Universidade de Aveiro Departamento de Biologia
2010

Susana Galante Correia Pinto de Oliveira

Factores determinantes para o uso do *imposex* na monitorização da poluição por TBT

Determinant factors for the use of *imposex* in TBT pollution monitoring

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Sr. Prof. Doutor Carlos Miguel Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro, e do Sr. Prof. Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio pela bolsa de referência SFRH/BD/18411/2004.

Dedico este trabalho ao Ser Humano mais bonito que conheço...

... a minha Mãe.

Que eu saiba sempre seguir-te as pegadas...

I dedicate this work to the most beautiful Human Being I know...

... my Mother.

That I may always follow your footsteps...

o júri

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agradecimentos

Ao Sr. Prof. Doutor Carlos Miguez, pelo contagiante amor à Vida, ao próximo e à Ciência, que fez destes anos de convívio um verdadeiro deleite que jamais esquecerei. Todas e quaisquer palavras seriam poucas para demonstrar o meu apreço pelo Sr. Prof. restando-me um agradecimento inveterado pela presença sempre atenta em tantos momentos (bons, menos bons e mesmo maus), pela amizade, sinceridade, compreensão e por toda a confiança que sempre depositou em mim e que, após a insegurança inicial, me permitiu crescer e chegar um pouco mais longe. O Sr. Prof. é, sem qualquer sombra de dúvida, uma das melhores pessoas que na vida tive e terei oportunidade de conhecer.

Ao Sr. Prof. Doutor Mário Pacheco agradeço o facto de ter aceite de imediato o convite para a co-orientação deste trabalho e a atitude sempre simpática e disponível na resposta a todas as minhas solicitações. Neste momento não posso também deixar de expressar a minha admiração pela invejável capacidade de análise e fluência de raciocínio do Sr. Prof., das quais sempre resultaram valiosas sugestões e conselhos que tantas vezes ilucidaram as minhas mais renitentes dúvidas.

Ao Sr. Prof. Doutor José António Santos, pela ajuda preciosa no tratamento dos dados deste trabalho, pelo entusiasmo e dedicação incansável à causa e por me ter feito entender de forma inequívoca e definitiva o quão importante é a complementariedade de áreas, à partida tão distintas, e a lógica da aplicação do pensamento matemático. Uma estratégia interdisciplinar é, sem dúvida, um dos caminhos para o sucesso!

Ao grupo de trabalho do Laboratório de Biodisponibilidade e Impacto de Contaminantes da *Marine Biological Association of the United Kingdom* na pessoa do Sr. Doutor William Langston, pela disponibilidade e preciosos ensinamentos. Particularmente a si, Doutor Langston: nunca esquecerei a sua atitude profissional como orientador e cuidado paternal como Ser Humano.

À equipa de trabalho do laboratório de Histologia e Embriologia do Instituto de Ciências Biomédicas de Abel Salazar na pessoa do Sr. Prof. Doutor Rogério Monteiro agradeço a disponibilidade incondicional e o auxílio prestado numa parte extensa deste trabalho e especialmente as prazerosas horas de convívio que me permitiram, desde muito criança, aprender o que são a ciência e a investigação, factos que despertaram em mim o gosto pelo estudo e pela carreira académico-científica pela qual acabei por enveredar.

À equipa de trabalho do laboratório de Histologia do Departamento de Biologia da Universidade de Aveiro na pessoa da Sr.ª Prof.ª Doutora Maria de Lourdes Pereira pela disponibilização de instalações e respectivos recursos e pela afável hospitalidade, não só para com a minha pessoa mas também para com todos aqueles que comigo colaboraram.

À equipa de trabalho do Laboratório de Biotecnologia Molecular do Departamento de Biologia da Universidade de Aveiro na pessoa da Sr.ª Prof.ª Doutora Sónia Mendo agradeço a atitude sempre afectuosa e a entreajuda tão salutar que pautou todos estes anos de trabalho intenso.

agradecimentos (cont.)

Aos colegas do Laboratório de Ecologia Marinha e Estuarina do Departamento de Biologia da Universidade de Aveiro na pessoa da Sr.ª Prof.ª Doutora Marina Cunha, não só pela partilha diária de instalações, recursos e conhecimento, mas também pela amizade e excelente ambiente de trabalho que transformaram as rotinas em repetidos júbilos.

Aos colegas de equipa, "colaboradores" e alunos com os quais tive o prazer de trabalhar, pela dedicação inesgotável e demonstração diária do que é o verdadeiro "amor à camisola" sem contudo esquecer o facto de que trabalhamos para viver e não o contrário. Esta nossa forma de encarar os deveres permitiu, mesmo perante alguma escassez de recursos, o tão desejado aumento saudável da produtividade. Mas embora neste momento enquanto olho "*(...) o mar, sonho sem ter de quê. Nada no mar, salvo o ser mar, se vê.*" *(Fernando Pessoa)*, peço que não deixemos de sonhar que esta filosofia irá um dia contagiar a sociedade em que vivemos, porque "*Eles não sabem, nem sonham, que o sonho comanda a vida, que sempre que um homem sonha o mundo pula e avança (...)*" *(António Gedeão)*.

À Sr.ª Doutora Ana Sousa – minha "Chefinha", minha Mestre(a), minha colega e amiga – começo por agradecer o ter-me apresentado o TBT, o *imposex*, os artigos científicos e o *Reference Manager*! Depois, agradeço o facto de me ter ensinado as mais variadas técnicas do "desenrasca", o que é o sal *SERA*, os filtros, as bombas e os aspiradores de água! Mas, e como se ainda não bastasse, também me ensinou o que são a determinação, o sacrifício, a resistência e a persistência. Obrigada por me teres aturado todos estes anos, o termos partilhado alegrias, dificuldades e frustrações; o sermos verdadeiras amigas e, mesmo do outro lado do mundo, o estares sempre presente.

À Mestre Isabel Oliveira por ter sido o meu braço direito... e o esquerdo... mas principalmente por ser uma das minhas almas gémeas. Uma amizade assim não se agradece... apenas se retribui! E, Beluxa, nunca esqueçamos que "*(...) Uma alegria é feita dum tormento, um riso é sempre o eco dum lamento, sabe-se lá um beijo de onde vem!*" *(Florbela Espanca)*. Por tudo o que és, pela vontade e consciência (e pelo teu horóscopo para o próximo ano, dado por um astrólogo marado qualquer!) não deixes de acreditar nem desesperes... o "*beijo*" há-de vir... se Deus quiser!

Ao Sr. Doutor Niels Jonkers pela *Palm experience* e pela alcunha; por me ter ensinado a, não só ouvir música, mas entender música; a sentir a Idade Média e a conhecer poesia estrangeira; e por ter acordado em mim a coragem para a mudança de rumo... Obrigada por seres quem foste, és e serás sempre...

À Sr.ª D. Joyce Easton, minha "mãezona Inglesa", agradeço a hospitalidade, a preocupação, o *Pears & Chocolate Crumble* em cima da cama, o amor e todos os ensinamentos: do *English accent* ao saber aceitar a solidão.

À Sr.ª Dr.ª Catarina Aresta agradeço a amizade sincera e a partilha constante que nos permitimos e que não me deixa nunca esquecer de onde venho e para onde quero ir! E, Catarinixa "*(...) a única dor que na Vida podemos evitar é provavelmente a que resulta da luta de tentar evitar a dor*" *(Robin Norwood)*.

agradecimentos (cont.)

Á Sr.ª Arquitecta Sara Ventura da Cruz agradeço todos os “planos”: os desenhados já edificados (exemplos que analiso atentamente e com os quais tento aprender) e os projectados para futura concretização (verdadeiras orientações que prometo tentar seguir)! Obrigada por teres cruzado o meu caminho, por alimentares diariamente esse nosso encontro e por sermos, portanto, a prova de que nem todas as verdadeiras amizades nascem na infância. És “*(...) o rochedo onde me apoio, (...) o sol que ilumina o meu dia; digam o que disserem sobre ela Senhor, ela aceitou-me e transformou-me naquilo que sou hoje.*” *(Robin Norwood)*. Que todos saibam reconhecer o que vejo em ti amiga: um coração de menina em toda e qualquer atitude de mulher!

Aos meus amigos, os do peito, intocáveis tesouros que guardo religiosamente, principais responsáveis pela manutenção do meu equilíbrio emocional. Obrigada por me permitirem “*(...) ser mais que a sombra duma sombra, por entre tanta sombra igual a mim*” *(Florbela Espanca)*.

Ao meu núcleo familiar agradeço o apoio incondicional, emocional e financeiro, a alteração de planos em prol das minhas necessidades imediatas sem qualquer interesse ou exigência, o amor e carinho, o facto de acreditarem nas minhas capacidades (as que tenho, as que achava não ter e as que efectivamente não tenho!), as palavras, conversas e discussões (que nos marcam mas aperfeiçoam), as “tempestades e bonanças” e os princípios que desde sempre me foram incutidos e que seguirei cegamente até ao fim dos meus dias. Mamã, Papá, Mano, Sandra e “Ervilhinha”: vocês são o meu bem mais precioso.

Aos meus “menos amigos” (uma vez que penso não ter inimigos) por despertarem em mim uma incrível vontade de lutar... e vencer!

E embora as únicas certezas que tenho neste momento sejam (i) a da morte e (ii) a de que, neste caso, não devo citar *Pessoa*; aqui fica a minha máxima, a título conclusivo (no que se refere a agradecimentos) e introdutório (relativamente a este trabalho)...

Pedras no caminho? Guardo todas.

Um dia vou construir um castelo...

acknowledgements

To Prof. Dr. Carlos Miguez, for his contagious love towards Life, the next Person and Science. You have made these years of conviviality a true and unforgettable delight. Any and all the words are not enough to show my esteem for you Professor. I can only express in these pages my inveterate thanks for your always attentive presence in so many moments (good, less good and even bad), the friendship, sincerity, understanding and all the trust you've had and have in me and, after an initial uncertainty, the way you allowed me to grow and reach a little further. You Prof., are, without any shadow of a doubt, one of the best person that, in life, I've had and will have the opportunity to meet.

To Prof. Dr. Mário Pacheco my thanks for having immediately accepted the invitation to this thesis co-supervision and for the friendly and available attitude in response to all my needs. At this moment I must also express my admiration for the Prof.'s enviable ability of analysis and thinking fluency from which always resulted valuable suggestions and advices that so many times elucidated my more reluctant doubts.

To Prof. Dr. José António Santos, for the valuable help in processing the data of this thesis, for the enthusiasm and diligent dedication to the cause and to have made me clearly and definitively understand how important are the areas complimentarily, so distinct from the beginning, and the logic in applying the mathematical thought. An interdisciplinary strategy is undoubtedly one of the paths to success!

To the working team of Bioavailability and Contaminants Effect Laboratory of Marine Biological Association of The United Kingdom through the person of Dr. William Langston, for the availability and valuable lessons. Particularly to you, Dr. Langston: I'll never forget your professional attitude as supervisor and paternal care as Human Being.

To the working team of Histology and Embryology Laboratory of Abel Salazar Biomedical Sciences Institute through the person of Prof. Dr. Rogério Monteiro. My thanks for the availability and unconditional assistance in an extensive part of this thesis and specially for the pleasant hours of conviviality that allowed me, since I was a little child, to learn what science and research were and are, facts that have woken up in me the joy of studying and the joy for the academic Career, which I ended up embracing.

To the working team of Histology Laboratory of Aveiro's University Biology Department through the person of Prof. Dr. Maria de Lourdes Pereira for your friendly hospitality, work space and facilities sharing, not only towards myself but also to all those who collaborated with me.

To the working team of Molecular Biotechnology Laboratory of Aveiro's University Biology Department, through the person of Prof. Dr. Sónia Mendo. My thanks for the always friendly and helping attitude throughout these years of intense and hard work.

acknowledgements (cont.)

To the colleagues from the Estuarine and Marine Ecology Laboratory of Aveiro's University Biology Department, through the person of Prof. Dr. Marina Cunha, not only for the daily knowledge and facilities sharing, but also for the friendship and excellent work environment, which transformed the day to day routine in repeated jubilation.

To my team work colleagues, "collaborators" and students, with whom I had the pleasure to work with. For the never ending dedication and daily demonstration of what true "love to the cause" is, without ever forgetting the fact that we work to live and not the other way around. This way of facing our duties, even with somewhat few resources, led to the always so wanted increase in productivity. But, nevertheless, at this moment looking at "*(…) the sea, I dream without having what to dream with. Nothing in the sea, except being it, is seen.*" (*Fernando Pessoa*), I beg of us not to stop dreaming that this philosophy will one day contaminate the society where we live in, because "*They don't know, or dream, that the dream commands life, that every time a man dreams the world jumps and goes forward (…)*" (*António Gedeão*).

To Dr. Ana Sousa – my "Boss", my Master, my Colleague and my Friend – first, my thanks for the introduction to TBT, *imposex*, scientific articles and the *Reference Manager*! Then, I thank her for endless teachings: several "do it yourself" techniques; what is the *SERA* salt; filters; pumps and water vacuum cleaners. But, and if it wasn't enough, also for having taught me what determination, sacrifice, resilience and persistence are. Thanks for putting up with me for all these years, the sharing of joy, difficulties and frustrations; being a true Friend and, although on the other side of the world, being always present.

To the BSc. Isabel Oliveira, for being my right arm… and my left… but most of all, for being one of my soul mates. A friendship like this one is only acknowledgeable by retribution. And, Beluxa, lets never forget that "*(…) A joy grows from a torment, a smile is always the echo from a lament, who knows where a kiss comes from!*" (*Florbela Espanca*). For everything you are, for the will and consciousness (and for your horoscope, read by some crazy astrologer!) never stop believing and never give yourself to despair… the *kiss* will come… God willing!

To Dr. Niels Jonkers for the *Palm* experience and the nickname; for teaching me how to, not only hear music, but understand music; to feel middle ages and to know foreign poetry; and for waking up in me the courage for change. Thanks for being who you were, are and will always be.

To Ms Joyce Easton, my English Mom. I thank You the hospitality, the concern, the *Pears & Chocolate Crumble* on my bed, the love and all the teachings: from the English accent to loneliness acceptance.

My thanks to Dr. Catarina Aresta for the honest friendship and the constant sharing, never allowing me to forget where I come from and where I want to go to. And Catarinixa "*(…) the only pain that, in life, we can avoid, probably results from the struggle in trying to avoid the pain.*" *(Robin Norwood)*.

acknowledgements (cont.)

To Architect Sara Ventura da Cruz my thanks for all the "plans": for those drawn and already edified (examples that I carefully analyse and learn from) and for those planed for the future (true orientations that I promise to try to follow)! Thank you for crossing my path, for feeding it daily and for being, therefore, the proof that childhood is not always the birth of real friendships. You are "*(…) the rock where I lean on, (…) the Sun that lights my day: no matter what they say about Her Lord, she accepted me and transformed me in what I am today.*" *(Robin Norwood)*. That everyone may acknowledge what I see in you, my Friend: a child heart in all and every woman attitude!

To my bosom Friends, untouchable treasures kept religiously in me, responsible for my emotional balance. Thanks for letting me "*(…) be more than the shadow of a shadow, between so many shadows just like me*" (*Florbela Espanca*).

To my family, my thanks for the unconditional emotional and financial support, for the change of plans in aid of my immediate needs without any interest or requirement, for the love and affection, for believing in my abilities (the ones that I have, the ones that I thought not to have and the ones that in fact I don't have!), for the words, conversations and discussions (which leave a mark but also improve), for all the "clouds and silver linings" and the principles that have always been instilling in me and that I'll blindly follow till the end of my days. Mommy, Daddy, Braddah, Sandra and "*Little Pea*", you are my very precious blessing.

To my "not so bosom friends" (as I think I don't have enemies) for awaken in me an incredible desire to fight ... and win!

And although at this moment in time (i) death and (ii) not quoting *Pessoa* are the only certainties I presently have, here is my thought, as an acknowledgments' conclusion and thesis introduction…

Stones in the way? I'll keep them all.

One day I will build a castle...

palavras-chave

Organoestânicos; Tributilestanho; TBT; *imposex*; biomarcador; *Nucella lapillus*; *Hydrobia ulvae*; bioindicador; costa Portuguesa; OMI;

resumo

Os compostos de tributilestanho (TBT) foram utilizados como biocidas em tintas antivegetativas (AV) e amplamente aplicados, durante décadas, de forma a evitar a bioincrustação em superfícies submersas, principalmente em cascos de embarcações. Os seus efeitos deletérios em organismos não-alvo tornaram-se evidentes após o aparecimento de gastrópodes prosobrâquios com *imposex* – sobreposição de caracteres sexuais masculinos sobre o tracto reprodutivo das fêmeas. Desde então, a expressão do *imposex* em prosobrânquios tem sido amplamente utilizada como biomarcador da poluição por TBT.

Um dos objectivos da presente tese é avaliar se as mais recentes restrições legais na utilização das tintas AV com compostos organoestânicos (OTs) resultaram numa redução da poluição pelos mesmos na costa continental Portuguesa. Para tal, foi levada a cabo a análise da variação temporal do *imposex* como biomarcador dos níveis ambientais de TBT e a validação de procedimentos, de modo a seguir de forma precisa a evolução da intensidade deste fenómeno ao longo do tempo.

O trabalho de investigação teve início no momento em que a ineficácia da legislação anterior (Directiva 89/677/CEE) na redução da poluição por TBT no litoral Português era reportada na literatura e quando estavam já agendados instrumentos decisivos para a diminuição definitiva deste tipo de poluição: o Regulamento (CE) N.º 782/2003 do Parlamento e do Conselho da União Europeia (UE) que bania as tintas AV baseadas em TBT na sua frota a partir de 1 de Julho de 2003; a "Convenção Internacional sobre o Controlo de Sistemas Antivegetativos Nocivos em Navios" (Convenção AFS), adoptada em 2001 pela Organização Marítima Internacional (OMI), que procurava erradicar os OTs da frota mundial até 2008.

Os níveis de *imposex* e as concentrações de OTs nos tecidos de fêmeas de *Nucella lapillus* foram medidos em 17 locais de amostragem ao longo da costa Portuguesa em 2003, a fim de se avaliarem os impactos do TBT nas populações desta espécie e de se criar uma base de dados para seguir a sua evolução futura. O índice da sequência do vaso deferente (VDSI), o índice do tamanho relativo do pénis (RPSI) e a percentagem de fêmeas afectadas por *imposex* (%I) foram utilizados na medição da intensidade deste fenómeno em cada local de amostragem e os seus valores variaram entre 0,20-4,04, 0,0-42,2% e 16,7-100,0%, respectivamente. Foram encontradas fêmeas estéreis em 3 locais de amostragem, com percentagens a variar entre 4,0 e 6,2%. As concentrações de TBT e dibutilestanho (DBT) nas fêmeas variaram respectivamente entre 23-138 e <10-62 ng $Sn.g^{-1}$ de peso seco, e o conteúdo em TBT nos tecidos revelou-se significativamente correlacionado com o *imposex* (nomeadamente com o RPSI e o VDSI). Os níveis de expressão do fenómeno e o conteúdo em OTs nos tecidos, foram superiores na proximidade

resumo (cont.)

de portos, confirmando as conclusões obtidas anteriormente por outros autores de que os navios e a actividade dos estaleiros são as principais fontes destes compostos no litoral Português.

As infra-estruturas associadas ás principais fontes de OTs – portos, estaleiros e marinas – estão geralmente localizadas no interior de estuários, motivo pelo qual estas áreas têm vindo a ser descritas como as mais afectadas por estes poluentes. Por esta razão, foi levado a cabo o estudo pormenorizado da poluição por TBT na Ria de Aveiro, como caso de estudo representativo da poluição por estes compostos num sistema estuarino em Portugal continental. *N. lapillus* foi usada como bioindicador para avaliar a tendência temporal da poluição por TBT nesta área entre 1997 e 2007. Foi registada uma diminuição da intensidade do *imposex* após 2003, embora as melhorias mais evidentes tenham sido observadas entre 2005 e 2007, provavelmente devido à implementação do Regulamento (CE) N.º 782/2003 que proibiu a aplicação de tintas AV com TBT em navios com a bandeira da UE. Apesar desses progressos, as análises ao conteúdo em OTs nos tecidos de fêmeas de *N. lapillus* e em amostras de água colhidas em 2006 indicaram contaminação recente por TBT na área de estudo, evidenciando assim a permanência de fontes de poluição.

A utilização de *N. lapillus* como bioindicador da poluição por TBT na Ria de Aveiro apresenta algumas limitações uma vez que a espécie não ocorre nas áreas mais interiores da Ria e não vive em contacto com sedimentos. Assim, a informação obtida a partir da sua utilização como bioindicador é fundamentalmente relativa aos níveis de TBT na coluna de água. Foi então necessário recorrer a um bioindicador complementar – *Hydrobia ulvae* – para melhor avaliar a evolução temporal da poluição por TBT no interior deste sistema estuarino e estudar a persistência de TBT nos sedimentos. Não foi registada uma diminuição dos níveis de *imposex* em *H. ulvae* na Ria de Aveiro entre 1998 e 2007, apesar da aplicação do Regulamento (CE) N.º 782/2003. Pelo contrário, houve um aumento global significativo da percentagem de fêmeas afectadas por *imposex* e um ligeiro aumento do VDSI, contrastando com o que tem sido descrito para outros bioindicadores na Ria de Aveiro no mesmo período. Estes resultados mostram que a diferente biologia/ecologia das espécies indicadoras determina vias distintas de acumulação de TBT, apontando a importância da escolha do bioindicador dependendo do compartimento a ser monitorizado (sedimento *versus* água). A ingestão de sedimento como hábito alimentar em *H. ulvae* foi discutida como sendo a razão para a escolha da espécie como indicadora da contaminação dos sedimentos por TBT. Foram também estudados os métodos mais fiáveis para reduzir a influência de variáveis críticas na medição dos níveis de *imposex* em *H. ulvae*. As comparações de parâmetros do *imposex* baseados em medições do pénis devem ser sempre realizadas sob condições de narcotização bem

resumo (cont.)

standardizadas uma vez que este procedimento provoca um aumento significativo do comprimento do pénis (PL) em ambos os sexos. O VDSI, a %I e o PL em ambos os sexos revelaram ser fortemente influenciados pelo tamanho dos espécimes: a utilização de fêmeas mais pequenas conduz à subestimação do VDSI, da %I e do PL, enquanto que diferenças no tamanho dos machos provocam variações no índice do comprimento relativo do pénis (RPLI), independentemente dos níveis de poluição por TBT. Existe, portanto, a necessidade de controlar algumas variáveis envolvidas na análise do *imposex* que mostraram afectar a fiabilidade dos resultados.

Uma vez que *N. lapillus* é o principal bioindicador dos efeitos biológicos específicos do TBT para a área da OSPAR, foi também estudada a influência de algumas variáveis na avaliação dos níveis de *imposex* nesta espécie, especificamente as relacionadas com o ciclo reprodutor e o tamanho dos espécimes. O estudo do ciclo reprodutor e a variação sazonal/espacial do comprimento do pénis do macho (MPL) incidiu num único local no litoral Português (Areão – região de Aveiro) de forma a avaliar se o RPSI varia sazonal e espacialmente na mesma estação de amostragem e se tais variações influenciam os resultados obtidos em programas de monitorização do *imposex*. Nos meses de Dezembro de 2005 a Junho de 2007, foram encontrados espécimes de *N. lapillus* sexualmente maturos e potencialmente aptos para a reprodução. Contudo, foi também evidente um padrão sazonal do ciclo reprodutor – o estado de desenvolvimento da gametogénese nas fêmeas variou sazonalmente e ocorreu uma diminuição do volume da glândula da cápsula e do factor de condição no final do Verão / início do Outono. Contrariamente, a gametogénese nos machos não apresentou variação sazonal significativa, embora os valores mais baixos do factor de condição, do comprimento do pénis e dos volumes de esperma e da próstata tenham também sido registados no final do Verão / início do Outono. Além disso, o MPL mostrou variar, no mesmo local de amostragem, inversamente com a distância aos ninhos de cápsulas ovígeras; um aumento dos valores do MPL foi também observado em espécimes de maior tamanho. Todas estas variações no MPL introduzem desvios nos resultados da avaliação do *imposex* quando é usado o RPSI. A magnitude do erro envolvido foi quantificada e revelou-se superior em locais com níveis mais elevados de poluição por TBT. Apesar do RPSI ser um índice que fornece informação importante sobre os níveis de poluição por TBT, a sua interpretação deve ser cuidadosa e realizada complementarmente com os outros índices, destacando-se o VDSI como índice mais fiável e com significado biológico mais expressivo.

Novas campanhas de monitorização do *imposex* em *N. lapillus* foram realizadas ao longo da costa Portuguesa em 2006 e 2008, e os resultados subsequentemente comparados com a base de dados criada em 2003,

resumo (cont.)

de forma a avaliar a evolução da poluição por TBT no litoral Português naquele período. Nestes estudos foram aplicados novos procedimentos na monitorização e tratamento dos dados, de forma a minimizar a variação nos índices de avaliação do *imposex* induzida pelos factores acima descritos, para seguir com maior consistência a tendência da poluição por TBT entre 2003 e 2008. Foi observado um declínio global significativo na intensidade de *imposex* na área de estudo durante o referido período e a qualidade ecológica da costa Portuguesa, segundo os termos definidos pela Comissão OSPAR, revelou melhorias notáveis após 2003 confirmando a eficácia do Regulamento (CE) N.º 782/2003. Não obstante, as populações de *N. lapillus* revelaram-se ainda amplamente afectadas por *imposex*, tendo sido detectadas emissões de TBT na água do mar ao longo da costa em 2006, apesar da restrição anteriormente referida. Estes *inputs* foram atribuídos principalmente aos navios que à data ainda circulavam com tintas AV com TBT aplicadas antes de 2003, uma vez que a sua utilização nas embarcações foi apenas proibida em 2008. Considerando que o Regulamento (CE) N.º 782/2003 constitui uma antecipação da proibição global da OMI que entrou em vigor em Setembro de 2008, prevê-se, por analogia, que haja uma rápida diminuição da poluição por TBT à escala mundial num futuro próximo.

Na sequência desta previsão, é apresentada uma discussão teórica preliminar relativamente ás possíveis estratégias usadas por *N. lapillus* na recolonização de locais onde, no passado, as populações terão aparentemente sido extintas devido a níveis extremamente elevados de TBT. Estes locais são tipicamente zonas abrigadas junto de infra-estruturas portuárias, cuja recolonização por esta espécie será provavelmente muito lenta dada a mobilidade reduzida dos adultos e o ciclo de vida não apresentar fase larvar pelágica. Foram então equacionadas duas hipotéticas vias de recolonização de zonas abrigadas por espécimes provenientes de populações de costa aberta/exposta: a migração de adultos e/ou a dispersão de juvenis. No entanto, em ambos os casos, estaria implicada a transposição de um problema amplamente descrito na bibliografia: as populações de *N. lapillus* de costa aberta podem apresentar um fenótipo muito diferente das de zonas abrigadas, podendo inclusivamente variar no número de cromossomas. A recolonização pode portanto não ter sucesso pelo simples facto dos novos recrutas não estarem bem adaptados aos locais a recolonizar. Para analisar este problema, foram estudados tanto a forma da concha de espécimes de *N. lapillus* ao longo da costa Portuguesa como o respectivo número de cromossomas. Embora a forma da concha tenha revelado diferenças, de acordo com o grau de hidrodinamismo entre as populações de *N. lapillus* avaliadas, o cariótipo 2n = 26, típico de zonas expostas, foi registado em todos os locais amostrados. Por outro lado, foi também testada em laboratório a possível mortalidade de juvenis em dispersão no interior menos salino dos estuários. Foi então verificada a ocorrência de mortalidade elevada de juvenis

resumo (cont.)

expostos a salinidades baixas (=100% após 1 hora a salinidades ≤9 psu), o que também pode comprometer a recolonização de zonas estuarinas menos salinas por esta via. Mesmo assim, os juvenis mostraram um comportamento de flutuação à superfície da água em condições laboratoriais, que pode ser considerado um benefício específico na colonização de áreas mais internas dos estuários, se tal facto vier a ser confirmado em estudos de campo.

