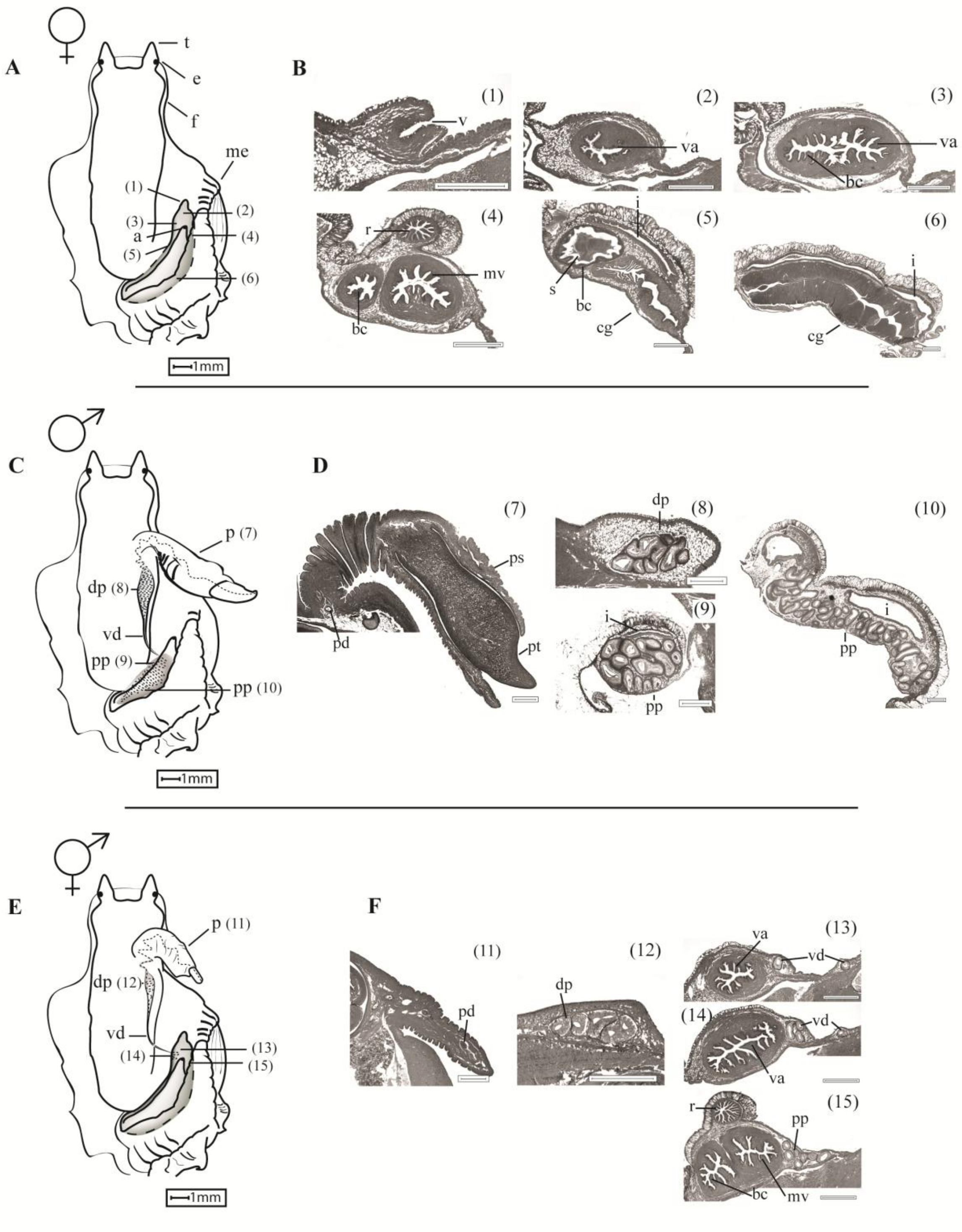

Figure 2.1. *Cantharus viverratus*. **A-** Female removed from the shell and mantle cavity opened mid-dorsally to display the anterior genital tract. **B-** Histological sections of organs indicated in A. **C -**Male removed from the shell and mantle cavity opened mid-dorsally to display the anterior genital tract. **D-** Histological sections of organs indicated in C. **E-** Female with imposex and exhibiting VDS stage 4*. **F-** Histological sections of organs indicated in E. The line on the bottom right hand side of each picture from B, D and F constitutes the respective scale and is always equal to 500 µm. Abbreviations: *a*, anus; *bc*, bursa copulatrix; *cg*, capsule gland; *dp*, distal prostate; *e*, eye; *f*, foot; *i*, intestine; *me*, mantle extremity; *mv*, muscular vestibulum; *p*, penis; *pd*, penis duct; *pp*, proximal prostate; *ps*, penis sheath; *pt*, penis tip; *r*, rectum; *s*, sperm; *t*, tentacle; *v*, vulva; *va*, vagina; *vd*, vas deferens.