As conclusões deste estudo contribuem certamente para a descrição do final da "história do TBT" dado que, uma vez controlados alguns factores determinantes no uso do *imposex* como biomarcador, a avaliação do declínio da poluição por estes compostos, agora esperado à escala global, se torna mais rigorosa.

keywords

Organotins; Tributyltin; TBT; *imposex*; biomarker; *Nucella lapillus*; *Hydrobia ulvae*; bioindicator; Portuguese coast; IMO;

abstract

Tributyltin (TBT) compounds were used as biocides in antifouling (AF) paints and largely applied, during decades, to prevent bioincrustation in submerged surfaces, mainly on vessels hulls. TBT deleterious effects on non-target organisms had only became apparent with the upsurge of prosobranch gastropods exhibiting *imposex* – the superimposition of male sexual characters onto females' reproductive tract. Since then, *imposex* expression in prosobranchs has been largely used as a biomarker of TBT pollution.

One of the objectives of this thesis is to evaluate if the most recent legal restrictions on the use of organotins (OTs) based AF paints resulted in a reduction on the pollution by these compounds in the Portuguese mainland coast. With this purpose, the analysis of *imposex* temporal trend was made, as a biomarker of the TBT environmental levels, as well as the procedures validation aiming to achieve a precise way to track the evolution of this phenomenon intensity over time.

The research work began at the moment when descriptions on the inefficacy of previous measures (Directive 89/677/ECC) in reducing TBT pollution in the Portuguese littoral were reported in the literature and when the most decisive instruments to diminish this kind of pollution were already scheduled: the European Union (EU) Council Regulation (EC) No.782/2003 banning TBT-based AF paints in their fleet from 1 July 2003; the "International Convention on the Control of Harmful Antifouling Systems on Ships" (AFS Convention), adopted in 2001 by the International Maritime Organization (IMO), seeking OTs eradication from the global fleet by 2008.

Nucella lapillus imposex levels and OTs females body burden (b.b.) were surveyed on the Portuguese open coast at 17 sampling sites in 2003 in order to assess the existing TBT impacts in this species populations' and to create a baseline for tracking its future evolution. The vas deferens sequence index (VDSI), the relative penis size index (RPSI) and the percentage of females affected with *imposex* (%I) were used to assess this phenomenon intensity at each site and were 0.20-4.04, 0.0-42.2% and 16.7-100.0%, respectively. Sterile females were found at 3 sites, with percentages varying between 4.0 and 6.2%. TBT and dibutyltin (DBT) females b.b. were 23-138 and <10-62 ng $Sn.g^{-1}$ dry weight, respectively, and TBT was significantly correlated with *imposex* (namely with RPSI and VDSI). *Imposex* levels and OTs in tissues were highest at sites located in the proximity of harbours, confirming ships and dockyard activities as their main sources in the Portuguese coast.

The infrastructures associated with OTs major sources – ports, dockyards and marinas – are usually located within estuaries, reason why these have been described as the most affected areas by these pollutants.

abstract (cont.)

Hence, a detailed study on the TBT pollution in Ria de Aveiro was performed, as a case study representing the pollution caused by these compounds in an estuarine system in Portugal. *N. lapillus* was used as bioindicator to evaluate the TBT pollution temporal trend in this area between 1997 and 2007. A decrease in *imposex* intensity has been registered after 2003. Nevertheless, the most evident improvements were observed between 2005 and 2007, probably due to the implementation of Regulation (EC) No.782/2003 prohibiting AF paints application in vessels carrying EU flags. Despite these progresses, OTs analyses in females' tissues and in water samples carried out in 2006 indicated TBT fresh inputs in the study area, thus evidencing the pollution sources persistency.

The use of *N. lapillus* as bioindicator of TBT pollution in Ria de Aveiro presents some limitations since the species is not present in the Ria innermost areas and does not live in contact with sediments. Therefore, the information obtained from its use as a bioindicator is mostly relative to TBT levels in the water column. It was then necessary to use a complementary bioindicator – *Hydrobia ulvae* – to better evaluate the temporal evolution of TBT pollution within this estuarine system and study the persistency of TBT in sediment. No decrease in *H. ulvae imposex* levels was registered in Ria de Aveiro between 1998 and 2007, despite the application of the Regulation (EC) No.782/2003. On the contrary, there was a global significant increase in the percentage of *imposex* affected females and a slight VDSI raise, contrasting with what has been described for other bioindicators in Ria de Aveiro in the same period. These results show that different biology/ecology traits determine distinct routes of TBT uptake and/or bioaccumulation, pointing the importance of choosing the bioindicator depending on the compartment being monitored (sediment *vs.* water). Sediment ingestion as feeding habit was discussed as the reason for choosing *H. ulvae* as a bioindicator of TBT pollution persistence in sediments. The most reliable methods to reduce the influence of critical variables on *H. ulvae imposex* levels to assess TBT pollution were also studied. Comparisons of *imposex* parameters based on penis measurements should always be performed under well standardized narcotization conditions as the procedure causes a significant increase in both genders' penis length (PL). VDSI, %I and both genders' PL were strongly influenced by specimens' size: smaller females induce VDSI, %I and females' PL (FPL) underestimation, whilst variation in males' size causes oscillations in the relative penis length index (RPLI), regardless of TBT pollution levels. These results prompted the need to control some variables involved in *imposex* analysis that were proved to affect its results reliability.

As *Nucella lapillus* is the main bioindicator of TBT-specific biological effects within the OSPAR area, the influence of those critical variables in this species *imposex* levels assessment were also studied, specifically those

abstract (cont.)

related with the reproductive cycle and specimens' size. The reproductive cycle and the male penis length (MPL) seasonal / spatial variation were studied at a single site in the Portuguese littoral (Areão – Aveiro region) to assess if RPSI varies seasonally and spatially in the same sampling site and if this can affect results obtained in *imposex* monitoring programmes. *N. lapillus* specimens potentially able to reproduce were found every month from December 2005 to June 2007. However, a seasonal pattern in the reproductive cycle was also evident – females' gametogenic maturation varied seasonally and a decrease in capsule gland volumes and condition factor occurred in late summer / early autumn. On the other hand, gametogenesis in males did not show a significant seasonal variation as in females but the condition factor, penis length, amount of sperm and prostate volume also diminished in late summer / early autumn. Besides, MPL has also showed to vary inversely with distance to egg capsules clusters at the same shore; increased MPL values in bigger specimens' were also observed. All these MPL variations introduce a bias on *imposex* assessment results when using RPSI. The magnitude of the error involved was also quantified and is greater at places with higher TBT pollution levels. Despite being an index that provides important information about TBT pollution levels, RPSI interpretation must be careful and accomplished complementarily with other indices, with the VDSI being the most reliable and biologically meaningful.

New monitoring campaigns of *N. lapillus imposex* were made along Portuguese coast in 2006 and 2008 and the results subsequently compared with the baseline created in 2003, in order to evaluate TBT pollution evolution in the Portuguese littoral in that period. New monitoring and data analysis procedures were applied in these studies, in order to minimize the variation in *imposex* assessment indices induced by the above described factors, for a consistent evaluation of the TBT pollution trend in the Portuguese coast from 2003 to 2008. A significant global decline in *imposex* intensity was observed in the study area during the referred period and the Portuguese coast ecological status, under the terms defined by the OSPAR Commission, has notably improved after 2003 confirming the effectiveness of the Regulation (EC) No.782/2003. Nevertheless, *N. lapillus* populations are still extensively affected by *imposex* and fresh TBT inputs were detected in seawater throughout the coast in 2006. These recent inputs are attributed to vessels still carrying TBT antifoulants applied before 2003, as their presence in vessels was only forbidden in 2008. Considering that Regulation (EC) No.782/2003 is an anticipation of the IMO global ban entered into force in September 2008, it is predicted, by analogy, that a rapid decrease of TBT pollution will take place on a global scale in a near future.

Following this prediction, a preliminary theoretical discussion is presented on *N. lapillus* possible strategies to recolonize sites where, in the past,

abstract (cont.)

populations were apparently extinct by extremely high TBT pollution levels. These places are typically sheltered areas near ports infrastructures, which recolonization by this species will probably be very slow since adults have reduced mobility and the life cycle has no pelagic phase. Thus, two hypothetical ways for the recolonization of sheltered areas by specimens from the open/exposed shore were equated: the adults' migration and/or the juveniles' dispersion. However, in both cases, the transposition of a problem widely described in the literature would be implied: *N. lapillus* open shore populations may present a phenotype much different than those at sheltered areas, a difference that may inclusively involve chromosomes number variation. Recolonization can be therefore unsuccessful because of the simple fact that the new recruits may not be well adapted to the site to be recolonized. To this problem analysis, *N. lapillus* shell shape was studied along Portuguese coast as well as the respective specimens' chromosomes number. Although shell shape has revealed differences regarding the hydrodynamics degree between the evaluated populations, the karyotype 2n=26, typical from exposed shores, was registered at all sampled sites. On the other hand, the possible mortality of juveniles in dispersion on the less saline inner estuary was also tested in laboratory. Then, it was verified the occurrence of high juvenile mortality exposed to lower salinity (=100% after one hour under salinity ≤9 psu), what can also compromise recolonization of less saline estuarine areas. Even though, juveniles showed a floating behaviour on the water surface under laboratory conditions, which can be considered as a specific asset regarding colonization of inner estuarine parts, if confirmed with data collected from field studies.

This study conclusions certainly contribute for the "TBT tale" final description, once the control of some factors responsible for the use of *imposex* as a biomarker allows a more thorough evaluation of these compounds pollution, now expected at the global scale.

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Acronyms

%I .... Percentage of *Imposex* Affected Females

%S .... Percentage of Sterile Females

AAS .... Atomic Absorption Spectroscopy

AF paints .... Antifouling paints

AF .... Antifouling

AFS Convention .... International Convention on the Control of Harmful Antifouling Systems on Ships

ASO .... Accessory Sex Organs

AV .... Antivegetativas

BTs .... Butyltins

CEMP .... Co-ordinated Environmental Monitoring Programme

CF .... Condition Factor

CYP19 .... Cytochrome P450-dependent aromatase

DBT .... Dibutyltin

DDT .... Dichlorodiphenyltrichloroethane

DECC .... Distant from egg capsules clusters

EAC .... Ecotoxicological / Environmental Assessment Criteria

EC .... European Community

ECC .... Egg Capsules Clusters

ECCDist .... Distance to Egg Capsules Clusters

EcoQ .... Ecological Quality

EcoQO .... Ecological Quality Objective

EDCs .... Endocrine Disruptor Chemicals

EU .... European Union

FPL .... Females Penis Length

GonadsMat .... Gonads Maturation

IMO .... International Maritime Organization

IOM .... Institute of Medicine

ISI .... Intersex Index

JAMP .... Joint Assessment and Monitoring Program

MBT .... Monobutyltin

MEPC .... Marine Environment Protection Committee

MicMat .... Microscopic Maturation

MicMatStg .... Microscopic Maturation Stage

MPL .... Males Penis Length

NAE............................	National Academy of Engineering
NAS............................	National Academy of Sciences
NECC..........................	Near Egg Capsules Clusters
NIH.............................	National Institutes of Health
NRC............................	National Research Council
OMI............................	Organização Marítima Internacional
OSPAR	Oslo and Paris Convention / Commission
OT	Organotin
OTs paints	Organotins based antifouling paints
OTs.............................	Organotins
PCI	Penis Classification Index
PL...............................	Penis Length
PMF	Penis morphogenic factor
POP............................	Persistent Organic Pollutant
PRF.............................	Penis Retrogressive Factor
PVC	Polyvinyl Chloride
RA	Retinoic Acid
RPLI............................	Relative Penis Length Index
RPSI	Relative Penis Size Index
RXR	Retinoic X Receptor
SexGlVol	Sexual Gland Volume
SH	Shell Height
SpermVolStg..............	Sperm Volume Stage
TBT.............................	Tributyltin
TPT.............................	Triphenyltin
VDS	Vas Deference Sequence
VDSI	Vas Deferens Sequence Index

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Part I

Introduction

Chapter 1

State of the Art

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1.1 TRIBUTYLTIN (TBT) POLLUTION

Pollution and contamination are concepts related to the environmental quality degradation. They are distinguishable by the fact that pollution causes a decrease of resources for humans as, for example, food sources from the aquatic environment (Clark, 1992). In Ecotoxicology, and referring to chemicals, contamination is defined as an artificial increase above the background, whilst pollution implies damage to living resources or risks to human health (Chapman, 1995). Therefore, a pollutant must be a contaminant but not all contaminants are pollutants (Chapman, 2001).

For a compound to be classified as a pollutant relatively to a given organism, the cause-effect relationship must be clearly determined (Clark, 1992). Tributyltin (TBT) compounds are definitely contaminants of the marine and estuarine environments and, because of their adverse effects on natural resources, they can also be considered pollutants.

1.1.1 TBT compounds – applications and history

The term “tributyltin”, as well as its acronym “TBT”, has been quite incorrectly accepted to represent a chemical compound. However, TBT is a chemical moiety, a part of an organotin (OT[a]) compound that comprises 3 *n*-butyl chains covalently linked to a single atom of tin (Sn-C covalent bonds; see Figure 1.1) and not a compound on its own right.

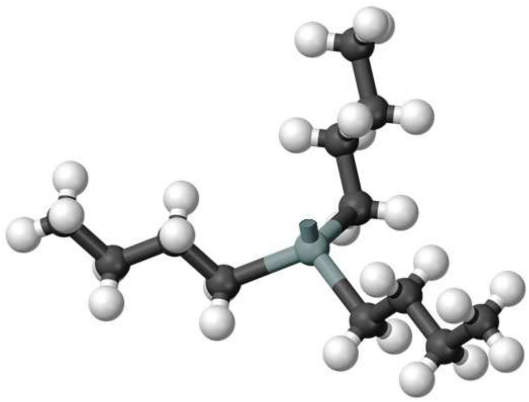


Figure 1.1 The TBT moiety. 3D image adapted after being generated as the “Tributyltin hydride model” by the open source software trial *Accelrys® Discovery Studio Visualizer 2.0*.

The tri-substituted OT species have been described as the most toxic substances ever deliberately introduced into the marine environment by mankind (Goldberg, 1986; Evans et al., 1995; Fent, 1996). TBT compounds act as broad spectrum biocides and, moreover, are endocrine disruptor chemicals (EDCs[b]) with androgenic activity (Matthiessen and Gibbs, 1998; Oetken et al., 2004; Oehlmann et al., 2007).

[a] Organometal characterized by a tin (Sn) atom covalently bound to one or more organic substituent groups (e.g. methyl, ethyl, butyl, propyl, phenyl, octyl) (Hoch, 2001).

[b] Exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations (ICPS, 2002).

Despite being used as polyvinyl chloride (PVC[c]) stabilizers since the 1940s (Appel, 2004; Wilkes et al., 2005), the industrial applications of OT compounds were followed by the recognition of the biocidal properties of their tri-substituted derivatives, essentially TBT and triphenyltin (TPT) compounds, in the early 50's (Bennett, 1996). They were applied in the preservation of wood, textiles and paper as disinfectants, fungicides, insecticides and antibiotics, preventing degradation (Bennett, 1996). Additionally, they were widely used as active ingredients in antifouling (AF) paints which, in this way, constituted TBT compounds' main source to the aquatic environment to date (de Mora, 1996; Yebra et al., 2004). TPT compounds have also been employed as TBT co-biocides in AF paints but its major application lied as fungicides in agriculture (Fent, 1996).

Antifouling paints prevent bioincrustation – unwanted settling and growth of biological materials on surfaces immersed in water – in vessels hulls (Figure 1.2 A), buoys, platforms and other structures (Figure 1.2 B and C) namely related to aquaculture (Yebra et al., 2004).

Figure 1.2 Pictures of marine biofouling on: (A) a ship hull, (B) a propeller of a vessel in dry dock and (C) the submerged portion of an anchor chain.

[c] Thermoplastic resin produced by the polymerization of vinyl chloride; used as an electrical insulator and in many other applications. PVC can be compounded into flexible and rigid forms through the use of plasticizers, stabilizers, fillers and other modifiers (Wilkes et al., 2005).

Bioincrustation, also known as biofouling, has been described for more than 2000 marine species belonging to different taxonomic groups including: diatoms, green algae, sponges, hydroids, crustaceans, molluscs, among others (Alzieu, 1996).

Data from the International Maritime Organization (IMO [d]) indicate that unprotected hulls may gather about 150 $kg.m^{-2}$ of biological incrustation in less than six months at sea (IMO, 1999). Therefore, a supertanker with a submerged area of 40,000 m^2 loads approximately 6,000 tonnes of biofouling in such a short period of time, which leads to a dramatic increase of the water resistance to movement and thus a fuel consumption rise of 40 to 50% (IMO, 1999). Hence, to a vessel owner, an AF system promotes the fuel consumption reduction and an increase in the time between paint repairs in dry dock, a procedure with huge costs and that substantially reduces the vessel operational time.

In the early days of navigation, products as quicklime, brimstone, rosin, pitch, tar and bitumen have been used to protect ships hulls (IMO, 1999; Yebra et al., 2004). Some of them continued to be used but over an extra wood sheath applied to the hull surface. This “sheathing method” allowed easier repairs, by the simple replacement of that last layer, in dry dock and at regular intervals.

Materials as iron, zinc, lead and copper were subsequently tested but, of them, the later performed very well in protecting the hull from biofouling once, in contact with water, it produces a kind of a “poisonous film” mainly of copper oxychloride (Kegley et al., 2008). Furthermore, as this film is slightly soluble, it is gradually washed away, leaving no way in which marine life can attach itself to the vessel submerged surface. Copper salts were the first agents to be integrated into biocidal AF systems and widely used until the 50's. In order to increase their effectiveness, compounds of

[d] Specialized agency of the United Nations with 168 Member States and three Associate Members, established by the Convention adopted in Geneva in 1948; is based in the United Kingdom with around 300 international staff members and the main task of develop and maintain a comprehensive regulatory framework for shipping including: safety, environmental concerns, legal matters, technical co-operation, maritime security and shipping efficiency.

arsenic, mercury and dichlorodiphenyltrichloroethane (DDT[e]) were then introduced in paints formulations (IMO, 1999).

During the 1960's, the chemical industry has produced effective and economic AF paints by using organometallic compounds, particularly the OT known as "TBT" (Crompton, 1997). In the 70's, due to their durability and biocidal efficiency, most of the maritime shipping vessels had their hulls coated with TBT-based AF paints (Yebra et al., 2004).

The first TBT-based AF systems were known as "free association" paints in which a high amount of biocide was dispersed in a matrix that could be insoluble – "contact leaching" paints (Figure 1.3a) – or soluble – "ablative" paints (Figure 1.3b). These coatings lixiviation, by the contact with water, released the active agent by diffusion preventing the fixation of biological material on the submerged area (de Mora, 1996). The initial rate of the biocide release was fast although suffering an exponential decrease to a loss of effect only after 18 to 24 months (IMO, 1999).

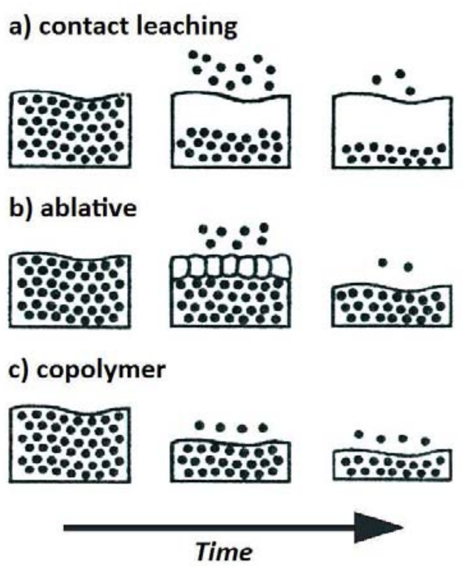


Figure 1.3 Diagram of the three types of TBT-based AF paints by the respective biocide dispersal mode over time: a) contact leaching, b) ablative c) copolymer. Adapted from de Mora (1996).

By the end of the 70's, new formulations were prepared using TBT paints copolymerized with methyl methacrylate (Yebra et al., 2004; Yebra et al., 2006). Thus,

[e] Pesticide first synthesized in 1874 and used in the second half of World War II to control mosquitoes spreading malaria and lice transmitting typhus. After the war, the compound was made available for use as an agricultural insecticide. In the 70s and 80s, and after being classified as a Persistent Organic Pollutant (POP) with damaging impacts to human and wildlife, DDT agricultural use was forbidden in most developed countries and, subsequently, worldwide banned under the Stockholm Convention in 2004, (van der Berg, 2008). Nevertheless, its limited use in disease vector control continues in certain parts of the world and remains controversial.

the leaching rate was controlled: the biocide was gradually released by the alkaline hydrolysis of the painting surface and the reaction only proceeded when the surface layer was depleted, resulting in a constant and relatively slow release of the biocide (Figure 1.3c). These systems reached a maximum life of 60 months (IMO, 1999).

In the marine environment, TBT degradation occurs by biotic and abiotic processes. In both cases, the compound sequential debutylation to its derivatives is involved: dibutyltin (DBT) and monobutyltin (MBT) to inorganic tin (Batley, 1996). The process has shown to be dependant mainly on temperature, organotins (OTs) concentration, salinity and light exposure. These compounds degradation was proved to be accelerated at higher temperature, lower TBT concentrations, higher salinity and under light exposure [see complete reviews on the subject by Batley (1996), Fent (1996), Meador (2000) and Burton et al. (2006)].

Nevertheless, TBT compounds high toxicity has resulted in numerous and widespread adverse biological effects (Oehlmann et al., 2007). Despite some initial scepticism, the problem of “TBT” as “large-scale pollutant” emerged as a result of the impact of public and political publications, specifically: (i) the description of shells malformations and reproductive failure in oysters by Alzieu et al. (1981) and (ii) reports of sexual abnormalities and decreased abundance of prosobranch gastropods in coastal ecosystems (discussed in detail in section *1.1.3.1*).

In the late 70’s, the oysters production at Arcachon Bay (France) was severely affected due to a process known as “shell thickening” or “chambering” – shells irregular calcification by hypersecretion of interlayer gel, overlap of a calcitic layer and disappearance of the gel, resulting in the formation of chambers that gave the shell a “ball shape” (Figure 1.4). Oysters’ shells chambering led to the animal’s body cavity reduction and to a break-down of the local production, with marked economic consequences. After several studies and field observations, the phenomenon was related to the TBT release into water at numerous marinas located in the bay. Besides chambering, TBT also revealed a negative effect on oysters larval growth and survival [see review by Alzieu et al. (2000)].

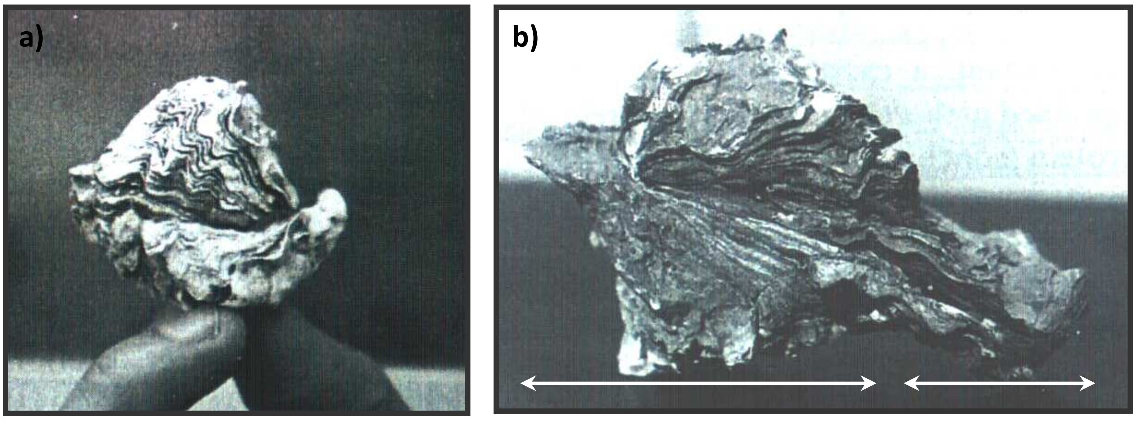


Figure 1.4 *Crassostreas gigas*. TBT effects on oysters: a) acute shells malformation – "ball shape" of a specimen collected in the Bay of Cádiz (Spain); b) shell from the Bay of Arcachon (France) with one type of growth during the TBT exposure period (left white arrow) and another after the TBT-based AF paints prohibition (right white arrow). Adapted from Alzieu (1996).

Oysters shells' thickening was first described in France, then in UK, and few years later in Australia and New Zealand (de Mora, 1996), being the cause of the first legislative measure prohibiting the application of TBT-based AF paints in 1982.

1.1.2 International regulation on the TBT use

Subsequent to the above mentioned descriptions of oysters shell malformations, and to avoid the collapse of the oysters' commercial industry from the Atlantic coast, the first legal restriction on the use of TBT compounds as biocides in AF paints was implemented by the French government in 1982. Such measure banned the use of AF systems with more than 3% of TBT on vessels <25 m in length, in areas of intense oysters' production. This regulation was then extended to the entire coastline, except for aluminium structures, as they would suffer severe corrosion when protected only with copper preparations (Stewart, 1996).

Similar restrictions were subsequently introduced in most industrialized countries, specifically: United Kingdom in 1987; United States and New Zealand in 1988; Canada, Australia, Norway and Japan in 1989/90 (IMO, 2002). In these countries, it was also mandatory to register all TBT-based products as pesticides (Stewart, 1996). These measures led to a certain decline of TBT pollution in water, sediment and molluscs tissues, being accompanied by the recovery from abnormal shell growth in oysters and *imposex* intensity in gastropods at some locations (Gibbs and Bryan, 1994).

Concern on environmental pollution caused by TBT-based AF paints was first raised at IMO's Marine Environment Protection Committee (MEPC) in 1988, when the Paris Commission[f] requested IMO to consider the need for measures under relevant legal instruments to restrict the use of TBT compounds on sea-going vessels (IMO, 2002). The MEPC concluded that, for economic reasons, it was not viable to drastically abolish the use of AF systems with TBT on large commercial vessels (Champ, 1999).