Histological sections revealed that many of these females were sterilised due to malformations and constrictions of the vulva, vestibule, vagina and bursa copulatrix due to growth of tumour-like masses of cells in the region corresponding to the proximal prostate of males (figure 2.2A and B). Histological sections of the ovary in these females with advanced stages of imposex did not reveal, however, any signs of spermatogenesis. Moving to the outside of the inlet, at the entrance of the Porto Grande Bay, the intensity of imposex decreased with females presenting short vas deferens tracts and smaller and unsheathed penises, which could be absent at all.

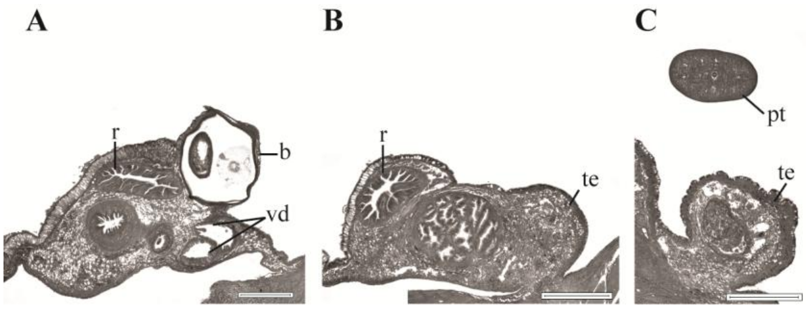

Figure 2.2. *Cantharus viverratus*. Abnormal growth of tumour-like excrescences and/or blisters in sterile females with advanced stages of imposex. **A-** Anterior genital tract showing blister and initial growth of the proximal prostate. **B-** Tumour-like excrescence growing in the anterior genital tract blocking the vagina. **C-** Tumour-like excrescence growing in the zone where distal prostate would develop in the female affected with imposex. The line on the bottom right hand side of each picture constitutes the respective scale and is always equal to 500 µm. Abbreviations: *b*, blister; *te*, tissue excrescences; other abbreviations as in figure 2.1.

Hence, a full range of imposex stages could be observed along a spatial gradient of naval traffic from outside to inside of Porto Grande Bay. We found that the morphological expression of imposex in *Cantharus viverratus* is somehow similar to the one described for *Cantharus cecillei* by Shi et al. (2005), but as this scheme was originally derived from the more general proposed by Stroben et al. (1992), both are intrinsically related and will be mentioned here in parallel. So, we used these classification systems to note imposex stages that were found (written in bold) or not found (written in italic) in *C. viverratus* in this study, though some specific differences and adaptations will be noted in due course (see figure 2.3):

Stage 1

type a: small penis, without a penis duct or penis sheath, behind the right ocular tentacle;

type b (not observed): a short distal vas deferens section behind the right tentacle;

type c, c*: a short proximal vas deferens tract beginning at (type c) or passing by (type c*) the vaginal opening;

Stage 2

type a: penis without a penis sheath but with a closing or closed penis duct, behind the right ocular tentacle;

type b (not observed): vas deferens with a short distal and also a short proximal tract;

type c, c (not observed): small penis (without penis duct), often as a small ridge and a short proximal vas deferens tract;*

Stage 3

type a: penis without penis sheath but with a duct continuing in a short distal tract of the vas deferens;

type b, b*: vas deferens running continuously from the right ocular tentacle over the bottom of the mantle cavity up to (type b) the vaginal opening or passing by (type b*) the vaginal opening;

type c, c*: penis without penis sheath but with a penis duct continuing in a distal portion of the vas deferens; additionally, a short proximal vas deferens portion arises from (type c) the vaginal opening or near (type c*) the vaginal opening;

Stage 4

type 4, 4*: penis frequently with a penis sheath and a continuous vas deferens extending from the penis (stage 4) up to the vaginal opening or (stage 4*) passing by the vaginal opening; the distal prostate starts to develop and become noticeable behind the penis; the proximal prostate also starts to develop beneath the vulva and capsule gland but is difficult to visualise, though easily detected in histological sections;

Stage 5

type a (not observed): the vagina is reduced and the vulva is absent; a more-or-less extended prostate gland can be found at the vulva.

type a (converted to stage 4 because the prostate development may not cause sterility in this species): a complete vagina and an open vulva with a more-or-less extended prostate gland near the vaginal opening;*

type b: the vaginal opening is occluded by proliferating vas deferens tissue, often forming "nodules" and "blisters";

type b*: an open vulva but a massive proliferation of cells in the region of the vagina creates nodules and blisters that cause blockage or contortion of the oviduct;

type c, c (not observed): the ontogenic closure of the pallial oviduct is incomplete, and a continuous vas deferens can be found up to (type c) or near(type c*) the vaginal opening.*

Stage 6

Types a, a*, b and b* differ from the corresponding types of stage 5 by the presence of aborted egg capsules in their oviduct; in the current study only one female was found with **stage 6b*.**

2.4. Discussion

Cantharus viverratus females collected in Porto Grande Bay exhibited different degrees of imposex that could be well described by the general classification scheme proposed by Stroben et al. (1992), after some adaptations. In this VDS scheme the vas deferens sequence may be ranked in seven levels, following from stage 0 to stage 6, and all these stages were observed in females of *C. viverratus*. In this scheme, the first appearance of the penis versus the vas deferens originates different sub-stages or pathways named "a, b and c". Via "a" means that the penis is the first organ to appear during the virilisation process while "b" and "c" refer to the condition where the vas deferens is the first to emerge; the difference between "b" and "c" regards the position where the vas deferens starts to develop, near the right ocular tentacle (distal tract) or near the vaginal opening (proximal tract), respectively.

The imposex expression in *C. viverratus* may follow a, b and/or c depending on the VDS stage but we admit that perhaps more paths could be found if more animals had been analysed. We believe that the precise path a, b or c through which imposex follows is not a random process, i.e., the initial eruption of the male characters may be synchronised and subjected to some kind of external control because imposex induced in the laboratory lead to the development of vas deferens without penis formation (to be published elsewhere).

One major discrepancy detected between the imposex expression in *C. viverratus* and the VDS scheme cited above is that the vas deferens frequently does not meet the vulva but, instead, it deviates and passes by the side of it. The general typical VDS scheme (Stroben et al. 1992) recognises this possible deviation at stage 4 for some species of prosobranchs and assigns the symbol "*" to this pathway but our results show that this condition is very common in *C. viverratus* and could be virtually observed in any VDS stage that embraces the proximal vas deferens tract. For stage 4 most of the females showed a vas deferens passing laterally to the vulva (VDS=4*). Other discrepancy between our results and the abovementioned VDS scheme is the fact that the proximal prostate development in females does not necessarily imply sterilization. In fact, histological sections of this region clearly demonstrate that the vas deferens at stage 4* does not run into the vulva, vagina or ventral channel. Instead, it passes by and convolutes, originating the primordium of an organ very similar to the male's proximal prostate (figure 2.1F15). This prostate development does not replace the vagina and does not cause female sterilization. We could observe the initial formation of this prostate in VDS stage 3b in some females, though it was predominant in stages 4, 5 and 6. Thus, the sterilisation of *C. viverratus* occurs by abnormal growth of tissues and blisters that block the vulva and constricts the vagina, vestibule and even the anterior region of the capsule gland (figure 2.2A and B). More rarely, the bursa copulatrix may become contorted and split, which may eventually impair fertilisation. It seems so that endocrine disruption triggers tissue growth for the development of male sexual organs, a process that becomes out of control and originates tumor-like excrescences that pushes or substitutes portions of the anterior tract of the gonoduct causing the blockage of the genital tract that impairs egg release and eventually copulation. Curiously these tissue excrescences were also observed behind the penis in females of advanced stage of imposex, in the region corresponding to the distal prostate of males, denoting an uncontrolled proliferation of cells in a very similar way as described above (figure 2.2C).

Some of these discrepancies from the typical VDS scheme were noted previously by Shi et al. (2005) regarding the expression of imposex in *C. cecillei* and, consequently, these authors proposed an updated scheme of imposex that incorporates, for instance, the deviation of the vas deferens near the vulva. The authors also proposed that stage 4* should no longer represent the end of imposex development as it conduces to female sterility by a similar process described here. Our results corroborate Shi et al. (2005) findings and so their updated VDS scheme is suitable to describe the morphological expression of imposex in *C. viverratus*, except for some details: (i) the penis sheath in *C. viverratus* only appears in advanced stages of imposex and (ii) females may produce a "male-like" distal prostate behind the penis after VDS stage 4.