Nevertheless, from the available descriptions and studies on biological effects of TBT on marine ecosystems, there were clear evidences that these compounds were being harmful to aquatic organisms. Even though, by that time, several countries had already restricted its use, it was obvious that international measures would need to be developed.

In February 1988, the European Commission proposed a legal restriction on the marketing and use of OT compounds. Therefore, the Council Directive 89/677/ECC[g] of 21 December amended, for the 8th time, Directive 76/769/EEC[h] on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations. Since then, "organostannic compounds may not be used as substances and constituents of preparations intended for use to prevent the fouling by micro-organisms, plants or animals of: (a) the hulls of boats of an overall length, as defined by ISO (International Standards Organization) 8666, of less than 25 metres; (b) cages, floats, nets and any other appliances or equipment used for fish or shellfish farming; (c) any totally or partly submerged appliances or equipment."

Under this Directive, OTs were also forbidden as substances and constituents of preparations used in industrial waters treatment, irrespective of their use.

[f] International organisation established by Treaty and concerned with the prevention of pollution of the North-East Atlantic. It is now part of the OSPAR (Oslo and Paris) Commission.

[g] *Ref. No. OJ L 398, pp. 19-23.*

[h] *Ref. No. OJ L 262, pp. 201-203.*

In April 1990, the "Third International Organotin Symposium", held in Monaco, recognized the IMO competence to carry out the regulation regarding the use of AF systems. During the same year, in November, MEPC adopted Resolution 46 (30) on "Measures to Control Potential Adverse Impacts Associated with Use of Tributyltin Compounds in Antifouling Paints". This resolution recommended that governments should adopt measures to eliminate the use of TBT-based AF paints on non-aluminium hulled vessels <25 m in length and to forbid the use of AF systems with an average leaching rate >4 $mgTBT.cm^{-2}.day$. These recommendations were intended to be interim measures until IMO could consider a possible large-scale prohibition of TBT compounds on ships (IMO, 2002).

The need for IMO to work on the AF issue was highlighted in the "United Nations Conference on Environment and Development", held in Rio de Janeiro – Brazil, in 1992 (Eco-92 Conference). As a result, a document called Agenda 21 was developed – document that established the importance of each country commitment to reflect, globally and locally, about the way governments, companies, non-governmental organizations and all sectors of society could cooperate in the study of solutions for social and environmental problems – and which Chapter 17 is entitled "States to take measures to reduce pollution caused by Organotin compounds used in Antifouling systems" (IMO, 1999, 2002).

At MEPC 42nd session in November 1998, and after instruction, in April of the same year, of a working group responsible for the layout and timing of the process of both application prohibition and TBT-based AF systems eradication, the success of Agenda 21 was confirmed. United Nations Member States were encouraged to adopt alternatives to AF paints using OTs as biocides, since the MEPC would work to develop a legal instrument to control its harmful effects by the 21st IMO Assembly, scheduled for November 1999 (Figure 1.5). On that date, and based on the MEPC working group Resolution A.895 (21), IMO established a legally binding global ban on the application and re-application of OT-based AF paints from 1 January 2003, and its total abolition from 1 January 2008 (IMO, 2001). The Assembly also approved the holding of a diplomatic conference in 2001 to adopt the proposed instrument.

Figure 1.5 Pictures of some Greenpeace activists' actions, carried out in September and October 1999, calling for a ban on the use of the TBT-based AF paints. Adapted from the organization international website www.greenpeace.org .

At that Diplomatic Conference, on 5 October 2001, MEPC adopted the "International Convention on the Control of Harmful Antifouling Systems on Ships" (AFS Convention; IMO, 2001), a canon aiming both to put an end to the use of OTs in AF paints and to develop mechanisms to prevent the introduction of new hazardous compounds in upcoming alternative formulations. Such document would be effective 12 months after being ratified by 25 countries, whose fleets represent not less than 25% of world's merchant shipping tonnage (IMO, 2001). For the application and re-application prohibition, the effective date was set for 1 January 2003, and the interdiction on the use and circulation, for 1 January 2008. The AFS Convention was opened for signature in 1 February 2002.

Also in 2002, and foreseeing the impossibility of implementing the convention by the scheduled dates, the European Community (EC) announced the intention of anticipate those restrictions in Member States, regardless the document entry into force date. To be precise, the EC intended to prohibit the application of OT compounds on ships flying flags of Member States from 1 January 2003, and the presence of such compounds on ships sailing to or from Member States ports from 1 January 2008. Nevertheless, and until the worldwide eradication, such regulation would be suspended for ships not flying the flag of a Member State, even though it could lead to competitive disadvantages for ship owners and shipyards of the European Union (EU), during the interim period (1 January 2003 to 1 January 2008).

However, such a measure would directly affect the functioning of the OTs internal market, being therefore necessary to approximate the laws of the Member States in this field. Hence, Directive 2002/62/EC[i] of 9 July adapted the already cited Directive 76/769/EEC for the 9th time, calling for a revision of provisions regarding OTs used in AF products (taking full account of developments within IMO and, in particular, the call of their MEPC that had endorsed the prohibition of these biocides in AF systems on ships by 1 January 2003). Specifically, the new Directive point (4) undoubtedly warned that a “regulation of the European Parliament and the Council” would “soon rule vessels bearing organostannic compounds”. EU Member States were compelled to adopt and publish the necessary provisions to comply with the new Directive no later than 31 October 2002 and after the 1st July 2003 obligated by the Regulation No.782/2003[j] from 14 April, to apply the AFS Convention restrictions to their national fleets and to any vessel flying their flags.

Nonetheless, in March 2002, anticipating both AFS Convention and the new EU policy, other countries – Sweden, Netherlands, United Kingdom and Finland – had already announced the cancellation of the production and registration of all TBT-based AF products. The United States had also begun, in 2002, the legal procedures necessary to implement the AFS Convention, so that, once ratified, it could immediately be applied.

[i] *Ref. No. OJ L 183, pp. 58-59.*

[j] *Ref. No. OJ L 115, p. 10.*

Although, in Europe, the application and re-application of these biocides on ships was not allowed from the 1st January 2003, only Denmark (1.24% of the world's merchant fleet tonnage) had completely ratified the AFS Convention by that date.

Afterwards, other countries have followed and, at the MEPC 49th session, in July 2003, five were those for which the convention had been ratified – Nigeria (0.07% of the world's merchant fleet tonnage), West Indies and Barbados (0.81%), Japan (2.53%) and Norway (3.93%) – together accounting for only 8.58% of the tonnage required for the convention worldwide application.

In addition, seven more countries had signed the document, and were pending the ratification as soon as the process of national legislation amendment was finished – Australia (0.33%), Belgium (0.03%), Brazil (0.64%), Finland (0.28%), Morocco (0.51 %), Sweden (0.51%) and USA (1.91%) – whose total contribution, together with the above indicated, would totalize 12.79%, that continued to be insufficient.

Other countries had formally announced the convention signing by the end of 2003 / early 2004 – Greece (4.99%), Spain (0.40%) and Italy (1.68%). United Kingdom (1.05%) and Panama (20.80%) had informally indicated their willingness to ratify the Convention in 2003 (IMO, 2009).

It was therefore clear that the AFS Convention would not enter into force before the end of 2005. By the end of 2006, the document had been ratified by eighteen countries – Antilles and Barbados, Bulgaria, Croatia, Cyprus, Denmark, Greece, Japan, Latvia, Luxembourg, Mexico, Nigeria, Norway, Poland, Romania, Saint Kitts and Nevis, Spain, Sweden and Tuvalu – totalizing 16.15% of the world's merchant shipping tonnage (IMO, 2009).

The AFS Convention entry into force was finally met on 17 September 2007, with the 25th State ratification – Panama – representing a total of 38.00% of the world's merchant shipping tonnage. Accordingly, the international ban on the use of TBT compounds as biocides in AF systems was scheduled for 17 September 2008. One year after that date (September 2009) the document had been ratified by 40 States representing a total of 67.83% of the world's merchant shipping tonnage (IMO, 2009).

Despite the need to verify all this measures effectiveness in reducing TBT pollution, TBT compounds monitoring is also required once they are considered priority hazardous substances in EU legislation namely under the Water Framework Directive 2000/60/EC (establishing a framework for the Community action in the field of water policy) and the Marine Strategy Framework Directive 2008/56/EC (establishing a framework for the Community action in the field of marine environmental policy). This last establishes a framework by which Member States shall take the necessary measures to achieve or maintain good environmental status in the marine environment by the year 2020 at the latest. The EU Environmental Quality Standards (EQS) on the release of hazardous substances into the seas are set by Directive 2008/105/EC and for TBT compounts in surface waters is 0.0015 µg TBT-Sn.l^{-1} (maximum allowed concentration).

The most recent European initiative regarding OTs is the Commission Decision 2009/425/EC of 28 May, restricting their use in consumer articles. Specifically:

(i) Tri-substituted organostannic compounds, such as TBT and TPT, are not allowed after 1 July 2010, when the concentration (in the article or part thereof) is greater than the equivalent of 0.1% by weight of tin;

(ii) DBT compounds are forbidden in mixtures and articles for supply to the general public after 1 January 2012, when the concentration (in the mixture or the article, or part thereof) is greater than the equivalent of 0.1% by weight of tin;

(iii) Dioctyltin compounds shall not be used after 1 January 2012 in textile articles, gloves, footwear, female hygiene products, childcare articles and nappies, when the concentration (in the article, or part thereof) is greater than the equivalent of 0.1% by weight of tin.

However, by way of derogation, points (i) and (ii) shall not be applied until 1 January 2015 to some articles and mixtures for supply to the general public, namely:

silicones, sealants, adhesives, soft PVC profiles, some items paints / coatings containing DBT compounds as catalysts, PVC coatings for outdoor applications containing DBT compounds as stabilisers, outdoor rainwater pipes, gutters and fittings, as well as covering material for roofing and façades.

1.1.3 Adverse effects on living organisms

Molluscs are amongst the most sensitive groups to TBT exposure, and the example of the oysters' shell malformations (mentioned in section *1.1.1*) was just the beginning of a long story. The high toxicity of TBT has resulted in numerous and widespread adverse biological effects to a wide range of organisms: from bacteria to mammals and from the molecular to the community level (some examples are presented in Table 1.1). Nevertheless, in terms of exposure concentrations and sublethal effects, it is now clear that molluscs are the TBT most sensitive taxon, initially due to the compound high uptake rate (Fent, 1996) but mainly because of their weak ability to metabolize TBT and slow rate of elimination via excretory organs (Oehlmann et al., 2007). As a consequence, molluscs attain higher bioaccumulation than other taxa, so that the pollutant exhibits negative impacts at lower environmental concentrations; because of the extended time for tissue residues to reach toxic levels, sublethal responses can also take a long time to develop (Meador, 2000).

Table 1.1 Summary of some ecotoxicological effects of TBT compounds indicated by taxonomic group.

Group	Effect	Important references
Bacteria	• Toxic for bacteria (gram-positive more sensitive);	ICPS, 1990 Mendo et al., 2003
Phytoplankton	• Reduction of marine microalgae growth; • Alteration of photosynthetic pigment content; • Changes in the community structure;	Petersen and Gustavson, 1998 Sidharthan et al., 2002 EC, 2005
Plants	• Impairment of macroalgae spores motility; • Reduction of several marine angiosperms growth; • Stress induction, by bioaccumulation, in terrestrial plants used for human consumption;	ICPS, 1990 Caratozzolo et al., 2007 Azenha et al., 2008 Lespes et al., 2009
Crustaceans	• Reproductive performance reduction; • Neonate survival decrease; • Juveniles growth rate decrease; • Community structure changes;	ICPS, 1990 Waldock et al., 1999 Dahllöf et al., 2001 Takeuchi et al., 2001 EC, 2005 Aono and Takeuchi, 2008
Molluscs	• Abnormal shell growth; • Females virilisation (*Imposex* / Intersex); • Sterility; • Increased mortality; • DNA damage; • Teratogenic effects – embryos malformations; • Community structure alterations;	Alzieu et al., 1981 Bryan et al., 1986 Bauer et al., 1995 Page et al., 1996 Matthiessen and Gibbs, 1998 Gabbianelli et al., 2006 Rank, 2009
Fish	• Growth inhibition; • Females masculinization; • Sperm abnormalities induction and reduced fecundity; • Liver vacuolation; • Teratogenic effects – larvae malformations; • Hyperplasia of the hematopoietic tissue; • Disruption of intracellular energy production by inhibition of ATPase and ion-pump activities; • Neurotoxic through the modulation of the glutamate signalling pathway; • Thymus atrophy and thymocytes apoptosis; • Cytochrome P450 system inhibition;	Fent, 1996 McAllister and Kime, 2003 Shimasaki et al., 2003 EC, 2005 Zheng et al., 2005 Zhang et al., 2008 Zhang et al., 2009 Zuo et al., 2009
Mammals	• Behavioural changes in rats; • Spermatogenesis reduction in mice; • Foetal gonad morphology alterations by changes in gene expression profiles; • Teratogenic effects – embryos malformations; • Inhibition of the basal and calmodulin-dependent Ca^{2+}-ATPase activity in rat brain synaptic membrane preparations; • Inhibition of mitochondrial oxidative phosphorylation or ATP synthesis; • Inhibition of natural killer cell cytotoxic function; • Suppression of osteoclastogenesis trough the retinoic acid receptor (RAR) pathway; • Adipose tissue differentiation rise – obesity induction; • Disturbance of the Ca^{+} homeostasis in human neutrophils; • Human lymphocytes inhibition;	Fent, 1996 Ema et al., 1997 Whalen et al., 2002 Grün et al., 2006 Aluoch et al., 2006 Ohtaki et al., 2007 Kishta et al., 2007 Yonezawa et al., 2007 Antizar-Ladislao, 2008 Chen et al., 2008 Yang et al., 2009 Grün and Blumberg, 2009

1.1.3.1 *The* Imposex *phenomenon*

Imposex is a morphological phenomenon defined as the superimposition of male sexual characters, such as vas deferens and/or penis, onto female's reproductive tract. It was firstly described by Blaber (1970) as the occurrence of a penis-like outgrowth behind the right tentacle in *Nucella lapillus* (L.) females, but the term was coined by Smith (1971) after the same abnormalities were observed in *Nassarius obsoletus* (Say).

Gastropods make use of an assortment of reproductive strategies including: gonochorism (unisex), protandritic hermaphroditism (a male phase precedes a female phase), protygynitic hermaphroditism (a female phase precedes a male phase), reversible hermaphroditism (successive changing between male and female phases), simultaneous hermaphroditism, and parthenogenesis (Sternberg et al., 2009). Nevertheless, *imposex* appears primarily to affect gonochoristic species, namely neogastropods[k] (Tyler-Walters, 2008).

In gonochoric prosobranch species, *imposex* is the main biological effect in response to TBT exposure, which, in extreme cases, can lead to the complete functional sterilization and death of the affected specimens (Schulte-Oehlmann et al., 1997). The apparent unique susceptibility of this taxon to TBT-induced *imposex* is probably a result from the difficulty in identifying this condition in hermaphroditic species (Sternberg et al., 2009).

The link between *imposex* and the exposure to TBT was determined by Smith (1981) a decade after the first description of this phenomenon. This work was then confirmed by Bryan et al. (1987), through a series of laboratory and field experiments

[k] Evolutionarily recent prosobranch gastropods in which individuals are dioecious, that is to say, of distinct sexes (Tyler-Walters, 2008).

using *N. lapillus*, the same working team that ended up proposing the use of this species as a bioindicator of TBT environmental pollution (Gibbs et al., 1987).

The phenomenon has been reported in more than 170 gastropod species (Shi et al., 2005) and has been used worldwide as the specific biomarker of TBT pollution.

Although it is accepted that, under field conditions, the phenomenon is induced almost exclusively by TBT (Oetken et al., 2004), the fungicide TPT has also proven to induce *imposex* in at least five species: *Thais clavigera* (Horiguchi et al., 1997), *Marisa cornuarietis* (Schulte-Oehlmann et al., 2000), *Nassarius reticulatus* (Barroso et al., 2002b), *Babylonia japonica* (Horiguchi et al., 2006) and *Bolinus brandaris* (Santos et al., 2006). Nonetheless the temporal coincidence of the TBT-based AF paints emergence and the first description of *imposex*, the good correlation between *imposex* levels and TBT concentrations in tissues and, especially, some populations recovery after the OT-based AF systems partial ban, strengthen the assumption that OTs have been the dominating cause of *imposex* observed in the field over the last decades (Oehlmann et al., 2007).

The *imposex* development is the result of profound changes in metabolic processes induced by TBT compounds. The mechanism(s) underlying the phenomenon induction and/or development are not fully clarified; however, several hypotheses have been proposed namely through three different pathways: (A) the steroid, (B) the neuroendocrine and (C) the retinoic.

(A) The Steroid pathway – TBT causing imbalance of sex steroid hormones ratios

The most widely referred mechanism for *imposex* induction by TBT was formulated after evidences that *imposex* affected females have consistently exhibited elevated free testosterone titres when compared with the non affected ones (Spooner et al., 1991; Bettin et al., 1996; Barroso et al., 2002b; Gooding et al., 2003; Barroso et al., 2005; Santos et al., 2005). Therefore, the disruption of steroid signalling and physiological balance was proposed as a potential driver for the phenomenon development.

According to the early findings of Spooner et al. (1991), and later experiments of Bettin et al. (1996), TBT may induce *imposex* in prosobranchs by inhibition of the cytochrome P450-dependent aromatase (CYP19), impairing the aromatization of androgens (androstenedione and testosterone) to estrogens (estrone and 17β-estradiol), (Figure 1.6). This theory was sustained by several studies (Barroso et al., 2002b; Santos et al., 2002b; Barroso et al., 2005) and investigations in fish (Zheng et al., 2005) and human cell lines (Heidrich et al., 2001; Saitoh et al., 2001) also confirmed a marked inhibition of CYP19 by TBT.

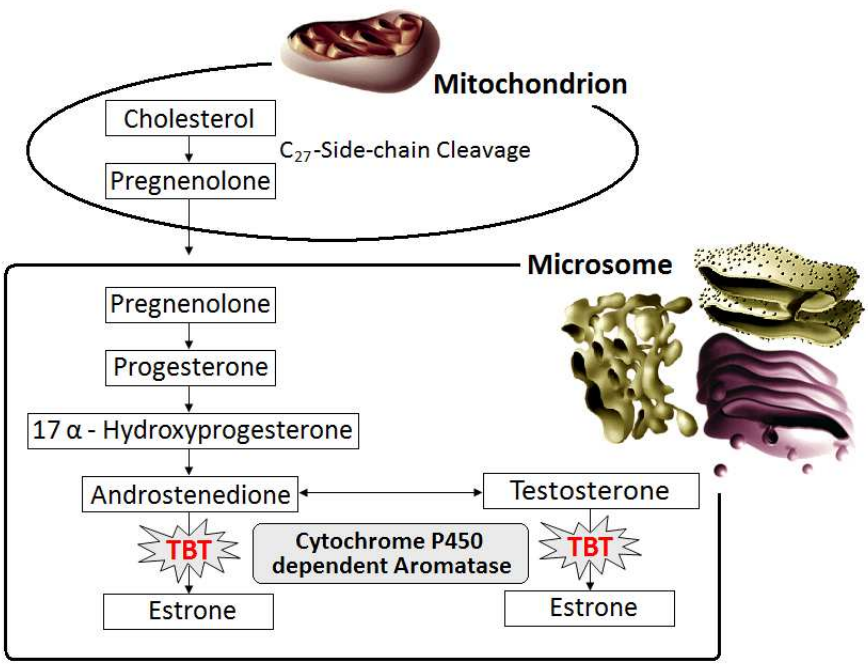


Figure 1.6 Scheme of the biosynthesis of steroid hormones indicating the possible TBT target. Adapted from Bettin et al. (1996).

Even though, several evidences have suggested that the sex steroid biosynthesis disruption might not be the primary mechanism involved in *imposex* development by TBT: (i) although to a lesser extent when compared with TBT, other aromatase inhibitors had also induced *imposex* (Tillmann, 2004; Santos et al., 2005; Horiguchi, 2006; Horiguchi et al., 2008); (ii) elevated testosterone levels were only found in females highly affected by *imposex* (Bettin et al., 1996) and (iii) other chemicals, that are proved to be aromatase inhibitors, had not induced *imposex* in the mud snail *Ilyanassa obsoleta* [McClellan-Green (2003) in Oberdörster et al. (2005)].

Another hypothesis for the imbalance of androgen / oestrogen ratios in *imposex* affected females was raised by Ronis and Mason (1996) considering the possibility of an inhibition of the testosterone excretion. Authors showed that: testosterone metabolization in *Littorina littorea* is made almost completely by water-soluble sulphur conjugates and TBT-exposed specimens retained more unmetabolized testosterone, facts that could lead to an increase of free testosterone levels in tissues. However, extremely high TBT concentrations were tested, during a short period of time (42h) and no reference was made to the endogenous testosterone levels. In addition, Gooding and LeBlanc (2001) showed that the metabolism of testosterone to polar sulphate conjugates is negligible in the *imposex* susceptible mud snail *Ilyanassa obsoleta* and that these organisms conjugate testosterone primarily to non-polar fatty acid esters. Following this research line, Gooding and co-authors (2001; 2003) demonstrated that TBT decreases the esterification of testosterone to fatty acids, leading to an increase in free testosterone, which could then induce *imposex*. These findings were then supported by Santos et al. (2005) for *N. lapillus* and by Janer et al. (2006; 2007) for *Marisa cornuarietis*.

(B) The Neuroendocrine pathway – TBT as toxicant for neuroendocrine factors

The molluscs' reproductive physiology stills poorly understood but it seems to be heavily dependent upon neurohormones, specifically neuropeptides (LeBlanc et al., 1999; Janer et al., 2005; Oehlmann et al., 2007).

The hypothesis that TBT could act as a neurotoxin was raised in the early 80s by Féral and Le Gall (1982; 1983). These authors showed that TBT inhibits the release of a neuroendocrine factor from the pleural ganglia. The factor was called Penis Retrogressive Factor (PRF) and was responsible for the suppression of penis formation in females; thus, inhibiting its release, TBT exposure would result in *imposex* development. However, these authors did not find any effect of TBT on the Penis Morphogenic Factor (PMF) formation – other neuropeptide, expressed in all prosobranch snails irrespective of their sex, responsible for the accessory sex organs

(ASO) initial growth. Some years later, after *imposex* induction in *Ilyanassa obsoleta* by administration of the neuropeptide APGWamide, and since this molecule is normally co-localized in the right pedal ganglia where PMF is thought to be produced, APGWamide was proposed as the PMF in this species (Oberdörster and McClellan-Green, 2000; Oberdörster et al., 2005). Nevertheless, the same working team had also found a reduction in CYP19 activity in TBT-dosed snails (Oberdörster and McClellan-Green, 2002) and an increase in females' penis length in TBT-dosed and testosterone-dosed specimens (Oberdörster et al., 2005). Based on the entire dataset, they concluded that a combination of changes in peptide and steroid hormones might be involved in *imposex* induction. Accordingly, a modulation of steroid and neuropeptides levels had been proposed as the biochemical mechanism for *imposex* (Figure 1.7).

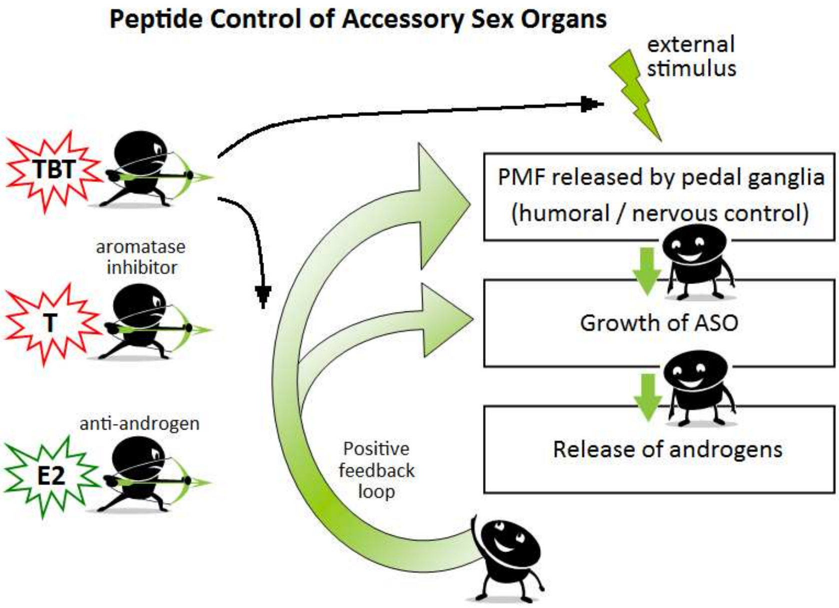


Figure 1.7 Mechanism of *imposex* induction by TBT and steroids. TBT acts in the nervous system, while steroids act on the positive feedback loop which maintains the accessory sex organs (ASO). Adapted from Oberdörster and McClellan-Green (2002). : *imposex* induction; : *imposex* inhibition; TBT: Tributyltin; T: Testosterone; E2: Oestrogen; PMF: Penis Morphogenic Factor.

Therefore, the neurohormone PMF induces initial ASO growth that, in turn, releases androgens (possibly testosterone) to maintain the ASO and spermatogenesis through a positive feedback loop. TBT acts as a neurotoxin that can stimulate the abnormal release of PMF, leading to ASO growth. Exogenously administered steroid hormones and their agonists could either induce or inhibit *imposex* by acting as components in the positive feedback loop. In summary, TBT would stimulate the PMF release inducing *imposex* in females, while testosterone (T) and oestrogen (E2) would act on the feedback loop.

Although this theory was supported by subsequent studies of the same working team (Oberdörster et al., 2005), APGWamide failed to promote *imposex* in some prosobranch gastropods: *Bolinus brandaris* (Santos et al., 2006) and in *Nucella lapillus* (Castro et al., 2007).

(C) The Retinoic pathway – TBT as agonist of Retinoic X Receptor (RXR) homologues

Sex differentiation is a complex process that is regulated by the action of many signalling pathways (Wilhelm et al., 2007). The retinoid signalling has been increasingly implicated in the regulation of male reproductive differentiation and development (Sternberg et al., 2008) and so suspected of being involved in *imposex* induction.

The involvement of the Retinoic X Receptor (RXR) on *imposex* induction mechanism was first raised by Nishikawa et al. (2004). These authors showed that TBT and TPT bind the human RXRs with high affinity and that the injection of 9-*cis* retinoic acid (9CRA), natural ligand of human RXRs, into *Thais clavigera* females induced *imposex*. The same working team has cloned an RXR homologue from *T. clavigera* which revealed a ligand-binding domain very similar to vertebrates RXR and also able to bound to both 9CRA and to OTs. RXR was then identified in the freshwater bloodfluke planorb *Biomphalaria glabrata* (Bouton et al., 2005), the dog-whelk *N. lapillus* (Castro et al., 2007) and the mud snail *I. obsoleta* (Sternberg et al., 2008).

All these findings, confirmed by Horiguchi et al. (2008) for *T. clavigera* and by Castro et al. (2007) for *N. lapillus*, provided the foundation for investigations into whether TBT could act via the signalling pathway involving RXR to induce *imposex*.