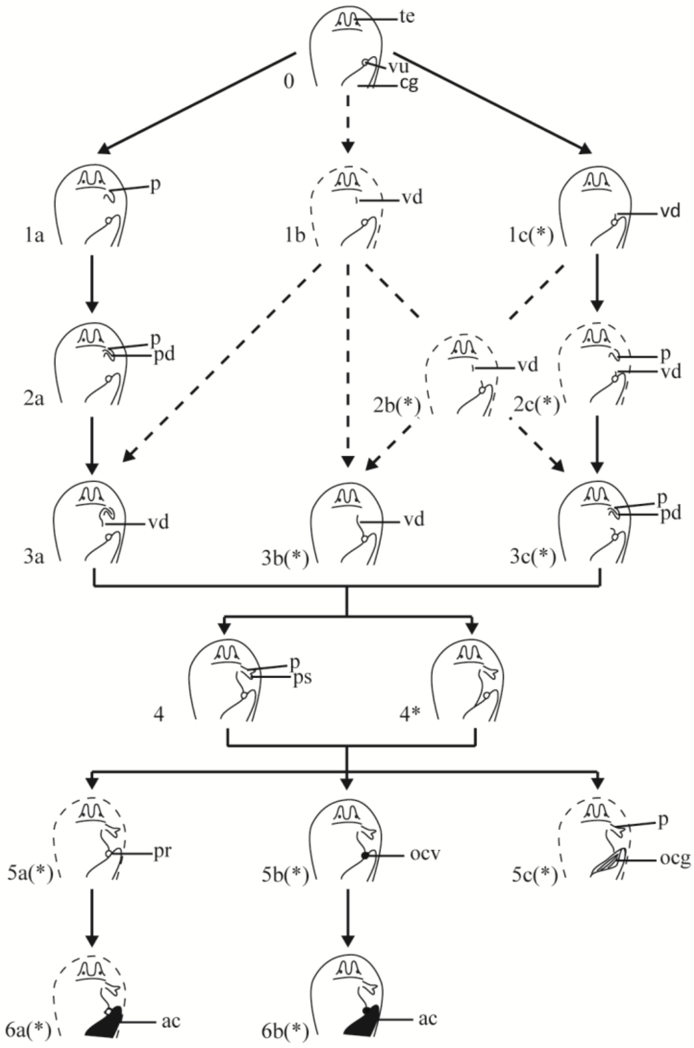

Figure 2.3. *Cantharus viverratus*. Imposex development scheme adapted from the scheme proposed by Shy et al. (2005) for *Cantharus cecillei*. The stages in dashed lines were not observed in the present study. Abbreviations: *ac*, aborted capsules; *cg* capsule gland; *obc*, open bursa copulatrix; *ocg*, open capsule gland; *ocv*, occlusion of the vulva; *p*, penis; *pd*, penis duct; *pr*, prostate; *ps*, penis.

Hence, figure 2.3 shows the VDS scheme of Shi et al. (2005) after modifications concerning the specificity of *C. viverratus*, in order to better describe the imposex expression in this species. This scheme describes well the full range of *C. viverratus* imposex intensities that occurred in females collected around the São Vicente Island, which ranged from normal condition, found at pristine sites, up to very high levels of VDS found inside the Porto Grande Bay where major sources of TBT pollution are located (see Chapter 3).

C. viverratus is a good candidate as a bioindicator for biomonitoring TBT pollution. During the course of the current work, as referred above, we could induce the development of imposex in *C. viverratus* in a just a couple of months after injecting females with very small doses of TBT chloride (to be published elsewhere). On the other hand, we also observed that imposex intensity in natural populations is positively associated with female TBT tissue contamination (see Chapter 3). So the VDS scheme shown in Figure 2.3 constitutes a tool to be used for biomonitoring TBT pollution levels, which is of great utility considering the scarcity of validated bioindicators for the West coast of Africa.

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Chapter 3

Assessment of Imposex and Butyltin Concentrations in *Cantharus viverratus* (Kiener, 1834), from São Vicente, Republic of Cabo Verde.

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3.1. Introduction

Imposex has been used worldwide as a biomarker in tributyltin (TBT) pollution monitoring surveys (Gibbs 2009, Galante-Oliveira et al. 2010, Sousa et al. In Press). It is described as the superimposition of male sexual characters, such as penis and vas deferens, on prosobranch gastropod females (Smith 1971) as a consequence of exposure to TBT (and, to a lesser extent, to triphenyltin) in the environment. Its effectiveness as a TBT biomonitoring tool is due to its strong specificity and sensitivity to TBT pollution, being triggered by concentrations of just a few nanograms per litre, and the fact that its degree of development is dose-controlled by the level of TBT in the environment (Gibbs et al. 1987, Schulte-Oehlmann et al. 1997, Barroso et al. 2000). Imposex has already been reported for more than 260 species of gastropods around the world, denoting the global dimension of this phenomenon and the wide scale of organotin pollution (Titley-O'Neal et al. 2011).

Several studies have shown a decline of TBT pollution in many countries, as a consequence of national and international restrictions on the use of antifouling paints containing organotins (Hoch 2001, Rees et al. 2001, Sousa et al. 2009, Galante-Oliveira et al. 2011, Castro et al. 2012, Choi et al. 2013). The major and most relevant legislative action is the “International Convention on the Control of Harmful Antifouling Systems on Ships”, known as the AFS Convention, issued by the International Maritime Organization (IMO), which totally bans the application of antifouling paints containing tributyl and triphenyltin in all kind of vessels since 2008, as an ultimate measure to counteract this global pollution problem (IMO 2001). However, the total lack of data regarding current organotin levels and ecological impacts in some world regions prevents the scientific community of inferring a truly global status for this type of pollution. One of the main gaps of information occurs in the western coast of Africa between Morocco and South Africa, where, to date, relevant studies have been undertaken only in Ghana (Nyarko and Evans 1997, Marshall and Rajkumar 2003, Lemghich and Benajiba 2007, Okoro et al. 2011, Okoro et al. 2012).

The Republic of Cabo Verde is constituted by an archipelago of 10 volcanic islands and 8 islets located in the eastern Atlantic Ocean at 600 Km off the West coast of Africa (Senegal), between latitude 14º 48’ and17º 12’ N and longitude 22º 40’ and 25º 22’ W. It belongs to the biogeographic region of Macaronesia, which also includes Madeira, Azores and the Canary Islands. The country 's main harbour is located at the Porto Grande Bay, in the São Vicente Island, close to the metropolitan area of Mindelo City. This harbour possesses a significant importance for the economy of country as it constitutes the major national commercial port (Porto Grande), which is registering an increasing activity, receiving more than 2000 large-draught ships yearly (Enapor 2013). Additionally, this harbour comprises a large shipyard industry, a marina for leisure boats and the island main fishing port.

The objective of the present work is to use the caenogastropod *Cantharus viverratus* (Kiener, 1834), proposed as a bioindicator of TBT pollution in Chapter 2, to perform (i) the first assessment of TBT pollution in the coast of São Vicente Island, (ii) to evaluate the impact of this kind of pollution on the populations of *C. viverratus* in the coast of São Vicente by surveying the occurrence imposex and female sterility in this area, and (iii) to provide additional validation on the use of this species as a TBT bioindicator for the Macaronesia and western coast of Africa.

3.2. Materials & Methods

3.2.1. Sampling

A total of 324 adults of *Cantharus viverratus* were collected by hand in the intertidal rocky shore of the coast of São Vicente Island between August and October 2012. Six sampling stations (Stn) were selected for this study, distributed along a gradient of naval traffic (see figure 3.1). One station was located in the west coast of the island, at a distance of 15 Km from the Porto Grande Bay (Calhau- Stn 1), other one was located in the northwest coast and 9 Km away from this harbour (Baía das Gatas - Stn 2), whilst the others were located very close or inside the Porto Grande Bay, namely, 1 km West of the harbour (Ninho de Guincho - Stn 3), at the harbour entrance (João Ribeiro - Stn 4) and inside the harbour (Matiota-Stn 5 and Praia da Galé-Stn 6).

3.2.2. Imposex analysis

In the laboratory the specimens were narcotized during 60 min using $MgCl_2$ 7% in distilled water. The shell height (distance from shell apex to lip of siphonal canal) was measured with vernier callipers to the nearest 0.1 mm. Shells were removed and animals were sexed. The penis length was measured in all animals using a 1 mm graduated graph paper under a stereo-microscope, whilst the vas deferens sequence (VDS) stage was assessed in females according to the scheme proposed in Chapter 2. The following imposex indices were determined for each sampling site: percentage of imposex-affected females (%I); mean female penis length (FPL); relative penis length index (RPLI = FPL / mean male penis length × 100) and vas deferens sequence index (VDSI).

3.2.3. Organotin Analysis

Tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) were measured in the whole tissues of 10-15 pooled females for each station. The analyses were performed at the laboratory UT2A (Pau, France). Butyltin compounds were extracted from 0.25 g of sample with 5 mL of acetic acid (CH_3COOH) using a microwave system (CEM Explorer, 3 min at 40 W). Tripropyltin was used as an internal standard and was added before the microwave irradiation procedure. A procedure blank was also performed in the same extraction run.

Butyltin species determination was performed by standard additions and this procedure was applied on a pooled sample by adding an appropriate volume of a standard solution containing TBT, DBT and MBT before the derivatisation procedure. Derivatisation was applied as follows: 1 mL of the supernatant was buffered to pH 4.5 with 5 mL of a 1 mol.L^{-1} acetic acid / sodium acetate solution, and the pH adjusted with ammonia. One mL of isooctane and 1 mL of freshly prepared 1 % aqueous sodium tetraethylborate ($NaBEt_4$) solution were added, the mixture shaken at 300 rpm for 20 min and after phase separation the organic layer was transferred into an amber Gas Chromatography (GC) autosampler vial and directly injected into the Gas Chromatography – Inductively Coupled Plasma – Mass Spectrometry (GC-ICP-MS) system. All extraction and dilution operations were performed taking the weight with an analytical precision balance. Extracts were kept in the cold (4°C) and dark, derivatisation was performed within maximum 6 hours after extraction. The derivatised extracts were stored at –20°C. Separation and determination of organotin compounds were performed by capillary GC-ICP-MS. Concentrations of butyltin compounds were calculated from application of the standard additions procedure. Mean blank values were eventually subtracted from the integrated peak areas prior to further concentration calculations. Mean values were formed from repeated sample analysis for each sub-sample. Limit of quantification was 0.5 $ngSn.g^{-1}$ for TBT and DBT, and 2 $ngSn.g^{-1}$for MBT, on a dry weight (dw) basis. Analysis performed in the Certified Reference Materials, BCR710 (oyster tissue) for TBT and DBT, and BCR646 (sediment) for MBT, gave the following results (expressed in $ngSn.g^{-1}$dw): 122 ± 8 for TBT (certified value of 133 ± 18); 72 ± 15 for DBT(certified value of 82 ± 18) and 671 ± 39 for MBT (certified value of 610 ± 120).