Subsequently, a speculative theory of *imposex* induction in *T. clavigera* via RXR was proposed by Horiguchi et al. (2007) referring the activation of a signalling cascade which would be dependent on the RXR activation / inhibition (Figure 1.8). These authors described a significantly higher RXR gene expression in the penises of males and *imposex* affected females than in the normal females penis-forming area (the region behind the right tentacle) and suggested that, for the differentiation and/or growth of the penis and vas deferens in *T. clavigera*, an interaction between OTs and RXR in this limited tissue area is primarily important.

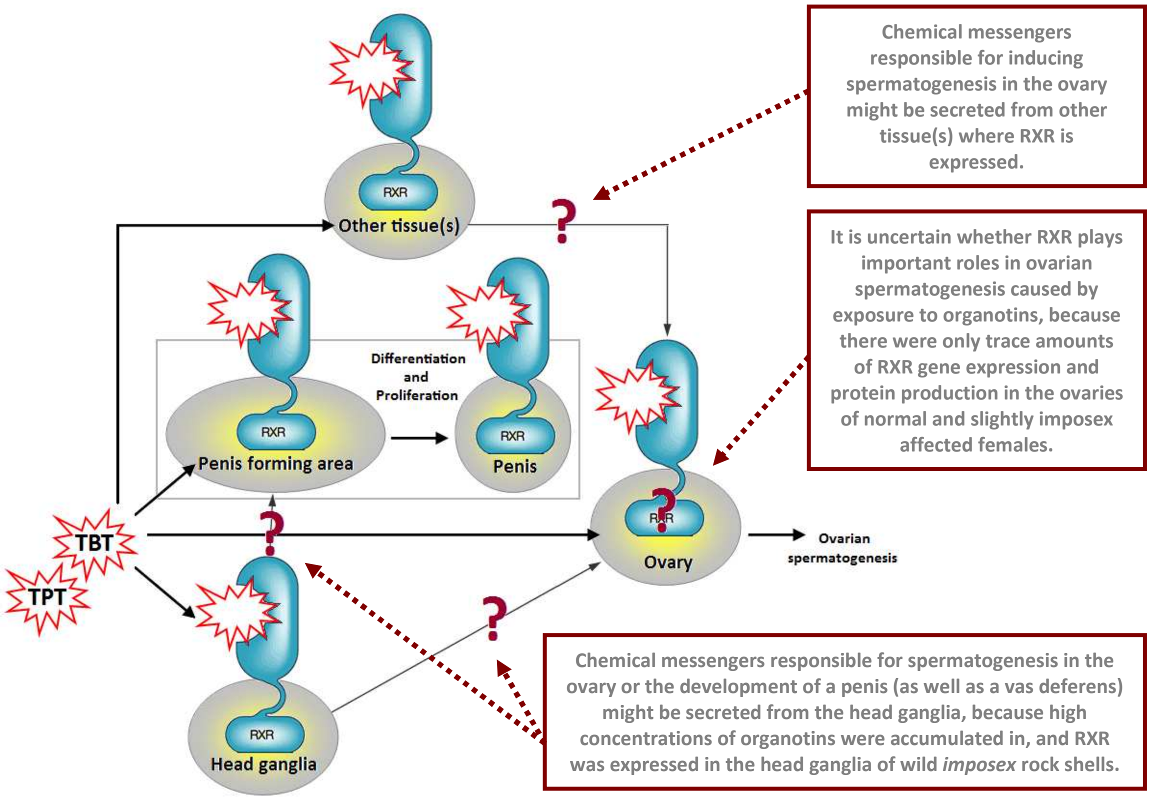


Figure 1.8 Speculative mechanism of *imposex* induction mediated by RXR interacting with TBT or TPT in gastropods. Adapted from Horiguchi et al. (2007).

Furthermore, the involvement of retinoid signalling via RXR in the anomalous development of male sex characteristics in females is strengthened by a study of Sternberg and co-authors (2008) suggesting that RXR-mediated signalling may be an important regulator of sex-specific recrudescence in gastropods, with the timing of RXR expression being critical to sex-specific differentiation. They proposed that early retinoid signalling via RXR may stimulate male recrudescence, whereas later signalling may stimulate female recrudescence and, if so, TBT may induce the development of male sex characteristics in females (*imposex*) by initiating RXR signalling prematurely in females during the temporal gap of normal male recrudescence.

The same working team has recently reviewed what is currently known on environmental and endocrine signals that regulate sexual differentiation / development in gastropods, as well as the known effects of TBT on relevant endocrine parameters in molluscs (Sternberg et al., 2009), and used the information to propose a rational model for the mechanism by which TBT causes *imposex* (Figure 1.9).

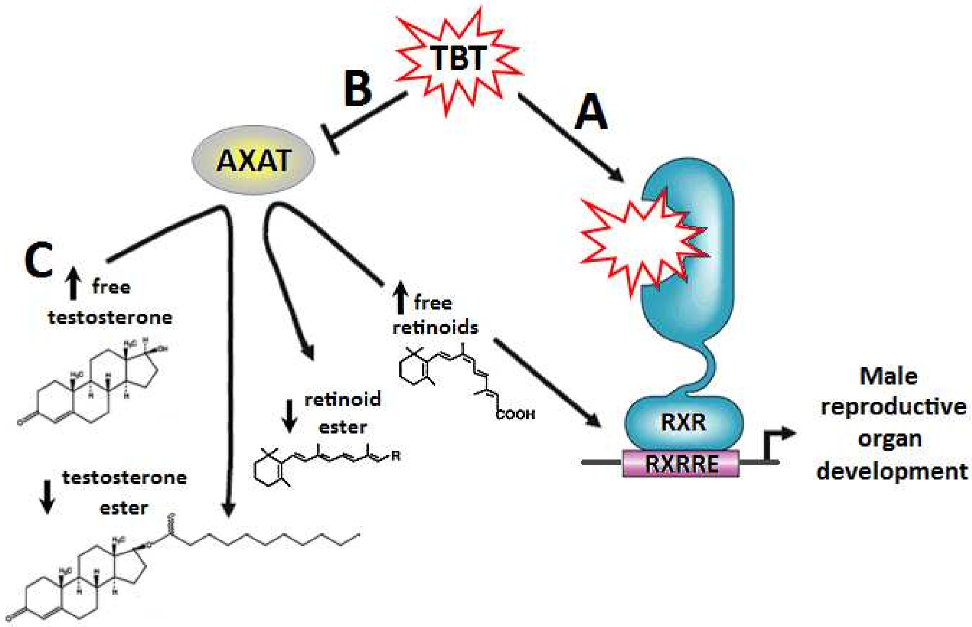


Figure 1.9 Proposed mechanism for *imposex* induction by TBT. Adapted from Sternberg et al. (2009). TBT: Tributyltin; AXAT: Acyl CoA-acyltransferase; RXR: Retinoic X Receptor; RXRRE: RXR Response Elements.

According to this theory, TBT activates the RXR signalling pathway to initiate the transcription of genes necessary for male reproductive organs development: (i) directly, binding to and activating RXR (**A** in Figure 1.9) or (ii) indirectly, by inhibiting acyl CoA acyltransferase (**B** in Figure 1.9), resulting in an increase in endogenous retinoids and testosterone levels. RXR is then activated by endogenous free retinoid that, in turn, stimulates gene transcription through interaction with RXR response elements. Similarly, exogenously-administrated testosterone (**C** in Figure 1.9) competitively inhibits retinoid esterification resulting in elevated endogenous free retinoids, capable of activate the RXR signalling pathway, leading to male reproductive organs development.

Nevertheless, several questions remain unanswered: (i) the exact role of steroid and neurohormones in molluscs' reproductive tract recrudescence; (ii) definite evidences whether retinoids and RXRs are responsible for the initiation or progression of the male reproductive system development and (iii) the natural ligand of RXR in gastropods. As concluded by Sternberg et al. (2009), "(…) only then can the TBT / *imposex* enigma be resolved".

1.2 BIOMONITORING

At the moment, there is an increasing effort to protect the natural environment, including estuaries and marine waters. Estuarine systems and coastal environments are located in territorial waters of neighbouring countries and, therefore, under their legal control and management (Kramer, 1994). Hence, environmental monitoring of these areas has the main objective of support the results of national and/or regional environmental policy.

Environmental monitoring of coastal areas has traditionally involved the analysis of physical / chemical parameters; biological variables were just occasionally considered (as, for instance, the assessment of benthic fauna changes in sediments monitoring; Lam and Gray, 2003).

The consciousnesses that some compounds could affect life on this planet led to the need of detect organic and inorganic micropollutants, in order to determine their geographical distribution, transport mechanisms, possible origin and fate. This detection can be much more valuable the sooner is made, as it can function as an early indication of some adverse biological effects occurrence.

Pollution monitoring, as it runs at present, began in the 70s and had as target the analysis of several ecosystem compartments as water, sediment and biota (Kramer, 1994). Biomonitoring, or monitoring of Biota, has been intensively developed, not only regarding the optimization of methods for the detection of chemicals in organisms (chemical monitoring of Biota) but also defining new parameters and developing new methods to assess the biological effects of many contaminants, namely by the identification and validation of biomarkers.

As can be noticed from the multiplicity of definitions found in the literature, the term "biomarker" has aroused much interest and not only amongst the scientific community. It is applied to a broad range of biological phenomena from normal cellular activity to disease states, toxicant-induced cell and also tissue changes (DeCaprio, 2006). Depending on the primarily focus of one's research, the selection of a working definition of biomarker may vary, as it can be perceived by the following examples:

- To be applied on drugs development, the Biomarkers Definition Working Group of the National Institutes of Health (NIH[I]) defined biomarker as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" (Atkinson et al., 2001);
- In Toxicology, biomarkers are usually defined as "*quantifiable biochemical, physiological or histological measures that relate in a dose- or time-dependent manner the degree of dysfunction that a contaminant has produced*" (DeCaprio, 2006).

Nevertheless, and in environmental monitoring, biomarkers are generally defined as "*quantitative measures of changes in the biological system that respond to either (or both) exposure to, and / or doses of, xenobiotic substances that lead to biological effects*" (Lam and Gray, 2003).

[I] Part of the U.S. Department of Health and Human Services and the primary Federal Agency for conducting and supporting medical research.

Although not explicitly contained in most definitions, the use of the term "biomarker" or "biomarker response" is often restricted to cellular, biochemical, molecular or physiological changes that are measured in cells, body fluids, tissues or organs within an organism. Changes that occur at the individual, population and higher levels of the biological hierarchy are more usually referred as "bioindicators" (Schlenk, 1999; Lam and Gray, 2003). One possible reason for limiting the term "biomarker" to sub-organismic changes is that one of the functions of biomarkers is actually to provide early warning signals of biological effects, and that it is generally believed that molecular, biochemical and physiological responses tend to precede those that occur at the individual or higher levels (Lam and Gray, 2003).

Biomarkers may be used to assess the exposure to / the effect of / the individual susceptibility to a chemical. Hence, in 1987, three main classes of biomarkers were proposed by the Committee on Biological Markers of the National Research Council (NRC[m]) in an attempt to classify their responses as (i) markers of exposure, (ii) of effect and (iii) of susceptibility; (National Research Council, 1987). So specifically we can consider:

(i) *biomarkers of exposure* – exogenous substances, or their metabolites or products of the interaction between xenobiotic agents and some target molecules or cells, that are measured in a compartment within an organism;

(ii) *biomarkers of effect* – measurable biochemical, physiological, behavioural or other alterations within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease;

(iii) *biomarkers of susceptibility* – indicators of an inherent or acquired ability of an organism to respond to the challenge of exposure to specific xenobiotic substances.

[m] NRC functions under the auspices of the National Academy of Sciences (NAS), the National Academy of Engineering (NAE) and the Institute of Medicine (IOM). The NAS, NAE, IOM, and NRC are part of a private, non-profit institution that provides science, technology and health policy advice under a congressional charter signed by President Abraham Lincoln that was originally granted to the NAS in 1863. Under this charter, the NRC was established in 1916, the NAE in 1964, and the IOM in 1970. The four organizations are collectively referred to as the U.S. National Academies.

As more biomarkers have been identified and characterized, it has become apparent that this tripartite classification has significant overlap, in that some biomarkers can be used in each of these capacities (Schlenk, 1999). Although some reviews on the subject were made (for a wider discussion on the topic see DeCaprio, 2006), this classification stills generalized and continue to be applied.

1.2.1 *Imposex* as a biomarker of TBT pollution

Imposex has been extensively recognized as the biomarker of exposure and effect of OTs in prosobranch gastropods (Picado et al., 2007; Hagger and Galloway, 2009).

1.2.1.1 Vantages vs. disadvantages

One of the advantages of using *imposex* as a biomarker of TBT environmental pollution is its specificity to this compound since there's no other definitive description of the phenomenon development by another agent, except for parasitism (Schulte-Oehlmann et al., 1997) and for TPT that occurs at very low environmental concentrations (Barroso et al., 2002a; Sousa et al., 2007; Sousa et al., 2009).

The high sensitivity of biomonitoring as a method to evaluate TBT pollution levels minimizes some of the problems related to the compound chemical determination such as: high variation of TBT levels in water (Galante-Oliveira et al., 2009; Galante-Oliveira et al., 2010) and the fact that the response may be initiated at TBT concentrations below the chemical detection limit (Gibbs and Bryan, 1986; Barroso et al., 2000).

Moreover, the *imposex* expression intensity is related to the TBT exposure concentration, i.e., by the phenomenon quantification it is possible to estimate the regime to which the animal was exposed (Gibbs, 1999). This quantification is of simple observation and determination using classification schemes. It is also a low cost and less time-consuming method, without requiring any sophisticated equipment, which shows the pollutant effects in a given ecosystem from the individual to the community level (Barroso et al., 2000).

However, some disadvantages have also to be referred. At first, *imposex* is an irreversible phenomenon, thus its expression intensity may indicate levels of TBT higher than those that actually occur at the sampling moment. The method limitations are also extended to observation: it has to be carried out on living animals, without being preserved / frozen (Minchin and Davies, 1999a).

There is also evidence of *Hydrobia ulva*e females showing signs of masculinization before TBT environmental pollution first descriptions (Schulte-Oehlmann et al., 1997). Krull (1935) and Rothschild (1938) described the presence of non functional small penises in *Hydrobia* specimens infested by parasites, although Schulte-Oehlmann et al. (1997) reported only a small increase of parasitism in *imposex* affected females when compared with non affected ones. These authors defend that, although the presence of parasites promotes females' masculinization, is not its only cause, because if it was, parasites must be observed at 100% of the *imposex* affected females. Therefore, for *imposex* analysis in the context of biomonitoring programs, parasitized specimens must be discarded.

1.2.1.2 Indicator species used in the current work

Imposex occurrence was already described in more than 170 gastropods species, representing 28 families, around the world (Shi et al., 2005). Many of them were used as bioindicators of TBT environmental levels by the quantification of the exhibited masculinization degree. The following are some of the species that have been proposed and validated in Europe: family Muricidae – *Nucella lapillus* (L.) (Bryan et al., 1986; Gibbs et al., 1987), *Ocenebra erinacea* (L.) (Gibbs et al., 1990; Gibbs, 1996) and *Ocinebrina aciculata* (Lam.) (Oehlmann et al., 1996); family Hydrobiidae – *Hydrobia ulvae* (Pennant) (Schulte-Oehlmann et al., 1997; Schulte-Oehlmann et al., 1998; Galante-Oliveira et al., 2010); family Littorinidae – *Littorina littorea* (L.) (Bauer et al., 1995; Bauer et al., 1997); family Buccinidae – *Buccinum undatum* (L.) (Hallers-Tjabbes et al., 1994; Mensink et al., 1996); family Nassaridae – *Nassarius* (*Hinia*) *reticulatus* (L.) (Stroben et al., 1992; Bryan et al., 1993; Barroso and Moreira, 1998; Barroso et al., 2002b; Rato et al., 2006), *Nassarius* (*Hinia*) *incrassatus* (Ström) (Oehlmann et al., 1998) and *Nassarius (Hinia) nitidus* (Jeffreys, 1867) (Rodríguez et al., 2009).

The indicator species should be abundant and native at the region being investigated and more than one species can be used. One example of species combination for TBT pollution monitoring was proposed by Barroso et al. (2000). These authors recommend the use of *N. reticulatus* and *H. ulvae imposex* levels to evaluate TBT pollution at moderately to highly polluted estuarine sediments, due to their complementary distribution regarding salinity, substrate type and geographical ubiquity along the European coastline.

In the current work two bioindicators are used: (A) *Nucella lapillus* and (B) *Hydrobia ulvae*.

(A) *Nucella lapillus*

To monitor TBT pollution biological effects in *N. lapillus*, the following parameters are recommended: mean females penis length (FPL), relative penis size index (RPSI), vas deferens sequence index (VDSI), percentage of *imposex* affected females (%I) and percentage of sterile females (%S), (Gibbs et al., 1987).

RPSI is calculated by the formula [FPL^3 / mean male penis length $(MPL)^3$] x100 and was proposed by Gibbs et al. (1987) to compare *imposex* expression in different *N. lapillus* populations. The *imposex* development can be followed by the vas deferens sequence (VDS) and its intensity quantified by using VDS classification schemes. In *N. lapillus*, VDS classification comprises 7 stages (from 0 to 6, increasing females' masculinization; see Figure 1.10) and two distinct pathways of completing the phenomenon development (A- and B-path).

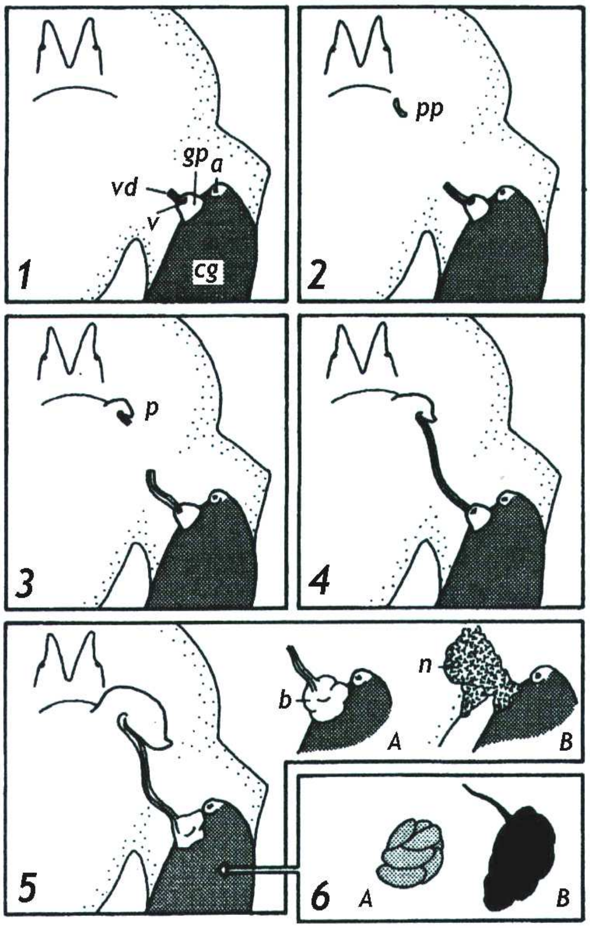


Figure 1.10 *Nucella lapillus.* Vas deferens sequence (VDS) classification scheme proposed by Gibbs et al. (1987) composed of 7 different stages: from *0* – normal female (not present in the figure) to *6* – sterilized female with aborted egg capsules mass inside the capsule gland. Stage 0 is not schematized. a – anus, b – "blister", cg – capsules gland; gp – genital papilla; n – "nodule"; p – penis; pp – penis primordium; v – vulva; vd – vas deferens. Adapted from Gibbs et al. (1987).

Stage 6 corresponds to the phenomenon complete development, i.e., a continuous vas deferens from the genital papilla to the penis base, which overgrowth covers the genital papilla and blocks the vulva, sterilizing the female and resulting in an accumulation of aborted egg capsules inside the capsule gland (Gibbs et al., 1987). The distinction between A and B paths is made since the vas deferens tissue proliferation, overgrowing the genital papilla (VDS stage 5), can form either blisters-like protuberances around the papilla (A-path) or nodules of hyperplasic tissue (B-path). Generally, *imposex* development in this species finalizes by the A-path (VDS stages 5 and 6). Moreover, among all the specimens sampled along the NW Portuguese mainland coast and analysed in the present work, a single case of VDS stage 5B was observed.

The morphological changes during *imposex* development in *N. lapillus* are described in Table 1.2.

Table 1.2 *Nucella lapillus.* Morphological changes during the *imposex* development, indicated by vas deferens sequence stage (VDS). For a schematic visualization see Figure 1.10.

VDS	Morphological changes
1	→ penis absent → formation of the vas deferens proximal section (commencing on the genital papilla)
2	→ penis primordium emergence → penis duct absent → development of the vas deferens proximal section towards the penis primordium
3	→ penis enlargement → penis duct formation → development of the vas deferens distal section at the penis base and towards the proximal section
4	→ penis enlargement to a size approaching that of the male → vas deferens completely formed from the penis base till the genital papilla (proximal and distal sections are now fused)
5	→ penis enlargement to a size approaching that of the male → vulva blockage by the excessive proliferation of the vas deferens tissue overgrowing genital papilla
6	→ penis enlargement to a size approaching that of the male → aborted egg capsules material forming a mass inside the capsules gland lumen as a consequence of the vulva occlusion

In *N. lapillus* the *imposex* development is initiated at TBT concentrations in water <1 ng TBT-Sn.l^{-1} (Bryan et al., 1987). Therefore, when compared with other species, as for instance *H. ulvae*, *N. lapillus* shows higher sensitivity to TBT (Barroso et al., 2000).

(B) *Hydrobia ulvae*

Schulte-Oehlmann et al. (1998) confirmed that *imposex* indices are significantly correlated with the TBT accumulation in *H. ulvae* tissues, a process that occurs at concentrations in water ≥20 ng TBT-Sn.l^{-1}. The following *imposex* assessment indices were recommended for the use of *H. ulvae* as bioindicador: FPL, relative penis length index (RPLI), VDSI and %I (Schulte-Oehlmann et al., 1997).

RPLI is calculated by the formula (FPL / MPL) x100, being therefore an adjustment made to the above referred RPSI. This later index was described to express *N. lapillus* female penis volume / size (and not only the length) as a percentage of that of the male of the same population and is calculated by (FPL^3 / MPL^3) x100 since the penis in this muricid is voluminous. However, *N. lapillus* specimens are examined without narcotization, a procedure that induces the tissue distension, being more pronounced in males and resulting in the RPSI underestimation. Although this effect is also observed in *H. ulvae*, RPLI is subjected to less variation being therefore indicated for species which specimens are observed after a narcotization period, or that have been cryopreserved (Gibbs, 1999).

Imposex intensity in *H. ulvae* is also quantified through a VDS classification scheme although comprising 5 stages, from 0 – "unaffected female" to 4 – "vas deferens completely developed" (Figure 1.11).

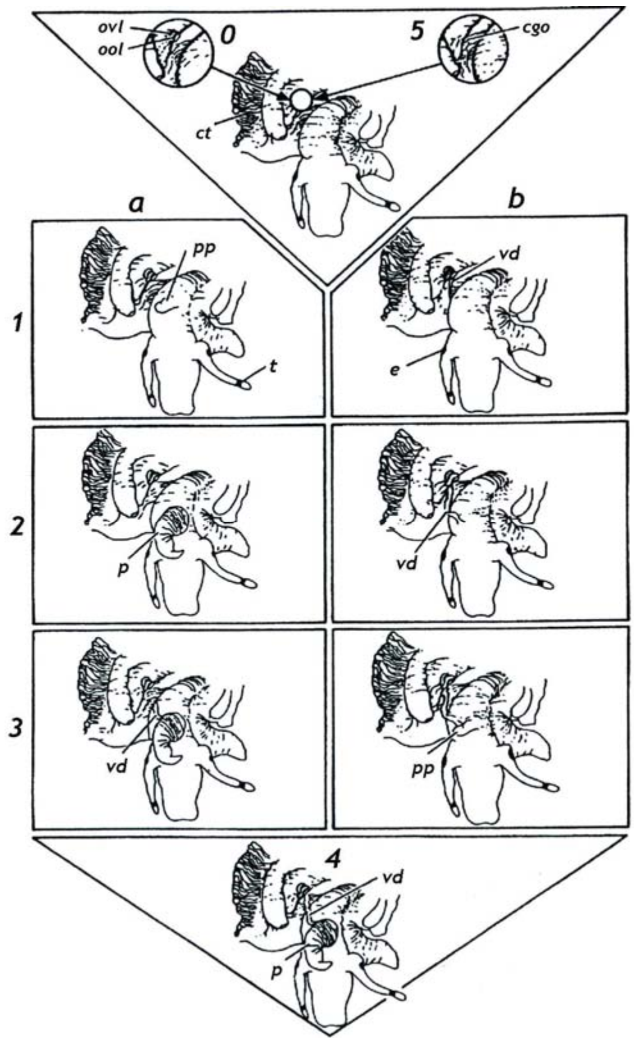


Figure 1.11 *Hydrobia ulvae*. Vas deferens sequence (VDS) classification scheme proposed by Schulte-Oehlmann et al. (1997): from *0* – normal female to *4* – female with a complete vas deferens. Stage *5* is also considered and represents a sterilized female without any males' sex characters. cgo – closed genital openings; ct – ctenidium; e – eye; ool – oviparous channel orifice; ovl – vaginal channel orifice; p – penis; pp – penis primordium; t – tentacle; vd – vas deferens. Adapted from Schulte-Oehlmann et al. (1997).

Stage 4 corresponds to the phenomenon complete development, i.e., a continuous vas deferens connects the penis base and the female genital orifices (Schulte-Oehlmann et al., 1997). As in *N. lapillus*, the gradual increase in *H. ulvae* females' masculinization can occur by two distinct pathways (see Figure 1.11) but, in this case, these paths are different ways of the process development from its beginning: in a-type females the penis is firstly formed and then the vas deferens gradually appears (by Stage 3a); while in b-type females a vas deferens portion initiates the process which is completed with a penis arise and enlargement (in Stage 3b) and the gradual extension of the vas deferens. In Table 1.3 are described the morphological changes occurring at each VDS stage following the a-path, the only observed in the NW Portuguese coast (Barroso et al., 2000; Galante-Oliveira et al., 2010).

Table 1.3 *Hydrobia ulvae*. Morphological changes occurring at each vas deferens sequence (VDS) stage during the a-path *imposex* development. For a schematic visualization see Figure 1.11.