3.3. Results

Levels of *Cantharus viverratus* imposex and female butyltin contamination are shown in figure 3.1 and table 3.1. Females collected outside the Porto Grande Bay (Stn 1, 2 and 3) were not affected by imposex but %I rapidly increased to 98% (Stn 4) and 100% (Stn 5 and 6) at the entrance and inside the bay, respectively. The other imposex indices presented the same spatial variation, though showing a more gradual progression. FPL, RPLI and VDSI at stations 1, 2 and 3 were zero, but entering the bay, as we move from the entrance (Stn 4) to the innermost side of the bay (Stn 6), they gradually rose from 1.3 to 5.2 mm in the case of FPL, from 11.4 to 54.6% in the case of RPLI and from 2.9 to 4.1 for VDSI.

Females normally presented a-type VDS stages, i.e. with simultaneous penis development, but some b-type females occurred at sampling stations 4 (14.3%) and 5 (12.9%) and c-type females occurred at Stn 4 (4.1%), (see Chapter 2 for more details regarding VDS expression). Sterile females were only found in Matiota (Stn 5) and Praia da Galé (Stn 6), with an incidence of, respectively, 3.2 and 21.4% in the sampled population (figure 3.1). These sterile females exhibited enlarged capsule glands and tissue excrescencies near the vulva, whilst only one female presented aborted egg capsules within the capsule gland at Stn 6.

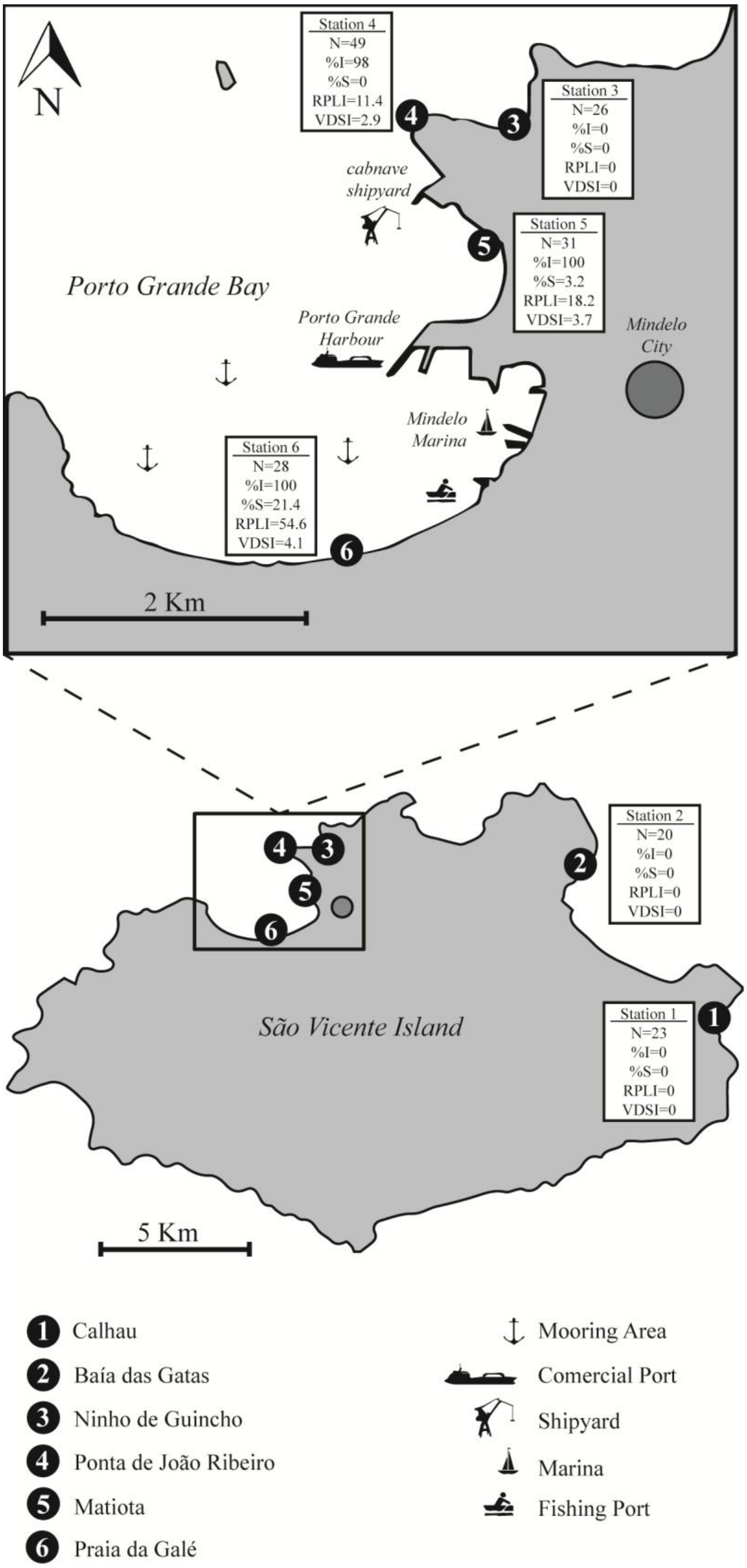

Figure 3.1. *Cantharus viverratus*. Map of the São Vicente Island showing the location of the sampling stations (1 to 6) and main harbour activities. Imposex levels are shown for each station: N - number of collected females; %I - percentage of imposex-affected females; %S - percentage of sterile females; RPLI - relative penis length index; VDSI - vas deferens sequence index. For more details compare with table 3.1.

Table 3.1. *Cantharus viverratus*. Data relative to each sampling site with the indication of the number of collected males (♂ N) and females (♀ N) by sampling station and the respective mean shell heights (mm), percentage of imposex-affected females (%I), female mean penis length (FPL), percentage of sterile females (%S), relative penis length index (RPLI) and vas deferens sequence index (VDSI). Mean tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) concentrations in female tissues (organotins) expressed in $ngSn.g^{-1}dw$; na: not analysed. For additional information compare figure 3.1.

Station Code and Name	Coordinates	♂ (N)	♂ Shell Height	♀ (N)	♀ Shell Height	%I	FPL	%S	RPLI	VDSI	Organotins ($ngSn.g^{-1}dw$)		
											TBT	DBT	MBT
1. Calhau	16º51'07"N-24º51'55"W	22	31.6±2.0	23	27.5±3.6	0	0	0	0	0	na	na	na
2. Baía das Gatas	16º54'09"N-24º54'15"W	23	33.3±2.2	20	29.9±1.1	0	0	0	0	0	na	na	na
3. Ninho de Guincho	16º54'19"N-20º59'31"W	20	30.2±2.1	26	28.0±2.9	0	0	0	0	0	5.0±0.1	3.9±0.1	2.4±0.3
4. João Ribeiro	16º54'18"N-24º59'56"W	27	29.3±3.5	49	29.2±3.4	98	1.3±1.4	0	11.4	2.9	21±5.0	27±5.0	12±0.8
5. Matiota	16º53'52"N-24º59'35"W	18	29.4±2.3	31	27.6±2.4	100	2.1±1.3	3.2	18.2	3.7	21±2.0	29±2.0	16±0.8
6. Praia da Galé	16º52'36"N-24º59'54"W	37	28.1±2.7	28	26.4±2.0	100	5.2±2.0	21.4	54.6	4.1	37±2.0	45±1.0	21±2.0

Levels of female TBT contamination ranged from a minimum of 5.0 $ngSn.g^{-1}dw$ near the Porto Grande Bay (Stn 3) and a maximum of 37 $ngSn.g^{-1}dw$ at the innermost side of the bay (Stn 6), accompanying fairly well the gradient of imposex described above. Levels of TBT represented 32 to 44% of the total butyltin (BT = MBT + DBT + TBT) concentration in the females tissues across stations, whilst DBT corresponded to 35 to 45% and MBT to 20 to 25%. The Butyltin Degradation Index (BDI), which gives the proportion of TBT in relation to its metabolites through the formula BDI = (MBT + DBT) / TBT (Diez et al. 2002), was 1.3 (Stn 3), 1.9 (Stn 4), 2.2 (Stn 5) and 1.8 (Stn 6).

3.4. Discussion

The results clearly indicate an increase of imposex levels and female TBT contamination with the proximity of the Porto Grande Bay and identify this area as the focus of TBT pollution in São Vicente Island. In fact, females collected out of the bay, at 1 km (Stn 3), 9 Km (Stn 2) and 15 Km (Stn 1) to the West, did not exhibited imposex, whereas those collected at the entrance (Stn 4) and inside the bay (Stns 5 and 6) presented increasing values of imposex as we moved to the inner side of the inlet, where hotspots of TBT pollution are located. Female TBT body burden accompanied approximately the same tendency described for imposex, i.e., near the entrance outside of the Porto Grande Bay (Stn 3), female TBT levels were relatively low (5 $ngSn.g^{-1}dw$) and increased towards the interior of the bay (21- 37 $ngSn.g^{-1}dw$).

The numerous anchored vessels inside the harbour and the occurrence of ships being repaired or repainted at the shipyard may be the cause of the input of TBT to the surrounding medium, while remobilisation of TBT stored in the bay sediments may also account for an additional source of TBT to the water and biota. The maximum values of imposex and TBT body burden were observed in Praia da Galé (Stn 6); despite this site being nearly as close as Stn 5 from potential hotspots of contamination, the higher levels observed here probably result from water currents converging into Praia da Galé as it has been reported that diverse materials suspended in the bay seawater accumulate preferentially in this innermost side of the inlet (Silva 2005). It seems plausible that a high retention and deposition of fine particles with adsorbed TBT may occur at this site, causing greater bioaccumulation of this compound in the local biota, including *C. viverratus*.

The Butyltin Degradation Index was proposed by Diez et al. (2002) for sediments and later used in molluscs (Ruiz et al. 2008, Sousa et al. 2009) to estimate fresh TBT inputs into the environment (BDI<1 indicate fresh inputs). The BDI values obtained in the current work for *C. viverratus* tissues ranged between 1.3 and 2.2, which is well above 1 and indicates that a considerable part of TBT inputs to the bay may not be very recent. These results interpretation is not always straightforward but suggests that, apart from TBT fresh inputs from local naval activities and/or resuspension from sediments, the predominance of debutylated species in the tissues can somehow be derived from past TBT contamination.