***a* - path**	
VDS	**Morphological changes**
1	→ penis primordium emergence → penis duct absent → vas deferens absent
2	→ penis enlargement → penis duct formation → vas deferens absent
3	→ penis enlargement to a size approaching that of the male → penis duct present → formation of a distal vas deferens section at the penis base
4	→ penis enlargement to a size approaching that of the male → vas deferens completely formed (connecting the penis base with the female orifices)

Although *H. ulvae* masculinization does not always lead to female sterility, other pathological conditions can develop morphological changes in female genital openings. In this species, the morphological appearance of the genital orifices is extremely diverse: high VDS stage females may exhibit opened genital orifices while females' genitalia without any signs of *imposex*, or very low levels, may show occlusion of the genital openings. Thus, in addition to the above indicated 4 stages, stage 5 represents the sterilized female without any male sex characters (Schulte-Oehlmann et al., 1997). However, the *imposex* development, and the consequent proliferation of the vas deferens tissue, may also lead to the oviparous / vaginal openings occlusion, but in general this situation occurs in b-path specimens. In these cases, sterility occurs at the beginning of masculinization process and individuals are also classified as VDS stage 5. In addition, when females present closed genital orifices by the initial vas deferens formation and its development is a VDS stage from 1 to 4, the value 5 is added to that stage (from a minimum of 6 to a maximum of 9) indicating a determined VDS stage although with genital openings occlusion (Schulte-Oehlmann et al., 1997). Nevertheless, even knowing that *H. ulvae* females can be sterilized by *imposex*, there is no evidence of a complete sex change in this species induced by this phenomenon development, as it was already described for the muricid *N. lapillus* (Oehlmann et al., 1991).

Schulte-Oehlmann et al. (1998) suggested that the sample size should be defined according to the *imposex* levels locally recorded: at highly contaminated areas (≈80% of affected females) it may be of 30 individuals, i.e., identical to the proposed for *N. lapillus* by Oehlmann (1994); at less contaminated sites (≈10% of affected females) an increased number of specimens should be analysed (>80 per sample) since the indices values variability increases as *imposex* expression decreases thus affecting results significance.

In general, the bioaccumulation of noxious compounds depends on the exposure and/or uptake types and rates. The TBT accumulation can occur by different ways: directly from water / sediment and by food, or after mobilization from contaminated sediments (Fent, 1996; Meador, 2000). Knowing that, the simultaneous use of *H. ulvae* and *N. lapillus* to monitor TBT pollution might be advantageous since the hydrobiid is directly dependent on sediments (Jackson, 2008) and the muriciid is a rocky shore inhabitant dependent on water (Crothers, 1985).

As recommended by OSPAR[n] Joint Assessment and Monitoring Program (JAMP[o]), *N. lapillus* is the elected species to monitor TBT environmental concentrations[p]. Is a widely distributed species, which life cycle development occurs within a restricted area

[n] Current legal instrument guiding international cooperation on the protection of the marine environment of the NE Atlantic. OSPAR Convention started in 1972 with the Oslo Convention against dumping; was broadened to cover land-based sources and the offshore industry by the Paris Convention of 1974. These two conventions were unified, up-dated and extended by the 1992 OSPAR Convention (OSPAR, 2009).

[o] Strategy adopted in 2003 at the OSPAR Commission Ministerial Meeting to prepare and produce a series of thematic assessments, leading to the next comprehensive Quality Status Report 2010.

[p] *OSPAR Ref. No: 2003-10 Technical Annex 3.*

(adults show reduced mobility and development takes place without a planktonic larval stage; Crothers, 1985); in addition, specimens are easily distinguishable at rocky shores where are inhabitants, fact that facilitates sampling, and are relatively resistant under laboratory conditions.

However, *N. lapillus* is absent from areas of low to intermediate salinity. Another disadvantage as a bioindicator is the species high longevity (from 5 to >10 years; Crothers, 1985). As *imposex* is largely irreversible and its intensity is declining, it is possible that *N. lapillus* populations can only show any masculinization reduction after several years (Gibbs, 1999), when older (and most affected) specimens are replaced by younger ones, exposed to lower TBT concentrations.

Finally, while at less contaminated sites and in a pollution reduction scenario *N. lapillus* high sensitivity to TBT constitutes an argument for its use as an indicator species, at severely polluted sites almost all females had possibly been sterilized, during the period of intense TBT-based AF paints usage and highest *imposex* incidence, fact that had certainly led to the species extinction near hotspots.

H. ulvae complements *N. lapillus* monitoring since: it is widely distributed in areas of middle, upper and riverine estuary; and longevity is ≈2 years, thus sampling in an annual basis can certainly show decreases in *imposex* intensity if they do occur, as the selected animals (adults of 1.5 to 2 years) are annually replaced by others, recruited in the previous year (Silva, 2002).

1.2.1.3 OSPAR Commission guidelines

Work under the OSPAR Convention is managed by the OSPAR Commission, made up of representatives of 15 Contracting Parties Governments and the European Commission, representing the European Union (OSPAR, 2009).

OSPAR Commission is responsible for the protection of the marine environment of the NE Atlantic and in 1994 agreed, for assessment purposes, to divide the NE Atlantic into five regions (I-V) which continue to serve as the geographical basis for future regional assessments (Figure 1.12).

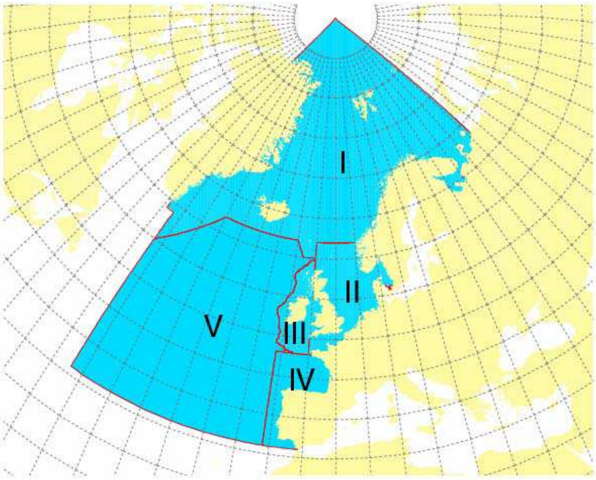


Figure 1.12 Geographical sectioning of the NE Atlantic by the OSPAR Commission: Region I – Arctic Waters; Region II – Greater North Sea; Region III – Celtic Seas; Region IV – Bay of Biscay and Iberian Coast; Region V – Wider Atlantic. Extracted from OSPAR (2009).

TBT compounds are on the OSPAR List of chemicals for priority action[q]. Monitoring OTs environmental concentrations and their biological effects are mandatory elements of the OSPAR Co-ordinated Environmental Monitoring Programme (CEMP[r]).[s]

The OSPAR Commission has adopted the JAMP Guidelines for monitoring contaminant-specific biological effects[t] and, specifically, the guidance to monitor TBT-specific biological effects (*imposex* / intersex) in some gastropod species: *Nucella lapillus*, *Nassarius reticulatus*, *Buccinum undatum*, *Neptunea antiqua* and *Littorina littorea*[u]. Inevitably, other species than *N. lapillus* had to be included since, as referred in section *1.2.1.2*, the dog-whelk is not present in some locations (e.g. the Baltic Sea or in many areas of the southern North Sea) and so, at least one other widespread and abundant bioindicator is required. Also arising through the CEMP, OSPAR has therefore developed the assessment criteria for use in assessing monitoring data on TBT-specific biological effects (Table 1.4).[v]

[q] *OSPAR Ref. No: 2002-18.*

[r] Monitoring program under the OSPAR, where the national contributions overlap and are co-ordinated through adherence to commonly agreed monitoring guidelines, quality assurance tools and assessment tools (instituted by OSPAR JAMP). It covers temporal trend and spatial monitoring programmes for concentrations of selected chemicals and nutrients, and for biological effects.

[s] *OSPAR Ref.[s] No.[s]: 2002-20 / 2005-5 and 2008-8.*

[t] *OSPAR Ref. No: 2003-10.*

[u] *OSPAR Ref. No: 2003-10 Technical Annex 3.*

[v] *OSPAR Ref. No. 2004-15.*

Table 1.4 Criteria for the assessment of TBT-specific biological effects prepared and agreed during the "The Hague technical TBT workshop, 6-7 November 2003". Overview of the six assessment classes (A-F) defined for the gastropods considered during the workshop as alternatives to *N. lapillus* as an indicator of TBT pollution. These assessment classes were adopted by OSPAR as provisional JAMP assessment criteria for TBT.[w] EAC – Ecotoxicological Assessment Criteria; VDSI – vas deferens sequence index; PCI – penis classification index; ISI – intersex index.

Assessment class	***Nucella***	***Neptunea***	***Nassarius***	***Buccinum***	***Littorina***
Criterion	**VDSI**	**VDSI**	**VDSI**	**PCI**	**ISI**
A Level of *imposex* close to zero	<0.3	<0.3	<0.3	<0.3	<0.3
B Level of *imposex* (≈30 to ≈100% of affected females) indicates exposure to TBT < EAC	0.3 – <2.0	0.3 – <2.0	<0.3	<0.3	<0.3
C Level of *imposex* indicates exposure to TBT > EAC	2.0 – <4.0	2.0 – 4.0	0.3 – 4.0	0.3 – 4.0	<0.3 – <0.7
D Populations reproductive capacity is affected as a result of the sterile females presence (but some capable females remain)	4.0 – 5.0	May occur beyond 4.0	May occur beyond 4.0	May occur beyond 4.0	0.7 – 2.0
E Populations are unable to reproduce; The majority, if not all females within the population, have been sterilized	5.0 – 6.0				>2.0
F Populations are absent / expired	-				

The criteria are indicated for *N. lapillus* as measured by the VDSI and are alongside with the equivalent VDSI / penis classification index (PCI) / intersex index (ISI) values for the alternative species. These relationships between species were estimated from correlations obtained by comparing *imposex* expression, in the field, in sympatric populations. This system enables consideration of the likely effects on *N. lapillus* based on effects in other species and allows the adoption of a consistent approach over the whole OSPAR region.[w]

The TBT-specific biological effects assessment criteria have been developed taking into account: (i) the existing OSPAR Ecotoxicological Assessment Criteria (EAC) for TBT in water, sediment and biota[x]; (ii) the objectives of the OSPAR Hazardous Substances

[w] *OSPAR Ref. No: 2004-15.*

[x] *OSPAR Ref. No: 1997-15.*

Strategy[y]; and (iii) the development, within OSPAR, of an Ecological Quality Objective (EcoQO) for *imposex* in dog-whelk *N. lapillus*[z].

This EcoQO is defined as a desired level of Ecological Quality (EcoQ) and was developed within OSPAR since the EcoQ reference level achievement is under the Commission duties – to protect and preserve the overall expression of the structure and function of the marine ecosystem, taking into account the biological community, natural factors and conditions, including those resulting from human activities.[aa] Accordingly, the reference level of EcoQ is the one to which the anthropogenic influence on the ecological system is minimal.[aa]

The justification for the development of this EcoQO for *imposex* in the dog-whelk is that it is particularly sensitive to TBT and so it would measure the effectiveness of the international agreements to phase out and prohibit the use of TBT-based AF paints, and the recovery progress of the marine environment from TBT presence.[z] Consequently, the EcoQO was developed assuming that the average *imposex* level, in a sample ≥10 females, should be consistent with the exposure to TBT concentrations below the EAC derived for TBT, that is to say <2.0 as measured by the VDSI. Where *N. lapillus* does not occur naturally, or where it has become extinct, the red whelk (*N. antiqua*), the whelk (*B. undatum*), the netted dog-whelk (*N. reticulatus*) or the periwinkle (*L. littorea)* should be used, with an exposure criteria on the same (or a correspondent) index of <2.0 (in *Neptunea*) and <0.3 (in *Littorina*, *Nassarius* and *Buccinum*).

In 2006, OSPAR adopted the agreement Ref. No: 2006-4 on the application of the EcoQO system in the North Sea and, in 2008, the "North Sea Pilot Project on EcoQO" first results were considered encouraging and extremely useful. The OSPAR Hazardous Substances Committee had considered the EcoQO an appropriate system to assess the

[y] *OSPAR Ref. No: 2003-21.*

[z] *OSPAR Pub. No: 247/2005.*

[aa] *HELCOM Ref. 2002 Doc. 5/2.*

link between pressure, implementation of measures (both OSPAR measures and the IMO AFS Convention) and impacts, since such impacts could be directly linked to the use of TBT.[bb]

To conclude, monitoring taking into account the EcoQO on *imposex* in dog-whelks is a mandatory commitment of Contracting Parties under the CEMP and should be carried out in accordance with Technical Annex 3 of the JAMP Guidelines for contaminant-specific biological effects monitoring.[cc]

After some considerations regarding the pilot project results, a simplified version of the above indicated Table 1.4 was presented by CEMP: a colour code was assigned to assessment classes aiming to ease, in any graphical representation, the recognition of the locations where the EcoQO was achieved (Table 1.5).[dd]

Table 1.5 Coloured scheme for the TBT-specific biological effects assessment classes in dog-whelks and other gastropods. In this scheme, green means that the OSPAR EcoQO on *imposex* is met. However, it should be taken into account that the EcoQO only applies to the species in the white columns.

Assessment class	***Nucella***	***Nassarius***	***Buccinum***	***Neptunea***	***Littorina***
	VDSI	**VDSI**	**PCI**	**VDSI**	**ISI**
A	< 0.3	< 0.3[1]	< 0.3[1]	< 0.3	< 0.3[2]
B	0.3 - <2.0			0.3 - <2.0	
C	2.0 - < 4.0	0.3 - <2.0	0.3 - <2.0	2.0 - <4.0[3]	
D	4.0 - 5.0	2.0 – 3.5	2.0 - <4.0		0.3 - < 0.5
E	>5.0[4]	> 3.5[4]	4.0[4]		0.5 - 1.2
F					> 1.2

[1] This species cannot be used to distinguish between class A and B. The assessment class is therefore by definition B.

[2] This species cannot be used to distinguish between classes A, B and C. The assessment class is therefore by definition C.

[3] This species cannot be used to distinguish between class C and higher classes. If a VSDI = 4.0 is reached, additional observations are required to determine the assessment class e.g. by using another species. If a VDSI = 4.0 is observed, the assessment class is by definition F.

[4] These species cannot be used to distinguish between classes E and F. Therefore, additional observations are required to determine the assessment class e.g. by using another species. If the VDSI (*Nassarius*) or the PCI (Buccinum) is >3.5, the assessment class is therefore by definition F.

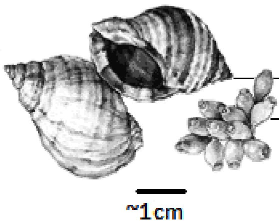
[bb] *OSPAR Ref. SIME 08/5/8-E(L).*

[cc] *OSPAR Ref. No: 2003-10.*

[dd] *OSPAR Pub. No: 378/2008.*

1.3 *Nucella lapillus* SISTEMATICS AND BIOLOGY

Kingdom **Animalia**
Phylum **Mollusca**
Class **Gastropoda** (Cuvier, 1795)
Subclass **Prosobranchia** (Milne-Edwards, 1848) / **Caenogastropoda** (Cox, 1960)
Order **Hypsogastropoda**
Suborder **Neogastropoda**
Superfamily **Muricoidea** (Rafinesque, 1815)
Family **Muricidae**
Subfamily **Ocenebrinae** (Cossmann, 1903)
Genus ***Nucella*** (Röding, 1798)
Species ***Nucella lapillus*** (Linnaeus, 1758)



(Gofas, 2009b)

N. lapillus is common on rocky shores and can be found in both sheltered and heavily wave exposed areas. Occurs within a salinity range from 18 to 40 psu and between 0 and 20^{0}C isotherms, throughout the North Atlantic littoral zone: from the Arctic to the south of Portugal in the east, including Iceland and the Faroe Islands, and from the south west of Greenland to the north of Long Island in the west (Crothers, 1985; Tyler-Walters, 2008) (Figure 1.13).

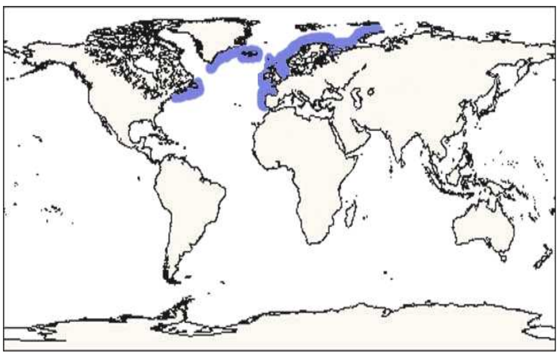


Figure 1.13 *Nucella lapillus*. Worldwide species distribution. Extracted from Tyler-Walters (2008).

The shell is conical and solid, usually of 17 to 47 mm in height (but may reach up to 60 mm), bearing spiral ridges and consisting of a short pointed spire dominated by the last whorl (Crothers, 1985; Tyler-Walters, 2008). Shell is very variable regarding colour – white, grey, yellow, brown, sometimes presenting spiral banding – and size, depending on the hydrodynamic exposure regime: at sheltered areas the growth rate is usually higher and so the shell is generally more elongated; though, at greatly exposed regions, the shell is smaller and more robust (Crothers, 1985). This morphological variation has been attributed to the species limited dispersal capacity once *N. lapillus* life cycle development does not include a planktonic larval phase and adults only show a crawling movement over short distances (Gibbs, 1999).

Similarly to the other neogastropods of the family Muricidae, *N. lapillus* specimens are active carnivorous, predators, which diet is mainly constituted by mussels (*Mytilus spp*) and barnacles (*Balanus spp.*), although they can also use some other bivalves and gastropods as preys (Crothers, 1985).

A rigorous method for determining the age of *N. lapillus* specimens stills undefined. Even so, some authors had suggested longevity from 5 to 10 years although, in sheltered areas, it might be superior (Gibbs, 1999).

Sexual maturity must occur within 2.5 years (Fretter and Graham, 1994a). Individuals can breed throughout the year but, in some places, reproduction is restricted to a few months (Gibbs, 1999). At those regions, spawning is associated with an agglomerative behaviour occurring from spring to early summer and involving 30 or more adults. In the winter, those spawning aggregates are difficult to distinguish since animals of all ages / sizes are grouped and may remain at the same location for 4 or 5 months (Tyler-Walters, 2008) exhibiting no significant movement, neither with feeding purposes (Crothers, 1985).

Similarly to *H. ulvae*, *N. lapillus* is a dioecious species with internal fertilization: the sperm is transferred to the female during copulation and the eggs are fertilized within the oviduct. Groups of ≈600 eggs are encapsulated by secretions produced in an organ known as the capsule gland. At the end of the process, egg capsules cross the oviduct and remain connected to the ventral pedal gland, where are shaped and hardened. *N. lapillus* egg capsules are vase shaped, of about 8mm high, usually yellow.

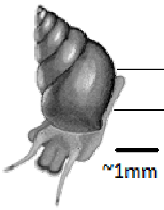
Under favourable conditions, masses of hundreds of capsules are deposited on hard substrata surface, in crevices and under overhangs (Figure 1.14).

Figure 1.14 *Nucella lapillus*. Pictures of specimens and egg capsules from the Portuguese mainland shoreline: A and B – adults laying egg capsules, forming clusters in a rock crevice (at Aveiro seashore); C – capsule recent laid under laboratory conditions (specimen collected at Nazaré seashore); D – recently hatched juvenile (at Aveiro seashore).

After 3 to 4 months, 15 to 30 juveniles emerge from the capsule apex (Figure 1.14D). Meanwhile, remained eggs are consumed providing a food source for the developing juveniles, being thus called “nurse-eggs” (Gibbs, 1999). Hence, there is no planktonic larval phase in *N. lapillus* life cycle: juveniles emerge from capsules as adults miniatures. One year old animals have approximately the adult shell height, being only recognized by a thinner shell outer lip that can become thickened and internally toothed with age (Crothers, 1985; Gibbs, 1999).

1.4 *Hydrobia ulvae* SISTEMATICS AND BIOLOGY

Kingdom **Animalia**
Phylum **Mollusca**
Class **Gastropoda** (Cuvier, 1795)
Subclass **Prosobranchia** (Milne-Edwards, 1848) / **Caenogastropoda** (Cox, 1960)
Order **Hypsogastropoda**
Suborder **Littorinimorpha** (Golikov and Starobogatov, 1975)
Superfamily **Rissooidea** (Gray, 1847)
Família **Hydrobiidae** (Simpson, 1865)
Genus ***Hydrobia*** (Hartmann, 1921)
Species ***Hydrobia ulvae*** (Pennant, 1777)



(Gofas, 2009a)

H. ulvae is an estuarine species, widely distributed in sandy and muddy banks of the tidal zone. Usually occurs at high densities, reaching 300,000 individuals per m^2 (Queiroga, 2003). These specimens can live and reproduce under water salinity between 5 and 40 psu. The species is widely distributed: through the Atlantic coasts, English Channel, North Sea, Baltic and Mediterranean (Jackson, 2008). *H. ulvae* feeding preferences are green algae – *Ulva* and *Enteromorpha* [Nicol (1935) in Fretter and Graham (1994b)] – as well as diatoms and bacteria that individuals graze from the sediment surface. For that, individuals exhibit both passive and active migratory capacity in the water column: they can be passively transported by other organisms or floating objects, especially algae (Queiroga, 2003), or enter the water column actively by a specific vertical migratory pattern (Figure 1.15).

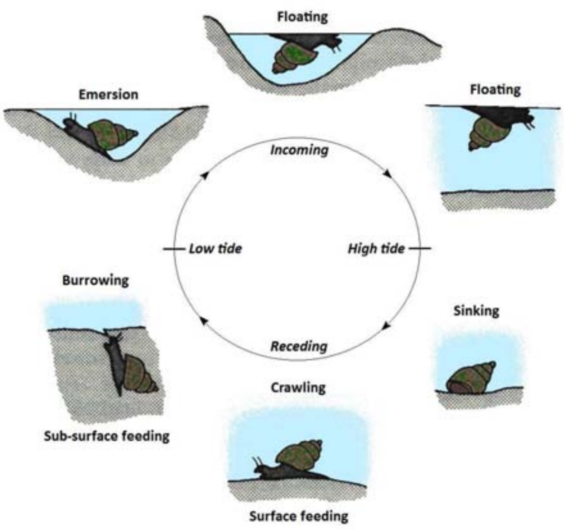


Figure 1.15 *Hydrobia ulvae.* Schematic representation of the vertical migration in the water column during one tide cycle proposed by Newell (1962). Adapted from Barnes (1974).

This behaviour was described by Newell (1962 in Barnes, 1974): at the low tide, snails crawl on the sediment surface, feeding on detritus and leaving characteristic marks on substrate; about two hours later, animals burrow in the sediment surface layer, burying themselves perpendicularly to the surface (with this orientation individuals feed on sub-surface material); during the incoming tide, organisms emerge and, by one of several mechanisms, rise in the water column and start to float on the water surface (upside down); this movement is eased by mucus secretion which also functions as a way of capture food particles during this phase and till high tide is achieved; during the receding tide, animals sink into the water column, reaching the sediment and completing the cycle. This behaviour was tested under laboratory conditions, confirming that *H. ulvae* is able to use all habitat compartments for their own benefit: sediment, interfaces sediment / air and sediment / water and water column (Barnes, 1974).

Although some authors consider that *H. ulvae* does not have a regular migration pattern (Jackson, 2008), their local floating capacity has also been interpreted as an important mechanism for the species dispersion (Armonies, 1992; Armonies and Hartke, 1995). Anderson (1971) had also observed juveniles (shell height <2 mm) floating on the surface and in different directions in the Scottish Ythan Estuary, using water flows, tides and currents. Armonies (1992) had described a high mobility of *H. ulvae* juveniles in summer – 98% of the floating individuals had shell height <2 mm – in the North Sea. Armonies and Hartke (1995) suggested that *H. ulvae* dispersion favours the exploration of new food sources, particularly at sites where the population density is very high.

H. ulvae is a dioecious species and has internal fertilization. Eggs diameter is of approximately 70 to 90 μm (Fretter and Graham, 1994b) and are laid in gelatinous masses known as egg capsules, often deposited on the shells of live individuals of the same species (Fish and Fish, 1974), on empty shells, over other animals or algae and even on sand grains (Anderson, 1971).

Geographic variability regarding the eggs laying period seems evident: in some places was described as occurring throughout the year (Fish and Fish, 1974) while in other areas the spawning period is annual (in spring) or bi-annual (in spring and autumn) (see Anderson, 1971).

Larval hatching occurs in a period of 8 to 31 days after spawning as a veliger [Stopford (1951) in Fish and Fish (1977)].

There are considerable conflicting evidences over the mechanism of larvae development in this species: Fish and Fish (1977) have found the planktonic stage to last up to four weeks and development to be entirely planktotrophic in animals from the Dovey estuary, however these results were obtained under laboratory conditions; in southwest England natural populations, Pilkington (1971) has found the planktonic stage to be completely absent with a non-feeding benthic larvae that metamorphoses after just two days of hatching.

Larval settlement occurs to a shell height of around 300 μm (Fish and Fish, 1974). *H. ulvae* maximum growth periods are spring and/or summer and a marked reduction, or even stop, is observed during winter (Sola, 1996).

The species growth rate seems to be unpredictable: at the end of the first year of life an organism can be 2 mm height (Anderson, 1971; Fish and Fish, 1974), 3 to 3.5 mm (Planas and de Mora, 1987) or 4 mm (Sola, 1996). Likewise, the age at which the animal reaches sexual maturity is also variable: in some places, maturation is reached during the first year of life (Bachelet and Yacinekassab, 1987), whereas in others, occurs after 2 years (Fish and Fish, 1974). Through several results it might be suggested that sexual maturation should occur during the first year for individuals whose growth rate is high and with longevity no longer than 2 years (Silva, 2002).

1.5 THE PORTUGUESE MAINLAND COAST

Coastal margins are the transition zone between land and sea. Its morphology reflects historical changes of seawater average level, currents, human activity, riverine discharges, coastal transport and sediment deposition.

The Portuguese mainland coast is oriented north to south along the 9^{o} W meridian in average, and around 40^{o} N latitude, except between latitudes 38.4^{o} N (Espichel Cape) and 39.4^{o} N (Peniche) where the land advances about 20 km westward (Figure 1.16). North of the Tagus estuary, the Estremadura rocky shoreline is the European western point, above which sandy rectilinear beaches receive direct northwest Atlantic ripple (Sauvaget et al., 2000).

The country continental shelf is relatively narrow (50 km in average) and steep bordered. Between the Espichel Cape and Peniche, the shelf widens forming the Tagus Plateau, which is delimited by two canyons: Setúbal on the south and Nazaré on the north. These are the coastline main irregularities and are proved to influence the ocean dynamics (Fortunato et al., 2002).

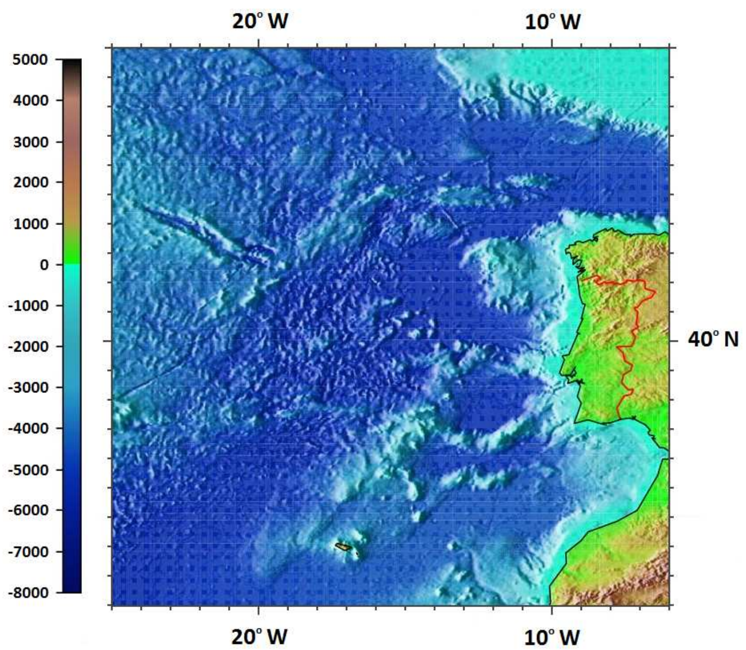


Figure 1.16 Bathymetry of Portugal and adjacent Atlantic Ocean seafloor. Adapted from Bischof et al. (2003).

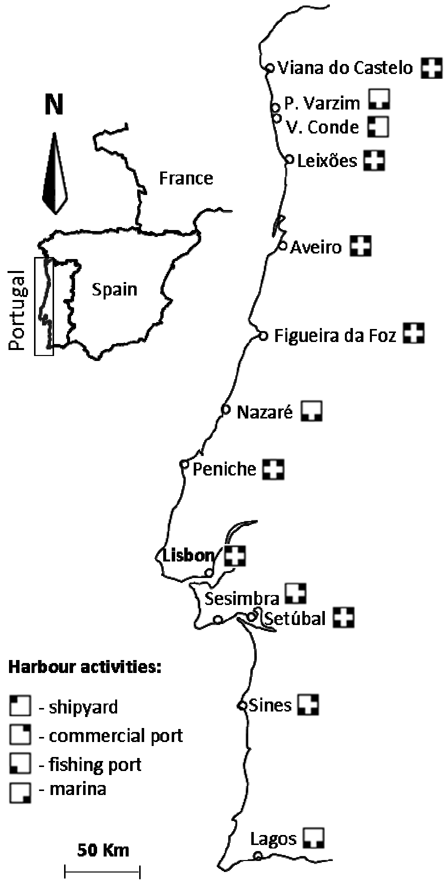
Together with Spain, Portugal forms the Iberian Peninsula. The country have around 787 km of Atlantic coast, most of it turned to the west (660 km), and the remainder (127 km) south.

Near 65% of the Portuguese territory is crossed by International River basins, whose sources are in Spain and estuaries are in Portugal – Lima, Douro and Tagus – or at common borders – Minho and Guadiana. In fact, about 46% of the Iberian Peninsula consists of basins shared between the two countries (A. Bordalo e Sá, *pers. com.*). Nevertheless, in the Portuguese territory, 13 estuaries and coastal lagoons with permanent access to the sea are registered. Eight of them are located geographically in the north – Minho, Cávado, Lima, Ave, Douro, Ria de Aveiro, Mondego and Tagus – and the rest in the south – Sado, Mira, Arade, Ria Formosa and Guadiana (Figure 1.17).

Figure 1.17 Portuguese inland hydrographical net. Adapted from INAG (2009).

Estuaries and coastal lagoons are interface systems between land and sea, having a main function in the transport regulation between the two environments. This interface position makes these ecosystems rich and diverse, also supporting important economic activities: from fishery and aquaculture to ports and dockyards, also including an assortment of industry facilities. For these reasons, estuarine water quality assessments should consider not only waste water effluents, and whatever discharged in as a result of many local activities, but also transferring phenomena between land, atmosphere and ocean (Kramer, 1994).

1.5.1 TBT pollution sources



Estuaries have been described as the TBT most polluted areas (Barroso et al., 2000; Santos et al., 2000; Santos et al., 2002a; Galante-Oliveira et al., 2006; Sousa et al., 2007; Rato et al., 2008; Galante-Oliveira et al., 2009; Sousa et al., 2009; Galante-Oliveira et al., 2010). At first, because AF paints were identified as these compounds major source for the aquatic environment (de Mora, 1996) and then, as it happens in Portugal, because estuaries and natural embayments usually enclose TBT pollution hotspots – the most important harbours, marinas and dockyards (Figure 1.18).

Figure 1.18 Map of the Portuguese continental coast indicating main shipping infrastructures location.

Concern about TBT pollution in estuaries was not only due to the input from vessels which had circulated and remained anchored at these sensible areas, leaching the compound from their AF systems during decades. Another problem is the TBT capacity to adsorb to particles, which contributed for its persistence in sediments (Langston and Pope, 1995; Ruiz et al., 2008). In the water column and in sediments surface layer (oxygenated), TBT half-life is estimated to be of 6 to 7 days at 28^{o}C (de Mora, 1996). However, in deeper anoxic layers, TBT degradation is much slower, with reported half-lives between 1.9 and 3.8 years (Batley, 1996). This extended degradation period in sediments, together with the wide-ranging and constant input by the TBT-based AF paints circulation during decades, implied that sediments have been acting as reservoirs, carrying on the compound slowly release (Langston and Pope, 1995; Burton et al., 2006; Ruiz et al., 2008).

Moreover, and in addition to those main harbours indicated in Figure 1.18, Portugal has also a number of smaller maritime infrastructures related to: (i) the recognition of the country as a nautical tourism destination and (ii) the fact that artisanal fishing activities continued to the present. Accordingly, several marinas and yachting docks, ports of refuge and minor anchorage areas for small fishing boats can be observed along the coast (Table 1.6).

Table 1.6 List of smaller maritime infrastructures and respective location (by coordinates indication confirmed using the open source software *©2009Google™earth*) along the Portuguese mainland coast (IPTM, 2009): (a) marinas and yachting harbours, (b) ports of refuge and (c) artisanal fishing boats moorings.

(a) Marinas and yachting harbours		*(b) Ports of refuge*		*(c) Fishing boats moorings*	
Name	**Coordinates**	**Name**	**Coordinates**	**Name**	**Coordinates**
Freixo riverine marina	08° 34' W 41° 08' N	Caminha	08° 50' W 41° 52' N	Portinho de Neiva	08° 49' W 41° 37' N
Angra do Douro marina	08° 28' W 41° 04' N	V. P. Âncora	08° 52' W 41° 48' N	Aguda breakwater	08° 39' W 41° 02' N
Ovar marina	08° 39' W 40° 51' N	Esposende	08° 47' W 41° 32' N	Espinho	08° 38' W 40° 59' N
Torreira marina	08° 42' W 40° 45' N	Douro	08° 38' W 41° 08' N	Porto das Barcas	09° 20' W 39° 13' N
Gramata marina	08° 44' W 40° 37' N	S. Martinho do Porto	09° 08' W 39° 30' N	Cascais port	09° 25' W 38° 41' N
Costa Nova marina	08° 45' W 40° 36' N	Foz do Arelho	09° 13' W 39° 25' N	Porto das Barcas	08° 46' W 37° 43' N
Bruxa marina	08° 44' W 40° 36' N	Berlengas	09° 30' W 39° 24' N	Portinho do Canal	08° 47' W 37° 44' N
Ribeira marina	09° 22' W 39° 21' N	Ericeira	09° 25' W 38° 57' N	Lapa das Pombas	08° 48' W 37° 38' N
Cascais marina	09° 25' W 38° 42' N	Portinho d'Arrábida	08° 59' W 38° 28' N	Entrada da Barca	08° 47' W 37° 33' N
Oeiras marina	09° 19' W 38° 40' N	Porto Côvo	08° 47' W 37° 50' N	Azenha do mar	08° 47' W 37° 27' N
Bom Sucesso marina	09° 13' W 38° 41' N	Portinho do Canal	08° 47' W 37° 44' N	Armona	07° 50' W 36° 59' N
Belém marina	09° 12' W 38° 41' N	Arrifana	08° 52' W 37° 17' N	Fuzeta	07° 48' W 37° 01' N
Santo Amaro marina	09° 10' W 38° 42' N	Baleeira	08° 55' W 37° 00' N		
Fontaínhas marina	08° 55' W 38° 30' N	Alvor	08° 35' W 37° 07' N		
Tróia marina	08° 54' W 38° 29' N	Albufeira	08° 15' W 37° 04' N		
Portimão recreatinal dock	08° 31' W 37° 07' N	Armona	07° 50' W 36° 59' N		
Albufeira marina	08° 15' W 37° 04' N	Fuzeta	07° 48' W 37° 01' N		
Vilamoura marina	08° 07' W 37° 04' N	Tavira	07° 37' W 37° 06' N		
Quarteira marina	08° 06' W 37° 04' N	Faro	07° 55' W 55° 00' N		
Faro recreational dock	07° 55' W 37° 01' N				
Olhão recreational dock	07° 50' W 37° 01' N				
Tavira recreational dock	07° 38' W 37° 07' N				
Guadiana marina	07° 24' W 37° 11' N				

Furthermore, maritime commercial transport in the Portuguese Exclusive Economic Zone (EEZ) is an important component of the international transportation system and several shipping corridors are contained in (Figure 1.19) being daily used by about 200 ships (CNADS, 2001; Delfaud et al., 2005; INE, 2009). Of these sea-lanes, the one considered as the most important runs parallel to the coastline and connects the Mediterranean, the North of Europe, Africa and America (Figure 1.19). It is positioned dangerously near the coast and was always considered a threat to coastal areas regarding environmental pollution (Delfaud et al., 2005; Portuguese Law Decree No.198/2006).

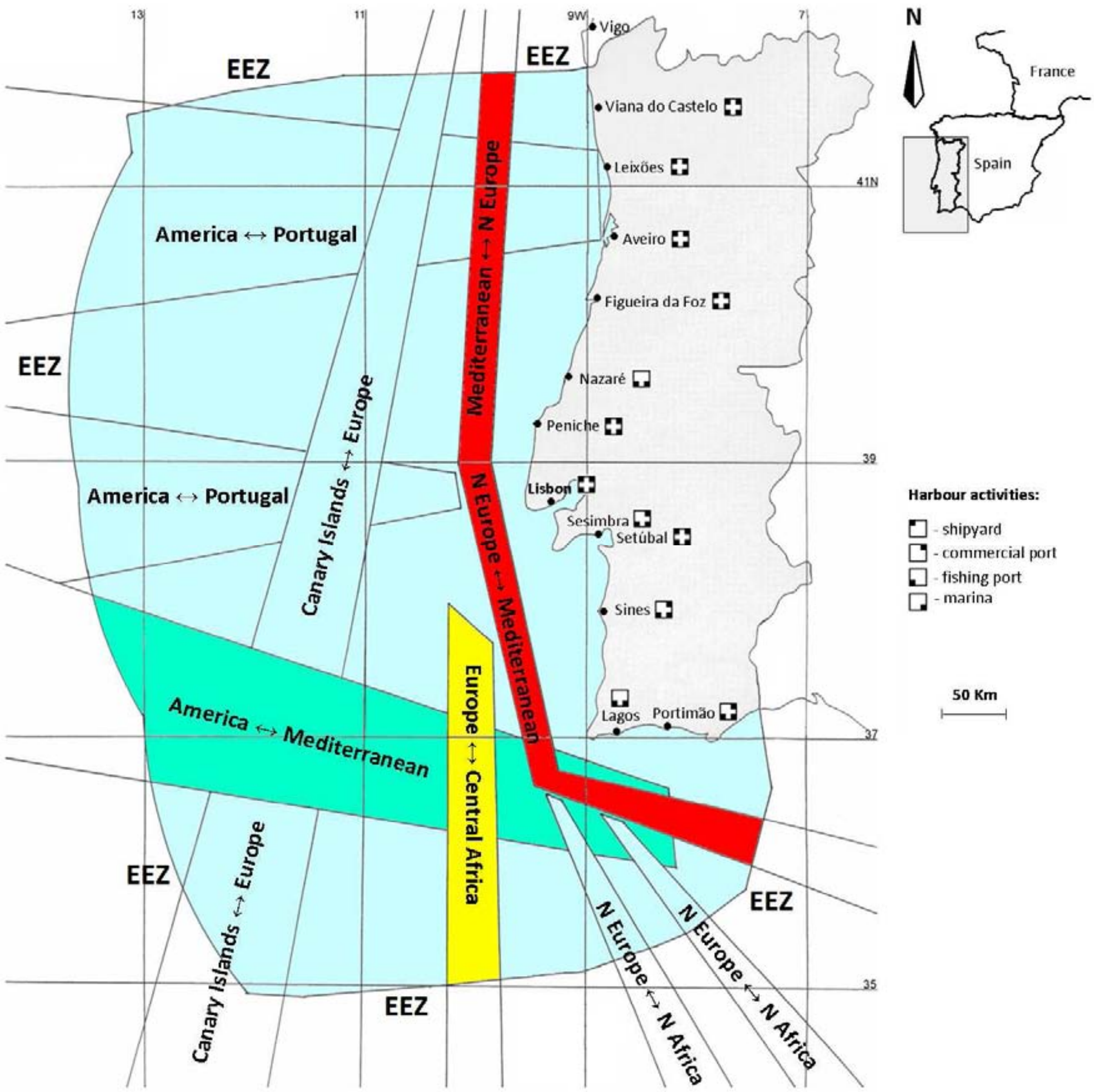


Figure 1.19 Traffic lanes within the Portuguese Exclusive Economic Zone (EEZ; light blue shaded). The threat for coastal environments is indicated by a colours scheme: green corresponds to a minor threat (3rd degree), yellow is an intermediate threat (2nd) and in red the higher threat is represented (1st degree). Updated from CNADS (2001) after modifications introduced by the Portuguese Law Decree No.198/2006.

Between Cape Finisterre (in Spain; north to Viana do Castelo harbour; see Figure 1.19) and Roca Cape (near Lisbon), although not far, this corridor was farther from the coast than between the Roca Cape and the São Vicente Cape (near Lagos harbour, in SW Portugal) where in 2003 it was positioned only 5-9 miles offshore. In 2006 new traffic separation schemes were adopted and this navigation line was repositioned to 14-15 miles offshore by the Portuguese Law Decree No.198/2006.

As referred above, all these areas were subjected to intense and continuous TBT inputs during the period when the use of OT-based AF paints was allowed. Therefore, these sites continue to be potential sources of TBT to the marine environment since these compounds were kept adsorbed in sediments and are now being slowly released.

The sea has been the common denominator of Portuguese identity over more than eight centuries in which the ports, shipping and maritime transport has always been of crucial importance, supporting the country's progress. For several reasons, the sea and the littoral have become a cultural and economical reference for Portuguese citizens, attracting men, activities and resources.

Partially influenced by the maritime heritage, some key elements of the Portuguese strategy for the national economy development are to strengthen the main ports facilities and to expand their connection to other European countries, being the continent geographic gateway for both goods and passengers.

Fortunately TBT compounds are finally banned and let us believe that competent authorities, supported by scientific development, would take the necessary measures to prevent other incidents alike the TBT pollution adverse impact in the environment.

We must not forget that *“whilst accidental spills from tankers can be spectacular, they account for only a minor fraction of all marine oil pollution”* (Ruiz, 2004) and that the *“gradual environmental degradation usually remains unperceived”* (Ruiz, 2004). In fact, *“if we consider that TBT input has occurred daily over the last few decades (and will occur for years to come), then we realise that”* an oil spill is indeed *“a mere drop in a glass of water”* (Ruiz, 2004).

Hence, the “TBT Tale” should be taken as an example of an early neglected warning, to avoid future threats to be no more than that, and so seas and all contained Life might not suffer similar aggressions.

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Chapter 2

The Dissertation

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2.1 AIMS AND RATIONALE

The present thesis aims at validating procedures to accurately follow *imposex* levels evolution in order to use this biomarker to assess the most recent legislation effectiveness to reduce tributyltin (TBT) pollution.

This work began at the moment when descriptions on the inefficacy of previous measures restricting TBT usage in the Portuguese coast appeared in the literature and, in turn, the most definite instruments intending to eradicate TBT-based antifouling (AF) paints were scheduled particularly: (A) the European ban by implementing the Council Regulation No.782/2003; and (B) the IMO global ban by the International AFS Convention entry into force. Thus, at that time, monitoring TBT biological effects and environmental concentrations assumed a crucial relevance in order to appraise if the upcoming legislation would finally be effective reducing TBT pollution levels.

Therefore, using *imposex* as a biomarker of TBT pollution and *Nucella lapillus* (L.) as a bioindicator, a baseline of *imposex* levels and organotins (OTs) in whole females' tissues along the Portuguese coast was created in 2003 at the moment when

Regulation (EC) No.782/2003 was implemented. *N. lapillus* was chosen because is the main bioindicator recommended by OSPAR to monitor TBT pollution. Similar surveys were repeated in following years to analyse the evolution of *imposex* and determine the effectiveness of the above legislation in reducing TBT pollution levels.

Nucella lapillus is particularly abundant in the open coast but is scarce in estuarine areas. Even so, populations could be found inside Ria de Aveiro (NW Portugal). Thus this system represents an exception of an estuarine area in the Portuguese coast where the species is relatively abundant. Similarly to other estuaries, Ria de Aveiro houses important naval infrastructures such as ports, dockyards and marinas, and so it was used as a case study to evaluate legislation efficacy near TBT main sources. However, given that *N. lapillus* is not present in the innermost areas of the estuary and is best suited to monitor TBT levels in the water column, *Hydrobia ulvae* was also used as a complementary bioindicator to monitor this system further upstream and to provide information regarding sediment contamination.

To reduce the influence of “confounding” variables on *imposex* levels assessment, new monitoring methods and data analysis were developed and applied to produce more reliable results of temporal evolution of TBT pollution.

A new baseline of *N. lapillus imposex* levels for the Portuguese mainland coast was carried out in 2008, right before the IMO global ban entry into force, to allow the future evaluation of this measure effectiveness to reduce TBT pollution.

Considering the amelioration of *Nucella lapillus imposex* levels along the Portuguese coast after the European ban on OTs AF paints, it was developed a preliminary study to analyse the possibility of dog-whelks to recolonize areas that were extremely affected by TBT pollution in the past and so where the species may had become extinct.

2.2 THESIS ORGANIZATION

This dissertation is organized into three main parts: (I) *Introduction*; (II) *Research Work*; and (III) *Final Remarks*. Each of these is in turn composed of different chapters, in a total of nine and organized as follows.

Part I – *Introduction.* Constitutes the basis to this project development. It starts by summarising the *State of the Art* (Chapter 1) regarding the tributyltin (TBT) pollution problematic and ends presenting *The Dissertation* (Chapter 2) aims, rationale and organization.

Part II – *Research Work*. It is presented into six chapters (Chapter 3 to 8) structured as scientific manuscripts, i.e. each of which containing its own theoretical introduction, specific aims, applied procedures, obtained results, respective discussion and conclusions. A brief summary per chapter is presented here.

- Chapter 3 describes the evolution of TBT pollution in the Portuguese coast from 2000 to 2003 and constitutes a baseline of *Nucella lapillus imposex* levels and OTs in whole females' tissues in 2003, the moment when the European ban took place by the implementation of Regulation No.782/2003.

- Chapter 4 presents two different approaches for the assessment of TBT pollution evolution in Ria de Aveiro, an area enclosing ports, dockyards and marinas and thus subjected to major TBT inputs. Data from short-term and long-term monitoring (*Nucella lapillus imposex* levels, OTs concentrations in females' tissues and water samples) are combined to track temporal trends of TBT pollution between 1997 and 2007 in order to verify the effectiveness of the above mentioned European ban.

- Chapter 5 addresses the temporal trends of *H. ulvae imposex* in Ria de Aveiro from 1998 to 2007 to evaluate TBT persistence in sediments in a period enfolding the Regulation No.782/2003 implementation. It also refers the most reliable methods to reduce the influence of critical variables, such as animals' size, on *Hydrobia ulvae imposex* levels assessment and provides a discussion on the influence of different biology / ecology traits in the response of different prosobranch species to TBT exposure, pointing the importance of choosing the bioindicator depending on the compartment that is being monitored (sediment *vs.* water).

- Chapter 6 describes *Nucella lapillus* reproductive cycle at its southern distribution limit in Europe (the Portuguese mainland coast) and quantifies the error introduced on *imposex* assessment results, when using "Relative Penis Size Index" (RPSI), caused by the male penis length seasonal / spatial variation at the same shore.

- Chapter 7 evaluates temporal trends of *Nucella lapillus imposex* intensity along the Portuguese coast from 2003 to 2008 using new monitoring and data analysis procedures, namely a new statistical approach to remove the effect of animals' size on *imposex* levels assessment. *Imposex* was measured in 2006 and 2008 and compared with available data for 2003 (Chapter 3) in order to evaluate the effectiveness of the European ban along the Portuguese coast. This chapter also represents a baseline of *Nucella lapillus imposex* levels in 2008, moment when the worldwide banishment on the use of TBT-based AF paints took place (that is the IMO AFS Convention entry into force).

- Chapter 8 discusses possible strategies of *Nucella lapillus* to recolonize estuarine inner areas where populations may had become extinct in the past due to extremely high TBT pollution levels. This chapter describes the phenotypic and genotypic ecotypes available in the Portuguese coast to recolonize the most sheltered shores and analyse juveniles' resistance to penetrate less saline estuarine areas.

Part III – *Final Remarks.* Since specific discussions from Chapter 3 to 8 are offered, this final part provides a general discussion and conclusions, framing the results obtained in a wider horizon – the global reality of TBT environmental pollution. Together with the framework of this study results, a personal view on the topic is also presented.

Part II

Research Work

Chapter 3

Imposex and organotin body burden in the dog-whelk (*Nucella lapillus* L.) along the Portuguese coast

Galante-Oliveira et al. (2006) *Applied Organometallic Chemistry* 20, 1-4.

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ABSTRACT

Nucella lapillus imposex – superimposition of male characters onto prosobranch (a subclass of gastropod molluscs) females – and organotin female body burden were surveyed on the Portuguese coast, from Vila Praia de Âncora (northern limit) to Praia da Luz (southern limit), at 17 sampling sites, between May and August 2003. The vas deferens sequence index (VDSI), the relative penis size index (RPSI), the percentage of females affected with *imposex* (%I) and the percentage of sterile females (%S) were used to assess the level of *imposex* at each site. VDSI, RPSI and %I were 0.20-4.04, 0.0-42.2% and 16.7-100.0%, respectively. Sterile females were found at site 2 (6.2%), 5 (4.0%) and 7 (5.0%). Tributyltin (TBT) and dibutyltin (DBT) female body burdens were 23-138 and <10-62 ng $Sn.g^{-1}$ dry weight, respectively. TBT female body burden was significantly correlated with RPSI and VDSI [Spearman rank order linear correlation: RPSI vs TBT body burden (b.b.) $r = 0.71$, $p < 0.01$; VDSI vs logTBT body burden $r = 0.71$, $p < 0.01$]. *Imposex* and TBT b.b. were highest at sites located in the proximity of harbours, where TBT leaching from antifouling paints is more intense owing to the high concentration of ships and dockyard activities.

3.1 INTRODUCTION

Tributyltin (TBT) compounds have been extensively used as biocide agents in ship antifouling paints since the mid 1960s (Bennett, 1996). Their deleterious effects on non-target organisms became apparent in the 1970s with the upsurge of prosobranch gastropod females with male characteristics, which was termed "*imposex*" by Smith (1971). After the mid 1980s many studies described TBT toxicity on organisms over a broad taxonomic spectrum, from bacteria to vertebrates, and its severe negative impacts on ecosystems. Legislation to ban the use of organotin (OT) antifouling (AF) paints on small boats (<25 m) was introduced for the first time in France in 1982, mainly motivated by the negative impact of TBT pollution on oyster farming. Latterly, similar legislation was applied throughout Europe; Portugal adopted this ban in 1993 but it was insufficient to reduce TBT pollution. Barroso and Moreira (2002) showed that TBT pollution increased in the Portuguese coast from 1987 to 2000 and suggested that could be linked to the increase of large ship traffic during that period. The International Marine Organization (IMO) adopted the "International Convention on the Control of Harmful Antifouling Systems on Ships" according to which OT AF systems cannot be applied or re-applied on any ship after 1 January 2003 and ships shall not bear such compounds after 1 January 2008.

The present work aims to assess the most recent evolution of TBT pollution on the Portuguese coast and to create a baseline for the IMO ban to allow future evaluation of its effectiveness.

The dog-whelk, *Nucella lapillus* (L.), was used as an indicator of the level of TBT pollution, as recommended by the OSPAR Joint Assessment and Monitoring Program (JAMP) guidelines (MEPC, 2008). *N. lapillus* is a common gastropod species of the Atlantic rocky shores, distributed in Europe from the north of Russia to the south of Portugal (Crothers, 1985). This species has a limited dispersion – a life cycle without a planktonic phase and with weak adult mobility – and develops *imposex* at very low levels of TBT in water (<0.5 ng Sn.l^{-1}; Gibbs et al, 1986). In advanced states of *imposex* the females may become sterilized owing to the overgrowth of the vas deferens, which blocks the vulva and prevents the egg capsules to be released, which has caused population extinctions at severely polluted sites throughout Europe (Bryan et al, 1986; Evans et al, 1996). For this reason the actual extent of female sterility was also surveyed in *N. lapillus* populations along the Portuguese coast.

3.2 MATERIAL AND METHODS

About 45-60 adult *Nucella lapillus* were collected by hand at the intertidal rocky shore from May to August 2003 at sites 1-17 along the Portuguese coast (Figure 3.1). The shell height (SH – apex to siphonal canal length) was measured with vernier callipers to the nearest 0.1 mm. After shell removal, the animals were sexed and analysed for *imposex* without narcotization.

The penis length was measured using a stereo microscope with a graduated eyepiece to the nearest 0.14 mm. The relative penis size index [RPSI = mean female penis length $(FPL)^3$ / mean male penis length $(MPL)^3$ x100], the vas deferens sequence index (VDSI), the percentage of *imposex* affected females (%I) and the percentage of sterile females (%S) were determined for each site, according to Gibbs et al. (1987). Parasitized specimens were discarded from the analysis.

TBT and dibutyltin (DBT) were measured by atomic absorption spectroscopy in homogenized whole tissues of 10-15 females from each site. The analytical procedures were largely based on the methods of Ward et al. (1981) and are fully described by Bryan et al. (1986). Recoveries of TBT and DBT were 100 and 92%, respectively, and were corrected by the use of standard additions in all samples. Detection limits for TBT and DBT were 10 ng $Sn.g^{-1}$ dry weight (Bryan et al, 1986).

3.3 RESULTS AND DISCUSSION

Nucella lapillus imposex and butyltins contamination was ubiquitous on the Portuguese coast (Figure 3.1; Table 3.1). RPSI, VDSI and %I were 0.0-42.2%, 0.20-4.04

and 16.7-100.0%, respectively. Sterile females were found at St. 2 (6.2%), 5 (4.0%) and 7 (5.0%), but these low levels presumably poses a low risk of population extinction at these sites. TBT and DBT female body burdens were 23-138 and 12-62 ng Sn.g^{-1} dry weight, respectively. RPSI and VDSI were significantly correlated with TBT female body burdens (Spearman rank order linear correlation: RPSI vs TBT body burden r = 0.71, p < 0.01; VDSI vs LogTBT body burden r = 0.71, p < 0.01).