The Republic of Cabo Verde has not signed the AFS Convention but most of the commercial naval traffic and shipyard activities in Porto Grande Bay are devoted to international vessels from states that ratified this convention, consequentially no longer using TBT antifouling systems, which could lead to a lessening of TBT inputs to the bay. This could also help to explain the relatively low levels of TBT body burdens (less than 37 ngSn.g^{-1}dw) observed in *C. viverratus*, despite the intense naval traffic and shipyard activities occurring in the Porto Grande Bay.

Comparing to other gastropod species and other geographical areas, before the 2008 IMO ban it would be common to find TBT body burdens in the range 100 to 1000 ngSn.g^{-1} dw or more and BDI<1 for gastropods collected in marine harbours across the world (Stroben et al. 1992, Mensink et al. 1996, Ruiz et al. 1998, Barroso et al. 2002). Howsoever, and after the ban, there has been a global and fast decrease in the TBT tissue contents to a range below 100 ngSn.g^{-1}dw and a BDI>1 (Barroso et al. 2002, Oliveira et al. 2009, Sousa et al. 2009, Choi et al. 2013). This seems to be happening in the Porto Grande Bay. The high levels of imposex found in *C. viverratus* inside the bay could be a consequence of major past contamination (as imposex is irreversible and reflect an historical exposure to TBT depending on the species longevity) or a high sensitivity of the species to this compound, to be confirmed in due course.

As seen above, the impact of TBT pollution in *C. viverratus* populations from São Vicente Island is confined to the Porto Grande Bay where almost all females are affected with imposex, whilst out of the bay populations are normal. Of most concern, though, is the fact that imposex lead to sterility of about 3% and 21 % of the females at Stns 5 and 6 where TBT contamination is more intense. This sterilisation originates from malformations of the oviduct as a consequence of abnormal mass cell proliferation and differentiation triggered by the advanced ongoing process of virilisation (see Chapter 2). The effect of sterility at the population level depends mostly on the percentage of females affected and the dispersive capacity of the species. In Porto Grande Bay the majority of the females was not sterile and so are still able to reproduce. On the other hand, *C. viverratus* presents planktonic veliger larvae (personal observations) that can travel in water. Although the length of the planktonic existence is not known, it is expected that recruitment of newborn individuals can be supplied from unaffected breeding females inside and outside the Porto Grande Bay. For these reasons it is supposed that TBT pollution is unlikely to pose the populations at risk.

The present work represents the first attempt to use *C. viverratus* as a bioindicator of TBT pollution. This species is abundant and ubiquitous in the São Vicente Island and easily collected by hand in small pools of the intertidal rocky shore, from sheltered to wave-exposed coasts. On the other hand, the expression of imposex presents good sensitivity to discriminate different levels of TBT pollution. Therefore, the species seems very promising to be used as a simple, inexpensive and efficient tool for TBT pollution biomonitoring in the Macaronesia and west coast of Africa.

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Chapter 4

Final Remarks

The present study fully described the anatomy of the reproductive system of the species *Cantharus viverratus* and how it is morphologically affected by TBT pollution, successfully validating the species as a new TBT pollution bioindicator. These findings constitute valuable new information to support and improve the current general VDS classification scheme that allows imposex intensity characterization. This is particularly relevant regarding the sterilization process of some species in which the proximal vas deferens section passes laterally to the vaginal opening: the previously described *Nassarius reticulatus* (Barroso et al. 2002) and *Cantharus cecilei* (Shi et al. 2005), and the current proposed bioindicator, *C. viverratus* (see Chapter 2).

In addition, it was revealed the occurrence of a distal prostate behind the penis in *C. viverratus* males. To our best knowledge, no records of such type of structure have been reported before for caenogastropods and, consequentially, because females acquire this organ during the imposex phenomenon development, it is a new character to be included in the available VDS schemes. Furthermore, other relevant data on *C. viverratus* biology were also obtained during this research work. New information on the species life cycle is now available, including the occurrence of a planktonic veliger stage (observed in laboratory conditions) and further studies are planned to deepen the knowledge on this new indicator species.

This thesis first objective of providing a valuable tool for TBT biomonitoring studies and ecological risk assessments in the West African coast was fully achieved, being now (and for the first time) available one bioindicator species indigenous within this geographical area – *Cantharus viverratus*. This species is very likely to have an important role in diminishing the gap of information regarding imposex in Africa and the rest of the world. The second objective regarding the first assessment of TBT pollution in the Republic of Cabo Verde was also successfully performed (see Chapter 3) applying the proposed bioindicator (Chapter 2). Given the crucial importance of the marine resources for the country, this assessment contributes with important data that can be used to implement regulatory policies in order to preserve its natural resources and also guarantee their righteous exploitation in favour of Cabo Verde development and progress.