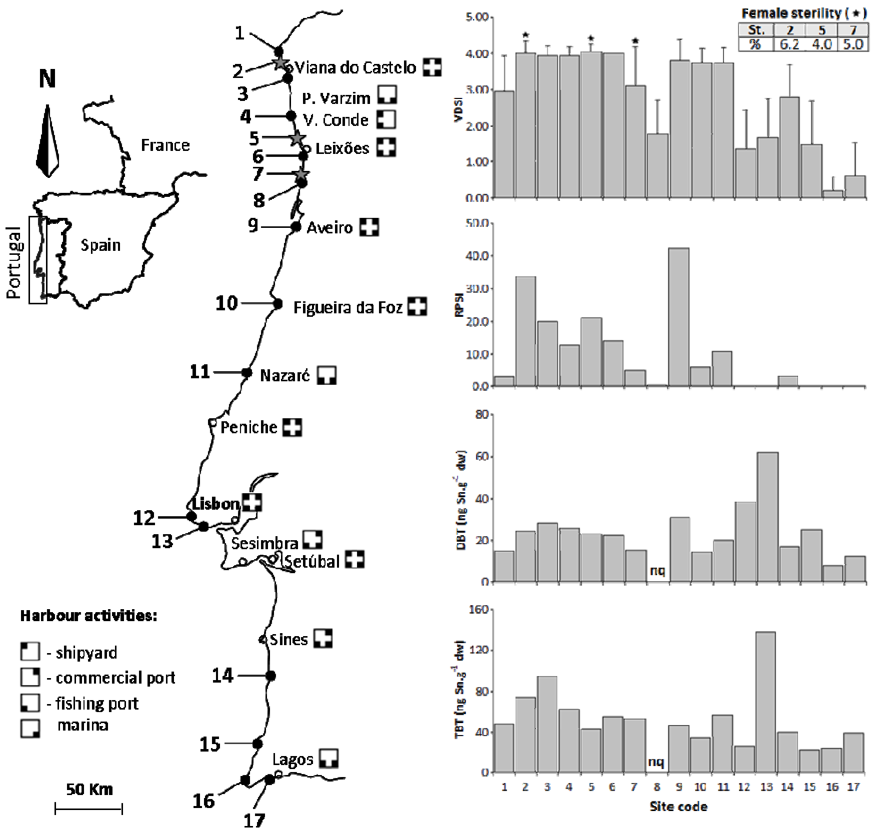

Figure 3.1 *Nucella lapillus*. Map of the Portuguese coast indicating sampling sites (St. 1 to 17) and main harbour activities. The histograms represent values for: vas deferens sequence index (VDSI), relative penis size index (RPSI), tributyltin (TBT) and dibutyltin (DBT) whole female body burden (ng Sn.g^{-1} dry weight). nq: not quantified. ★: occurrence of female sterility.

Table 3.1 *Nucella lapillus*. Number of specimens analysed at each site (n), males and females, with the indication of mean shell heights (♂SH and ♀SH, respectively) and the percentage of *imposex* affected females (%I). Standard deviations (s.d.) are rounded off to unity and given next to the mean value in the format "mean $^{(s.d.)}$". Time comparisons of *N. lapillus* imposex indices and organotin female body burdens between 2000 (Barroso and Moreira, 2002) and 2003 (current study) at common sites along the Portuguese coast. For additional data compare Figure 3.1.

Site code and name	Coordinates (EUR 50)	♂ n	♂SH (mm)	♀ n	♀SH (mm)	RPSI		VDSI				%I		TBT (ng Sn.g^{-1} dw)		DBT (ng Sn.g^{-1} dw)	
						2000	**2003**	**2000**	**2003**	***U***	***p***	**2000**	**2003**	**2000**	**2003**	**2000**	**2003**
1. Vila Praia de Âncora	41°48.93N 8°51.94W	22	21.0$^{(2)}$	28	22.3$^{(3)}$	11.70	2.70	3.81	2.96	44.0	***	100.0	100.0	70	48	50	15
2. Praia Norte	41°41.85N 8°41.13W	20	19.2$^{(1)}$	32	20.9$^{(2)}$	21.70	33.80	4.04	4.00	401.0		100.0	100.0	90	74	51	24
3. Praia da Amorosa	41°38.72N 8°49.31W	12	20.5$^{(2)}$	14	20.2$^{(2)}$	33.20	19.80	3.95	3.93	150.0		100.0	100.0	135	94	99	28
4. Póvoa do Varzim	41°23.18N 8°46.40W	19	22.5$^{(1)}$	31	23.1$^{(2)}$	8.70	12.70	3.96	3.94	378.0		100.0	100.0	63	62	38	26
5. Praia de Leça	41°12.21N 8°42.82W	31	22.4$^{(2)}$	25	23.2$^{(2)}$	14.70	21.00	4.00	4.04	240.0		100.0	100.0	77	42	66	23
6. Praia da Foz	41°09.78N 8°41.10W	25	20.9$^{(2)}$	25	21.1$^{(2)}$	28.40	14.10	4.00	4.00	250.0		100.0	100.0	117	55	112	22
7. Aguda	41°03.09N 8°39.18W	37	21.2$^{(2)}$	20	21.0$^{(2)}$	-	4.80	-	3.10	-	-	-	100.0	-	53	-	16
8. Espinho	41°00.44N 8°38.71W	20	21.0$^{(2)}$	25	21.9$^{(3)}$	4.30	0.60	3.77	1.84	17.5	***	100.0	92.0	37	-	34	-
9. Aveiro	40°38.71N 8°44.82W	25	23.0$^{(4)}$	25	23.6$^{(4)}$	32.90	42.20	4.00	3.80	230.0		100.0	100.0	95	47	98	31
10. Fig. Foz	40°10.18N 8°53.26W	20	19.4$^{(2)}$	31	19.6$^{(1)}$	4.40	5.80	3.55	3.74	410.0		100.0	100.0	-	34	-	14
11. Nazaré	39°36.26N 9°04.49W	20	18.2$^{(1)}$	31	19.1$^{(2)}$	18.70	10.70	4.08	3.74	264.5	**	100.0	100.0	-	57	-	20
12. Praia do Guincho	38°43.74N 9°28.46W	20	19.2$^{(2)}$	33	20.1$^{(1)}$	0.10	0.10	1.35	1.36	506.0		48.6	85.0	30	26	120	38
13. Praia das Avencas	38°41.21N 9°21.27W	23	20.5$^{(3)}$	32	21.9$^{(2)}$	30.70	0.04	4.00	1.66	22.0	***	100.0	84.4	147	138	180	62
14. Vila nova de Mil Fontes	37°43.30N 8°47.25W	20	21.1$^{(1)}$	25	22.4$^{(2)}$	8.40	3.00	3.15	2.80	206.0		95.5	100.0	77	40	48	17
15. Zambujeira do Mar	37°33.20N 8°47.44W	20	18.3$^{(1)}$	31	19.5$^{(2)}$	-	0.00	-	1.48	-	-	-	83.9	-	23	-	25
16. Praia do Amado	37°15.22N 8°38.45W	17	21.6$^{(2)}$	18	22.9$^{(3)}$	-	0.00	-	0.20	-	-	-	16.7	-	24	-	< 10
17. Praia da Luz	37°05.21N 8°43.64W	20	20.8$^{(2)}$	31	22.3$^{(2)}$	0.00	0.00	1.43	0.61	383.5		38.5	41.9	nd	39	nd	12

U: Mann-Whitney *U*-test result; **: $p < 0.01$; ***: $p < 0.001$; -: not analysed; nd: not detectable.

Site 13 was excluded from the correlation analysis since the population at this site is possibly affected by the Dumpton syndrome. This syndrome was first described and coined by Gibbs (1993) for a dog-whelk population in SE England and consists of a genetic deficiency that causes the underdevelopment of the genital system, leading to a lack of penis or undersized penis and incompletely developed gonoducts in males. The syndrome seems to be advantageous to the populations living at highly TBT polluted sites since females carrying the deficiency may not become sterilized (Gibbs, 1993).

Nucella lapillus was only collected from sites of the open coast, some of them very close (less than 1 mile) to main harbours (St. 2-3, 5-6, 9-11, 13), others close to small boat anchorage places (St. 1, 4, 7-8, 14) and others located at pristine areas (St. 12, 15-17). As a consequence, *imposex* and butyltins body burden levels were higher in the two former groups (Figure 3.1), which points out the link between TBT pollution and the proximity of ships or boats, most of which are known to still bear TBT based antifouling coatings.

Nucella lapillus was found at all sites already sampled in 2000 by Barroso and Moreira (2002). Considering that female maturation occurs at about 2-3 years, the three-year period elapsing between 2000 and 2003 surveys is large enough to depict temporal trends in TBT pollution. There was a significant decrease in the VDSI between 2000 and 2003 at St. 1, 8, 11 and 13 but no significant changes were observed in the remaining sites (Mann-Whitney *U*-Test; see Table 3.1). The nature of the data regarding the RPSI and TBT body burden does not allow a statistical comparison between the two surveys. Nevertheless, the simple analysis of the data suggests that the RPSI has a less consistent change, increasing at some sites and decreasing at others, while the TBT female body burden has apparently decreased at all sampling sites, despite different analytical methods being employed in the two surveys (Table 3.1). Although inconclusive, there are signs of a possible slight slowing down tendency of *imposex* and TBT pollution that has to be confirmed by further studies. Hopefully, after the IMO ban there will be a global consistent decrease in TBT that will be easily detected in future research using similar methods.

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Chapter 4

Imposex levels and tributyltin (TBT) pollution in Ria de Aveiro (NW Portugal) between 1997 and 2007: evaluation of legislation effectiveness

Galante-Oliveira et al. (2009) *Journal of Environmental Monitoring* 11, 1405–1411.

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ABSTRACT

Nucella lapillus imposex levels and organotin (OT) concentrations in water and female tissues were measured in samples collected from the Ria de Aveiro (NW Portugal) between 1997 and 2007. Vas deferens sequence index (VDSI), relative penis size index (RPSI), mean female penis length (FPL) and percentage of *imposex* affected females (%I) were used to determine *imposex* levels at each site. A significant temporal decline in *imposex* intensity was observed during the assessed period. *Imposex* decrease was evident after 2003 although improvements were most notable from 2005 to 2007, probably due to the implementation of the EU Council Regulation No.782/2003 forbidding further application of tributyltin (TBT) antifouling (AF) on vessels carrying EU flags. Despite these improvements, OT analysis in *N. lapillus* female tissues and water indicate there are still recent TBT inputs into the study area.

4.1 INTRODUCTION

Environmental concern about organotin (OT) compounds has increased strongly due to the worldwide use of tributyltin (TBT), since the mid 1960s, as a biocide in antifouling (AF) paints applied to boat hulls and other submerged structures to prevent bioincrustation (de Mora, 1996). Although it was extremely efficient as a biocide, several harmful effects on non-target organisms were described as a consequence of its intense usage. One of the best documented effects is the disruption of the endocrine and reproductive functions in some prosobranch gastropod molluscs, expressed by the development of *imposex* – the superimposition of male sexual characters in females (Smith, 1971; Smith, 1981). *Imposex* in the dog-whelk *Nucella lapillus* (L.) has been intensively used as a biomarker of TBT pollution levels on North Atlantic rocky shores (Birchenough et al., 2002). In 1998, the use of this species as a bioindicator was recommended in OSPAR Joint Assessment and Monitoring Program (JAMP) guidelines (MEPC, 2008), mainly due to the species widespread distribution (from northern Russia to southern Portugal), restricted power of dispersion (absence of a planktonic larval phase), limited adult mobility (Crothers, 1985) and high sensitivity to TBT pollution (*imposex* induction occurs at very low concentrations in water: <0.5 ng $Sn.l^{-1}$; Gibbs and Bryan, 1987). During the 1980s, extreme cases of complete female functional sterilization, population declines and extinctions were among the reported consequences of TBT pollution (Bryan et al., 1986; Gibbs and Bryan, 1986).

In response to these and other deleterious impacts, the European Council Directive 76/769/EEC was amended by the Directive 89/677/EEC that prohibited the use of OT compounds in AF systems on vessels <25 m in length. This latest Directive was transposed into Portuguese internal law in 1993, but it was insufficient to reduce TBT pollution (Barroso and Moreira, 2002; Santos et al., 2002). In 2001 the International Maritime Organization (IMO) adopted the "International Convention on the Control of Harmful Antifouling Systems on Ships" (AFS Convention, 2001). This resolution called for a worldwide prohibition on the application of OTs as biocides in

AF paints on ships by the effective date of 1 January 2003, and a complete banishment by 1 January 2008. However, the Convention could only enter into force, legally, 12 months after 25 States representing 25% of the world's merchant shipping tonnage had ratified it. Until then, the legal effect of 1 January 2003 would be suspended. Meanwhile, based on the IMO resolution to implement the Convention as a matter of urgency, EU countries banned the marketing and use of OT compounds in AF systems under Directive 2002/62/EC. Subsequently, the EU adopted Regulation No.782/2003, prohibiting the application or re-application of TBT coatings on Member States' national mercantile fleets and on ships operating under their authority, from 1 July 2003. The AFS Convention entry into force date was finally met on 17 September 2007, with the 25th State ratification, representing a total of 38% of the world's merchant shipping tonnage (IMO, 2009). As a result, the international ban on TBT was scheduled for 17 September 2008.

The current study aims to assess the evolution of TBT pollution in Ria de Aveiro (NW Portugal) between 1997 and 2007 considering the above legislative scenario. This estuarine system has a high economical potential, supporting fishery and aquaculture activities, as well as ports, dockyards and industry facilities. In addition it is classified as a special protected area by the EU nature and biodiversity policy ‘Natura 2000 Network’. This identifies the Ria de Aveiro as an ecosystem of considerable importance (Lopes et al., 2007), requiring active management of its environmental and ecological quality.

4.2 MATERIAL AND METHODS

4.2.1 Sampling

Two sampling strategies were adopted in the current work. One centred on the selection of 4 sampling sites (St. 2-3, 9 and 11; Figure 4.1) covering an extensive area from the open coast to inside the estuarine system where TBT potential sources are located (port terminals, dockyards and marinas). These sites were used for the assessment of long-term evolution of *imposex*, here designated as “Long-term monitoring”; dog-whelks were sampled at these sites in 1997, 2003, 2005, 2006 and 2007 (from June to November) and *imposex* levels were assessed. The other strategy, designated as “Short-term monitoring”, aimed to check if an eventual reduction in *imposex* levels could be detected within a one-year period and focused on a larger number of sites spread over a widened geographical area of the Ria de Aveiro and its adjacent coast. For that purpose, dog-whelks were sampled in August 2005 and 2006 at St. 1-5, 9, 11, 16 and 20 (Figure 4.1) for *imposex* assessment.

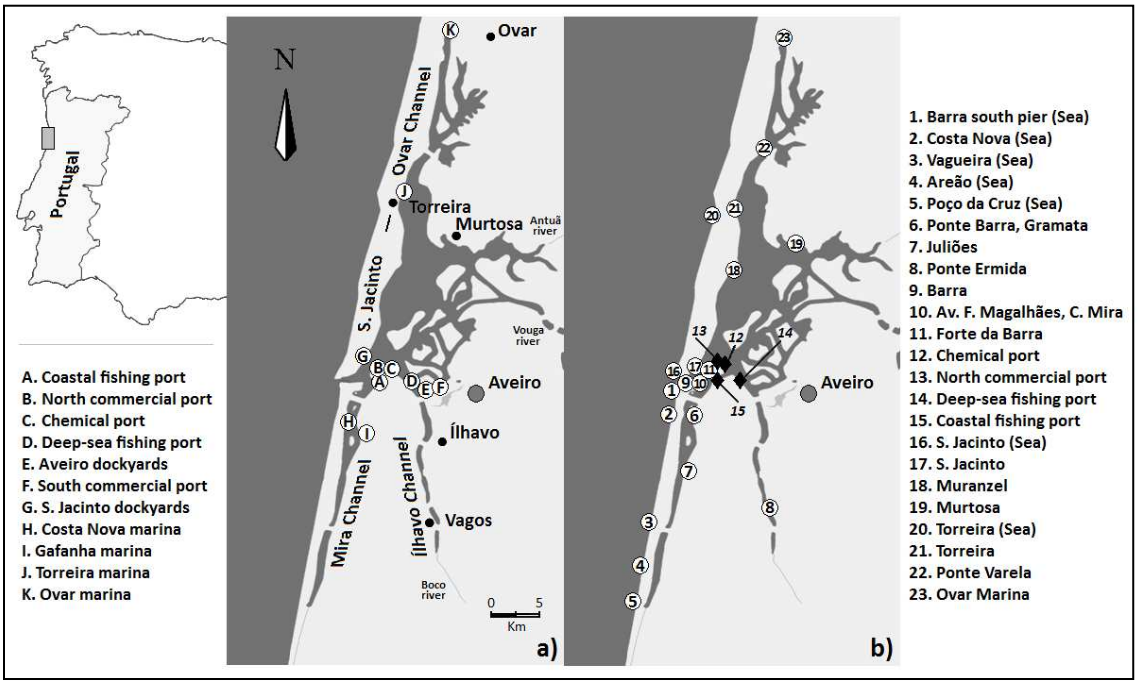

Figure 4.1 Ria de Aveiro and adjacent coastal area map indicating: (a) main TBT pollution sources (A–K); (b) sampling sites (St.) location, code and name (1-23; ○ and ◆, outside and inside port terminals, respectively).

Additionally, in order to verify if very recent inputs of TBT still occur in this estuarine system, OTs concentrations were determined in female dog-whelk tissues and water. Animals were collected in August 2006 (St. 1-5, 9, 11, 16 and 20) and water samples were obtained in the same period at St. 1-23 (Figure 4.1). Water collection covered most of the area of Ria de Aveiro to allow a representative image of OTs water contamination: sampling sites were spread across the main channels (including the navigation channel) and the adjacent Atlantic coast. Water sampling was performed twice, with one week interval, on the same lowest-tide level.

4.2.2 *Imposex* analysis

N. lapillus were collected randomly by hand at each site. The shell height (SH – length from the apex to the siphonal canal) was measured with vernier callipers to the nearest 0.1 mm. After shell removal, animals were sexed and analysed for *imposex* without narcotization. The penis length (PL) was measured using a stereo microscope with a graduated eyepiece to the nearest 0.14 mm and the mean female penis length (FPL) was determined for each sample. The relative penis size index [RPSI = FPL^3 / mean male penis length $(MPL)^3$ x100], the vas deferens sequence index (VDSI) and the percentage of *imposex* affected females (%I) were also determined for each site according to Gibbs et al. (1987). Parasitized specimens were discarded from the analysis.

4.2.3 Female OT body burdens

After being examined for *imposex*, 10-15 female dog-whelks from each site were preserved at $-20\ ^0$C for chemical analysis. TBT and dibutyltin (DBT) were measured by atomic absorption spectroscopy (AAS) in homogenized whole tissues. Although this technique does not perform the complete OTs speciation (of butyl-, phenyl- and octyltins), previous studies have shown that butyltins (BTs) represent the dominant OT fraction in the study area and, of these, TBT is the most abundant compound (Barroso et al., 2000; Rato et al., 2006; Sousa et al., 2007). Analytical procedures were largely based on those of Ward et al. (1981) as modified and described fully by Bryan et al. (1986). Recoveries of TBT and DBT were ≈100 and 92% respectively, and were corrected by the use of standard additions in all samples. Detection limits for TBT and DBT were about 10 ng $Sn.g^{-1}$ dw.

4.2.4 Hexane-extractable tin concentrations in water

Samples of 2l of sub-surface (15 cm depth) water were collected in two 1l glass bottles, previously washed in 0.5% hydrochloric acid (HCl). Immediately before each sample collection, bottles were rinsed with local water. Afterwards, samples were acidified with 5 ml concentrated HCl litre^{-1}. Methods used for extraction (from unfiltered water) and OT analysis are those described by Bryan et al. (1986) providing a detection limit of about 0.2 ng Sn.l^{-1}. However, washing of hexane extracts with 1 N sodium hydroxide (NaOH) to separate DBT from the TBT fraction was not performed; hence we report values as hexane extractable tin, as we only aim to depict any recent OT inputs.

4.2.5 Statistical data analysis

SigmaStat v2.0 software was used to perform the statistical analysis of the data. The analysis of dog-whelk *imposex* evolution between 1997 and 2007, at St. 2-3, 9 and 11 (“Long-term monitoring”), was performed using two different approaches depending on whether sampling sites were analysed together or separately. In the first approach, the subjects were the four sampling sites and the observations were the VDSI or the FPL at each site; the purpose of the analysis was to test if the VDSI and FPL of all four sites changed significantly over the 10 year period, using a Friedman test followed by the post-hoc Dunn's test for multi-comparisons. The second approach was applied to evaluate the change in *imposex* intensity at each site over the 10 year period. In this case the subjects were the females analysed per site and the observations were the VDS stage exhibited by each specimen, i.e. samples were assumed to be independent since specimens collected randomly over time were not the same; a non-parametric Kruskal-Wallis test, followed by the post-hoc Dunn's test for multi-comparisons, was applied in this case. Statistical comparisons using RPSI were not performed because this index depends on SH, which varied not only within but also between genders and in some cases in dissimilar directions.

The assessment of *imposex* evolution between 2005 and 2006 for the “Short-term monitoring” analysis was performed using the two different statistical approaches described above. Firstly, the subjects were the nine sampling sites and the observations were the VDSI or the FPL at each site; the purpose of the analysis was to test if the combined VDSI and FPL changed significantly within a single year, using a Wilcoxon signed-rank test. In the second approach, the Mann-Whitney *U*-test was applied to assess differences in VDS and PL in females at each site between 2005 and 2006.

4.3 RESULTS

4.3.1 Long-term monitoring

Nucella lapillus FPL, RPSI, VDSI and %I observed in 1997, 2003, 2005, 2006 and 2007 at St. 2-3, 9 and 11 are registered in Table 4.1. When sampling sites are analysed together, it is clearly detected a general decline from 1997 to 2007 in both VDSI (Friedman's test: $s = 16.000$, $p < 0.01$) and FPL ($s = 15.400$, $p < 0.01$). Dunn's multi-comparisons tests between different years show a significant reduction of VDSI and FPL levels from 1997 to 2007 and also of FPL from 2003 to 2007; the *imposex* decline was evident only after 2003. When females are analysed at each site, it is found a decrease in VDS along time (Figure 4.2) at the four locations (Kruskal-Wallis tests gave always significant differences with $p < 0.001$). Multi-comparisons show that the *imposex* decline is evident from 2003 onwards and most marked between 2005 and 2007, being accompanied by an increase in the values dispersion (Figure 4.2). However, a high percentage of females with *imposex* was observed, even in 2007, showing that it remains a common phenomenon in the study area.

Table 4.1 *Nucella lapillus imposex* long-term monitoring. Male and female mean shell heights (mm), standard deviation (SD) and number of analysed specimens (n) are presented in the format mm ± SD (n), per site and year. Indication of: mean female penis length (FPL), relative penis size index (RPSI), vas deferens sequence index (VDSI) and percentage of *imposex* affected females (%I). For additional data on site locations see Figure 4.1.

		Site code			
	Year	**2**	**3**	**9**	**11**
Shell height ♂±SD (n)	1997	26.5±1.6 (10)	23.5±2.1 (24)	24.9±2.6 (19)	27.5±2.2 (17)
	2003	19.1±1.8 (20)	20.5±2.0 (22)	23.0±4.0 (25)	26.5±2.2 (29)
	2005	23.2±2.1 (36)	23.5±2.0 (35)	26.4±2.2 (59)	27.1±2.5 (21)
	2006	22.4±1.6 (20)	23.6±2.3 (30)	26.1±2.1 (31)	26.1±2.0 (30)
	2007	25.2±1.7 (21)	25.8±2.2 (20)	26.3±1.4 (20)	25.1±2.1 (20)
Shell height ♀±SD (n)	1997	26.2±1.7 (13)	23.6±1.7 (18)	25.8±1.1 (17)	26.4±2.4 (13)
	2003	19.3±1.9 (27)	21.5±2.8 (25)	23.6±4.0 (25)	27.3±3.0 (17)
	2005	24.3±1.8 (62)	23.8±1.7 (38)	26.5±2.1 (30)	25.2±3.7 (24)
	2006	22.8±1.8 (24)	23.5±2.0 (30)	26.4±2.2 (27)	26.1±2.0 (30)
	2007	25.3±1.3 (25)	26.0±1.9 (25)	26.4±2.9 (23)	25.9±1.8 (25)
FPL (mm)	1997	2.22	2.07	2.22	2.28
	2003	1.76	1.58	2.18	2.45
	2005	0.99	0.86	1.93	1.44
	2006	0.81	0.45	1.00	0.45
	2007	0.16	0.08	0.62	0.31
RPSI (%)	1997	23.5	21.4	25.3	21.5
	2003	31.3	12.5	42.2	31.6
	2005	1.9	1.6	6.7	2.7
	2006	1.1	0.1	1.6	0.2
	2007	0.0	0.0	0.2	0.0
VDSI	1997	3.92	4.00	4.00	4.00
	2003	3.81	3.36	3.88	3.82
	2005	2.82	2.53	3.77	3.30
	2006	2.54	2.00	2.96	1.80
	2007	1.28	0.96	2.13	1.48
%I	1997	100	100	100	100
	2003	100	100	100	100
	2005	97	100	100	100
	2006	100	100	100	100
	2007	100	80	100	100

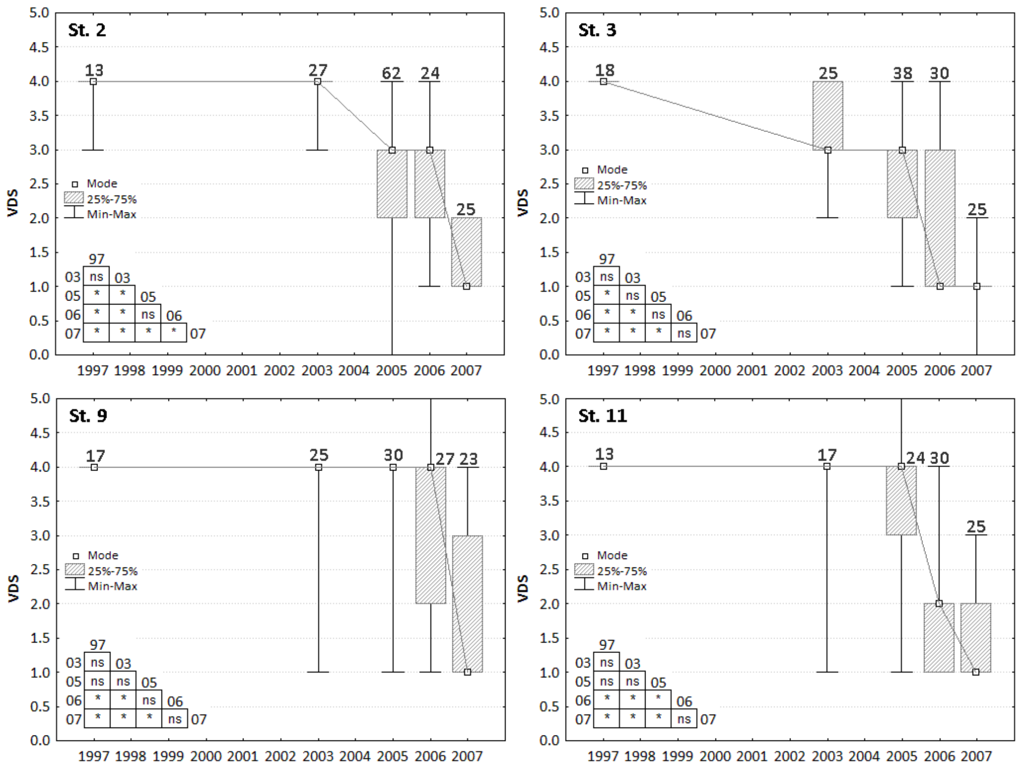

Figure 4.2 *Nucella lapillus* long-term monitoring (separated sites). Temporal variation of *imposex* exhibited by specimens collected in Ria de Aveiro (NW Portugal) at St. 2-3, 9 and 11. For each site, the VDS mode, percentiles (25% - 75%), minimum and maximum values (Min - Max) registered in 1997, 2003, 2005, 2006 and 2007 are indicated. The number of analysed specimens is shown above the respective mode point. The significance of the Dunn's test for multi-comparisons between years is shown on the respective plot. *: $p < 0.05$; ns: not significant.

4.3.2 Short-term monitoring

N. lapillus FPL, RPSI, VDSI and %I observed in 2005 at St. 1-5, 9, 11, 16 and 20 ranged between 0.28-1.93 mm, 0.0-8.7%, 1.43-3.77 and 91-100%, respectively. In 2006 the same indices varied between 0.10-1.00 mm, 0.0-1.6%, 0.71-2.96 and 46-100% (Table 4.2). Combining all sites together, there was a significant reduction between 2005 and 2006 in both VDSI (Wilcoxon test: $W = -45.000$, $p < 0.01$) and FPL ($W = -45.000$, $p < 0.01$). Similarly, the per site analysis indicates that females VDS and PL decreased in all sampling sites from 2005 to 2006, though the decline was significant only for about $^2/_3$ of the sites (see Table 4.2).

Table 4.2 *Nucella lapillus imposex* short-term monitoring. Male and female mean shell heights (mm), standard deviation (SD) and number of analysed specimens (n) are presented in the format mm ± SD (n), per site and year. Indication of: mean female penis length (FPL), relative penis size index (RPSI), vas deferens sequence index (VDSI) and percentage of *imposex* affected females (%I). Statistical comparisons of results between 2005 and 2006 are given next to the 2006 FPL and VDSI values (Mann-Whitney *U*-test significance). Organotins female body burdens are also indicated: TBT and DBT (ng Sn.g^{-1} dw). For additional data on site locations, see Figure 4.1.

		Site code								
	Year	**1**	**2**	**3**	**4**	**5**	**9**	**11**	**16**	**20**
Shell height ♂±SD (n)	2005 2006	23.5±2.0 (38) 22.9±1.7 (23)	23.2±2.1 (36) 22.4±1.6 (20)	23.5±2.0 (35) 23.6±2.3 (30)	23.6±2.4 (32) 22.8±1.8 (24)	23.3±2.0 (32) 23.6±1.9 (26)	26.4±2.2 (59) 26.1±2.1 (31)	27.1±2.5 (21) 26.1±2.0 (30)	23.2±2.1 (28) 22.9±2.2 (23)	24.0±2.0 (34) 23.5±1.8 (31)
Shell height ♀±SD (n)	2005 2006	23.8±2.3 (31) 23.4±2.1 (30)	24.3±1.8 (62) 22.8±1.8 (24)	23.8±1.7 (38) 23.5±2.0 (30)	24.0±2.0 (35) 23.6±2.0 (28)	24.0±2.2 (33) 23.6±2.1 (31)	26.5±2.1 (30) 26.4±2.2 (27)	25.2±3.7 (24) 26.1±2.0 (30)	23.4±2.1 (36) 24.2±2.1 (43)	23.9±2.2 (35) 23.8±2.0 (28)
FPL (mm)	2005 2006	0.28 0.10	0.99 0.81	0.86 0.45 **	1.67 0.96 ***	0.74 0.19 ***	1.93 1.00 ***	1.44 0.45 ***	0.90 0.82	0.28 0.10
RPSI (%)	2005 2006	1.5 0.7	1.9 1.1	1.6 0.1	8.7 1.1	0.6 0.0	6.7 1.6	2.7 0.2	0.7 0.8	0.0 0.0
VDSI	2005 2006	2.94 2.43	2.82 2.54	2.53 2.00 *	3.71 2.96 **	2.73 1.23 ***	3.77 2.96 **	3.30 1.80 ***	2.61 2.53	1.43 0.71 **
%I	2005 2006	100 100	97 100	100 100	100 100	100 68	100 100	100 100	95 100	91 46
TBT (ngSn gdw^{-1}) **DBT (ngSn gdw^{-1})**	2006	46 11	41 15	34 < 10	134 12	49 11	10 < 10	126 18	47 13	75 < 10

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

OTs concentrations in female *N. lapillus* tissues are also presented in Table 4.2. TBT concentrations varied from 10 to 126 ng $Sn.g^{-1}$ dw and a much higher proportion of TBT in relation to DBT was recorded. Hexane-extractable tin values in water are presented in Table 4.3 and were highly variable over the one week period between the two sampling occasions in 2006, probably as a consequence of the changeable hydrodynamic regime of this estuarine system. The arithmetic mean of values observed within a week interval at each site ranged from <0.6 to 38.5 ng $Sn.l^{-1}$.

Table 4.3 Organotins in water (ng $Sn.l^{-1}$ as hexane extractable tin) in 2006: values for two sampling occasions (1^{st} and 2^{nd} samp) and the respective mean value is indicated. For additional data on sites location see Figure 4.1.

	Hexane extractable tin (ng $Sn.l^{-1}$)		
St. code	**1^{st} samp**	**2^{nd} samp**	**mean**
1	1	4	2.5
2	2	2	2.0
3	1	3	2.0
4	1	1	1.0
5	1	< 0.2	< 0.6
6	74	3	38.5
7	4	7	5.5
8	8	2	5.0
9	1	6	3.5
10	3	10	6.5
11	37	1	19.0
12	5	9	7.0
13	3	4	3.5
14	11	6	8.5
15	7	13	10.0
16	1	1	1.0
17	4	3	3.5
18	1	4	2.5
19	2	3	2.5
20	3	1	2.0
21	9	1	5.0
22	4	5	4.5
23	5	1	3.0

4.4 DISCUSSION

OSPAR developed provisional assessment criteria for *Nucella lapillus imposex* (OSPAR, 2004). These criteria define 6 assessment classes (A–F) through VDSI intervals (from VDSI < 0.3 to VDSI ≥ 5) that were developed taking into account the objectives of the OSPAR Hazardous Substances Strategy and the existing Ecotoxicological Assessment Criteria (EAC) for TBT in water (upper EAC = 0.04 ng Sn.l^{-1}), sediment and biota (OSPAR, 1997). The Ecological Quality Objective (EcoQO) for *imposex* in *N. lapillus* corresponds to values lower than VDSI = 2 (the limit between assessment classes B and C), (OSPAR, 1997; OSPAR, 2004).

In retrospect, the ecological quality of the Ria de Aveiro in 1997 (current study) and in 1998 (survey performed by Barroso et al., 2000) fall into class D (4.0 ≤ VDSI ≤ 5.0) as *N. lapillus* exhibited VDSI between about 4.0 and 4.4 at all surveyed sites (2-3, 9 and 11-12); it should be noted that sterile females were observed only at St. 12 in 1998 but this population had probably become extinct afterwards as no animal could be found recently, namely during the 2005 and 2006 sampling campaigns. The present study shows that there was an evident decline in TBT pollution in the following years and in 2006/2007 the ecological status of the Ria de Aveiro had improved significantly (see Figure 4.2) since the VDSI at the same sites dropped to values between 1.0 and 3.0, corresponding to the OSPAR classes B–C. The current study also shows that an *imposex* decline could be detected over a short period of time between 2005 and 2006, which suggests that TBT pollution is diminishing rapidly. As *imposex* in *N. lapillus* is an irreversible phenomenon (Bryan et al., 1986), these results also suggest that the species has a high population renewal rate in the study area; i.e. older animals with higher levels of *imposex* are rapidly substituted by less affected younger ones.

The temporal trend described above is corroborated by the amelioration of *imposex* in *Nassarius reticulatus* in the Ria de Aveiro between 2000 and 2005 (Sousa et al., 2007). Hence, in a decade, the ecological status of the Ria de Aveiro has changed considerably. The ecological enhancement of the ecosystem was accompanied by enrichment of the economical potential, confirmed by the example of the oyster production activities in the study area: in the 10 year period of this study, shell length measurements showed an increase of 36% in the oysters growth rate, accompanied by a 77% decrease in shells chambering (S. Galante-Oliveira, unpublished data) – a phenomenon caused by exposure to TBT (Alzieu et al., 1986; Chagot et al., 1990). According to the shellfish farmers, this amelioration was noted only after 2004 and has resulted in a two-fold net increase in export value (discounting inflation) of the oysters from 2004 to 2007.

It is important to analyse the possible causes for the recent decline in TBT pollution in the area, as these considerations are useful for future management of similar pollution problems elsewhere. It is known that the first ban on the use of AF paints on small boats was ineffective in reducing TBT pollution along the Portuguese continental coast (Barroso and Moreira, 2002; Santos et al., 2002). In fact, the transposition of the 89/677/EEC Directive into national law occurred in 1993 but in 2000 (Barroso and Moreira, 2002) and 2003 (Galante-Oliveira et al., 2006) *N. lapillus* populations were still severely affected by *imposex* along the coast and in 2003 a VDSI $\geq$ 4.0 was still evident at ≈60% of the sampled sites. The very same pattern was also observed in the current study within the Ria de Aveiro (see Figure 4.2). The major change in *imposex* levels in the Ria de Aveiro appears to have occurred only after 2003, which was coincident with the implementation of the EU Regulation No.782/2003. Specifically, our data show a statistically significant decline in the FPL in the successive surveys performed from 2003 to 2007 and a progressive significant decline of VDSI between 2005 and 2007. During the latter period, a decrease in the RPSI and %I was also evident. Hence, the main cause for this change was most probably the implementation of the EU Regulation No.782/2003 in 1 July 2003. It is important to stress that during this period there was no decrease of naval or commercial traffic calling at the Port of Aveiro, which suggests a decline in the use of TBT-based AF paints by ships and/or a reduction of inputs from dockyards after the EU regulation. Interestingly, the proportion of ships that were entering into Aveiro's Port from countries where TBT could still be applied (from states outside the European Union and countries where the AFS Convention has not been ratified) also decreased from 50% in 2003 to 23% in 2005 and to 19% in 2007 (C. Oliveira, Aveiro's Port Administration, *pers. com.*).

Despite the substantial recovery of *N. lapillus imposex* over the studied period, the VDSI level recorded at St. 9 in 2007, located in the major traffic lane in the study area, still falls into class C. This fact indicates that at sites close to identified sources there is still a risk of adverse effects, such as reduced growth and recruitment in the most sensitive taxa of the ecosystem, caused by long-term exposure to TBT. Moreover, as *imposex* levels continue to be higher at sites located in the vicinity of maritime traffic lanes and port terminals (St. 9 and 11; Figure 4.1 and Figure 4.2), ships may still represent the major sources of TBT to the environment.

Further evidence that TBT pollution continues, despite the current amelioration, comes from the analysis of OTs in *N. lapillus* tissues and water. Recent contamination of *N. lapillus* is suggested by the high proportion of TBT in relation to DBT (primary degradation metabolite of TBT) in the tissues. Additionally, OTs were present in sub-surface waters with mean concentrations varying from <0.6 to 38.5 ng $Sn.l^{-1}$, the major fraction of which is known to be TBT and DBT. These data are corroborated by prior descriptions of fresh TBT inputs in the study area: for the same period Sousa et al. (2007) described a high proportion of TBT in sediments as well as in *Nassarius reticulatus* and *Mytilus galloprovincialis* tissues, in relation to the other BTs [DBT and monobutyltin (MBT)]. These fresh inputs were expected since TBT-based AF paints legislation was not fully effective until September 2008. Furthermore, it is well documented that TBT is persistent within sediments, with half-lives in the range of years (de Mora, 1996; Saeki et al., 2007), and can be continuously released into the environment (Sheikh et al., 2007). Thus, *N. lapillus* may still accumulate TBT from the above contamination sources.

Nevertheless, if *imposex* decline in *N. lapillus* observed in the recent years continues at a similar rate in the future (see Figure 4.2) it is predicted that the phenomenon might reach almost undetectable levels by the beginning of next decade.

4.5 CONCLUSIONS

The current study shows that all *N. lapillus* populations in the study area are extensively affected by *imposex* and there are still recent inputs of TBT into this estuarine system. Nevertheless, we observed a general reduction in TBT pollution in the last 10 years. This decline was mainly noticed after 2005, for which the EU Council Regulation No.782/2003 was certainly determinant. The ecological status of the Ria has improved, confirming the effectiveness of legislation and demonstrating benefits in an estuarine system where the maintenance of environmental quality is highly important.

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Chapter 5

Hydrobia ulvae imposex levels at Ria de Aveiro (NW Portugal) between 1998 and 2007: a counter-current bioindicator?

Galante-Oliveira et al. (2010) *Journal of Environmental Monitoring* (in press).

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ABSTRACT

Imposex expression in prosobranch gastropods has been widely used as a biomarker of tributyltin (TBT) pollution. Estuaries have been described as the most affected areas by this problem since they usually enclose the main TBT sources – ports, dockyards and marinas – resulting from these compounds application as biocides in antifouling (AF) paints on ships. Using *Hydrobia ulvae* as a bioindicator, the current work addresses the most reliable methods to reduce the influence of critical variables, such as the animals' size, on *imposex* levels assessment for TBT pollution monitoring and presents its temporal trends from 1998 to 2007 in Ria de Aveiro (NW Portugal) to evaluate the effectiveness of recent legislation applied to reduce TBT environmental levels. *H. ulvae imposex* levels did not decrease in this estuarine system during the last decade despite the implementation of the EU Regulation No.782/2003. Instead, there was a global significant increase in the percentage of *imposex* affected females (%I) and a slight increase of the vas deferens sequence index (VDSI), contrasting with what has been described for other bioindicators in the same study area. These results show that different biology / ecology traits determine distinct routes of TBT uptake and/or bioaccumulation, pointing the importance of choosing the bioindicator depending on the compartment that is being monitored (sediment *vs.* water). Sediment ingestion as feeding habit is discussed and pointed as a reason to choose *H. ulvae* as a bioindicator of

TBT pollution persistence in sediment. It is therefore predicted that the response of different prosobranch species around the world may diverge according to the compartment that is being monitored and that female masculinization may not be completely eradicated in the near future due to TBT persistence in sediments.

5.1 INTRODUCTION

Ria de Aveiro is a shallow estuarine system located in NW Portugal covering an area of ≈66 to 83 km^2 depending on the tide level (Dias, 2001). This system has a high economical potential, supporting fishing and aquaculture activities and providing conditions for the established industry, shipyard, ports and marinas facilities. In addition, Ria de Aveiro is classified as a special protected area by the European Union (EU) nature and biodiversity policy “Natura 2000 Network” (Lopes et al., 2007). Hence, this is an ecosystem of considerable importance, requiring active management of its environmental quality.

Tributyltin (TBT) pollution has been monitored in Ria de Aveiro in a regular basis since 1997 in order to evaluate its ecological impact and temporal evolution in response to successive legislative actions to banish the use of these organotins (OTs) from antifouling (AF) formulations (Barroso et al., 2000; Barroso et al., 2005b; Rato et al., 2006; Sousa et al., 2007; Galante-Oliveira et al., 2009). The first EU action was to forbid the application of OTs based AF paints (hereafter designated as “OTs paints”) on boats <25 m in length through the Directive 89/677/EEC. However, this measure was ineffective in many areas where the naval traffic included larger vessels, as it was the case of Ria de Aveiro in which TBT levels remained almost unaltered for several years after the Directive’s transposition in 1993 (Barroso et al., 2000; Barroso and Moreira, 2002; Santos et al., 2002). In 2001, the International Maritime Organization (IMO) adopted the “International Convention on the Control of Harmful Antifouling Systems on Ships” (AFS Convention, 2001), which called for a worldwide prohibition on the OTs paints application in all kind of vessels by the effective date of 1 January 2003, and a complete banishment by 1 January 2008. However, this convention could only be applied 12 months after being ratified by 25 States, representing at least 25% of the world's merchant shipping tonnage, and so it only entered into force on 17 September 2008 (IMO, 2009). Previewing the process delay, the EU anticipated the AFS Convention for the 1st July 2003 through the Regulation No.782/2003. Therefore, it is essential to verify if this legislation is being effective to reduce TBT pollution.

A well-known consequence of this pollution is *imposex* – the superimposition of male sexual characters onto prosobranch females (Smith, 1971) – which has been used as a biomarker of TBT environmental concentrations (Bryan et al., 1986; Bryan et al., 1987; Morcillo and Porte, 1998; Picado et al., 2007). Three key indicator species have been used to monitor TBT pollution in Ria de Aveiro, namely: *Nucella lapillus* (L.), *Nassarius reticulatus* (L.) and *Hydrobia ulvae* (Pennant). In 1998, Barroso et al. (2000) recorded high levels of *imposex* in these species and the *N. lapillus* decreasing abundance from the system mouth inwards was attributed to the females' sterility caused by TBT. From 2003 to 2007 a decrease of *imposex* levels in *N. lapillus* and *N. reticulatus* has been observed in Ria de Aveiro (Sousa et al., 2007; A. Sousa, unpublished data; Galante-Oliveira et al., 2009) as a probable consequence of the Regulation No.782/2003 implementation, and the present work intends to evaluate if the same temporal trend occurred in *H. ulvae.* This species was proposed as a TBT pollution indicator by Schulte-Oehlmann et al. (1997) so that the evolution of *imposex* levels can be used to describe the progress of TBT environmental levels.

The current work establishes the most reliable methods for *imposex* monitoring programmes using *H. ulvae*, particularly those not focused previously in the literature, such as the influence of animal's size on *imposex* assessment. Subsequently, it addresses three key objectives: (i) the evolution of *imposex* in this species from 1998 to 2007 in Ria de Aveiro in order to track pollution trends; (ii) the evaluation of the legislation effectiveness to reduce TBT environmental levels; and (iii) the comparison of this temporal trend with other indicator species for the same study area.

5.2 MATERIAL AND METHODS

The effect of animals' size on *imposex* parameters was studied in *H. ulvae* specimens of different shell heights (SH) collected at St. 1 and St. 3 (Figure 5.1) in February 2003, 2004 and 2007.

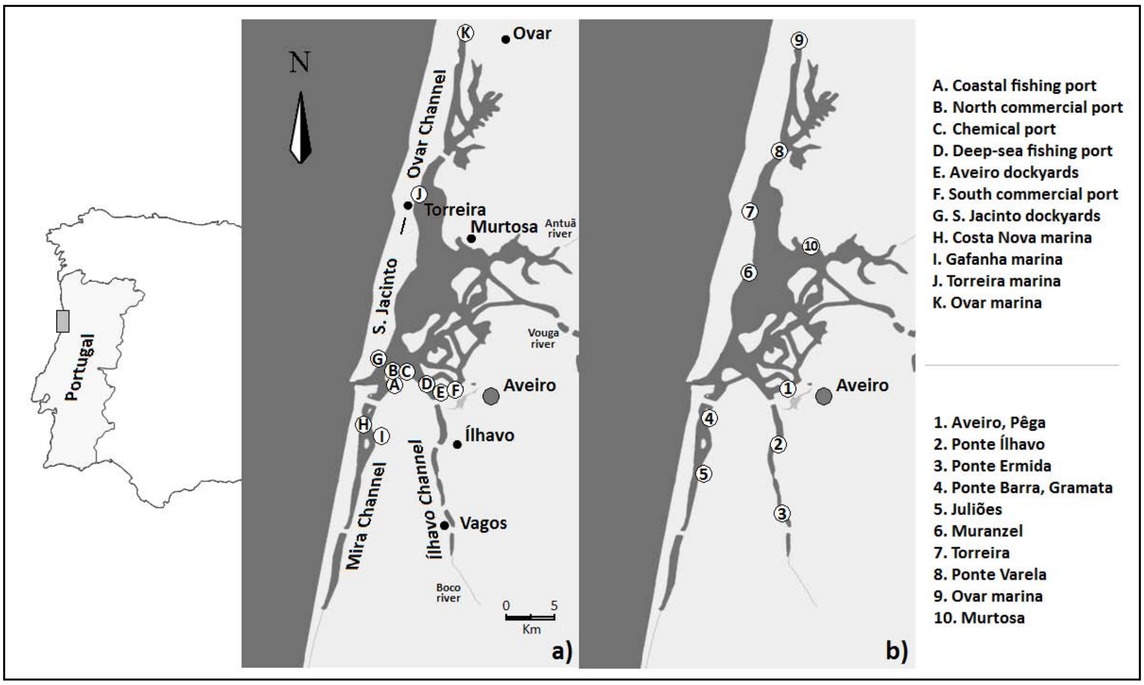

Figure 5.1 Ria de Aveiro and adjacent coastal area map indicating: a) potential TBT pollution sources represented by ports, dockyards and marinas (A–K); b) sampling sites location, code and name (1-10).

The *imposex* parameters analysed were the mean female penis length (FPL), the relative penis length index [RPLI = FPL / mean males penis length (MPL) x100], the vas deferens sequence index (VDSI) and the percentage of *imposex* affected females (%I), (Schulte-Oehlmann et al., 1997). In the laboratory, the SH (distance between shell apex and the base of the aperture) was measured using a stereo microscope with a graduated eyepiece to the nearest 0.14 mm. Shells were removed and animals were sexed. The presence of parasites was carefully checked under the stereo microscope. Although parasitized specimens were not used for the *imposex* monitoring surveys, as recommended by Schulte-Oehlmann et al. (1997; 1998), they were analysed separately to look for any apparent relationship between parasitism and female masculinization. Specimens were narcotized during 60 min using $MgCl_2$ 3.5% in distilled water (according to the average local salinity of around 15 psu) and the penis length (PL) was measured in all animals using a stereo microscope with a graduated eyepiece to the nearest 0.06 mm, whilst the vas deferens sequence (VDS) stage was assessed in females according to the scheme proposed by Schulte-Oehlmann et al. (1997). For females the FPL, VDSI and %I were calculated per SH class (1.50-2.75; 2.76-4.00; 4.01-5.25; 5.26-6.50 mm) and analysed graphically. For males a multiple linear regression (using *Statistica v6.0* software) was conducted to determine if animals' SH have a significant effect on male penis length (MPL) and if this relationship varies with sampling sites or dates.

The effect of narcotization on *H. ulvae* penis length (PL) was studied in a sample of specimens representing a wide range of SH collected in 2003 at St. 3 (Figure 5.1). After shells removal, animals were sexed and PL was measured. Specimens were narcotized during 60 min using the $MgCl_2$ 3.5% solution and PL was measured again. The difference between penis length measurements before (PL_b) and after (PL) narcotization was assessed by regression analysis (after checking homoscedasticity, linearity, and normally distributed error terms assumptions) and by the Wilcoxon signed-rank test.

For the purpose of assessing the evolution of *H. ulvae imposex* levels in Ria de Aveiro, specimens with an average SH around 4.0-6.0 mm were collected at St. 1-10 (Figure 5.1) between November 2002 and March 2003 (here referred as “2003” survey), between April and May 2004 (“2004” survey) and in August 2007 (“2007” survey). About 60-80 individuals were collected at each site, which is well above the minimum of 30 recommended by Schulte-Oehlmann et al. (1998) for study areas with a high *imposex* incidence. The *imposex* parameters FPL, RPLI, VDSI and %I were determined for each sample, as described above. Statistical comparisons of SH, PL and VDS stage between the different surveys were performed for each site using the Kruskal-Wallis test followed by Dunn's post-hoc tests, taking the specimens randomly sampled along time as observations. These comparisons included also data obtained previously in 1998 by Barroso et al. (2000) for the same locations, using exactly the same methods as in the current study. An additional analysis was conducted for the whole study area with the Friedman test to verify if the combined females SH, FPL, VDSI or %I of the 8 common sites (where animals were found in all sampling occasions) changed significantly over the period from 1998 (data from Barroso et al., 2000) to 2007.

5.3 RESULTS

Imposex parameters are determined for a sample of adult animals from a given site but frequently the animals' size varies between different locations or sampling dates and therefore it is important to assess how this factor may influence *imposex* measurements. The observation of Figure 5.2 and the multiple regression analysis reveal that male penis length depends on shell height (t = 25.84, p < 0.001) and this relationship varies with sampling site (t = −5.76, p < 0.001) and sampling occasion (t = 4.45, p < 0.001).

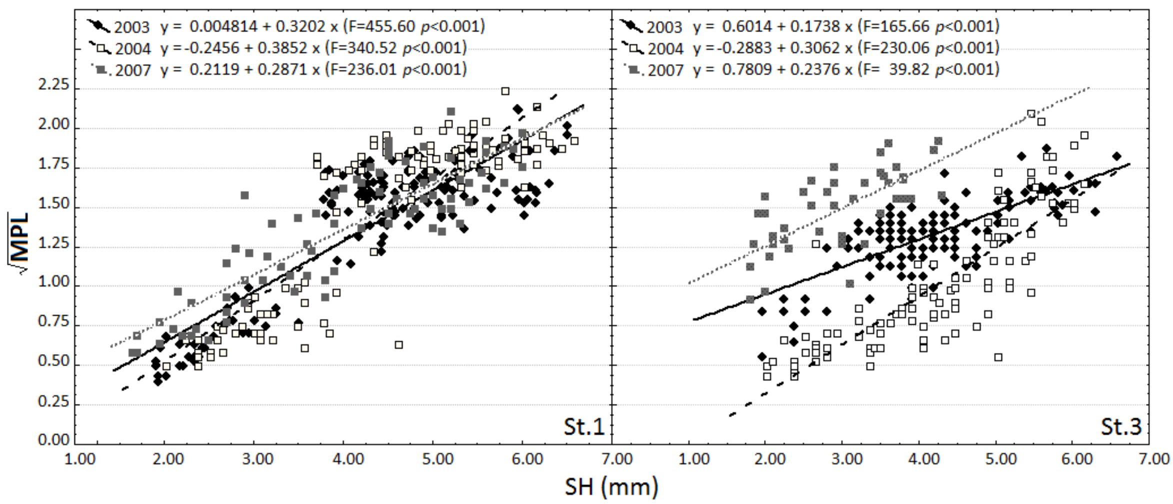

Figure 5.2 *Hydrobia ulvae*. Relationship between male penis length (MPL) square route and shell height (SH), both expressed in millimetres (mm), at Ria de Aveiro (St. 1 and 3). For each site, three sampling occasions were compared: 2003, 2004 and 2007. Regression equations are indicated in each plot.

Regarding females it is also clear that FPL, VDS and %I are strongly influenced by animal's size (Figure 5.3).

	St. 1				St. 3			
SH(mm)	1.50 - 2.75	2.76 - 4.00	4.01 - 5.25	5.26 - 6.50	1.50 - 2.75	2.76 - 4.00	4.01 - 5.25	5.26 - 6.50
n (2003)	46	48	61	31	41	70	86	44
n (2004)	14	22	32	27	9	25	43	36
n (2007)	34	29	32	15	47	48	11	0

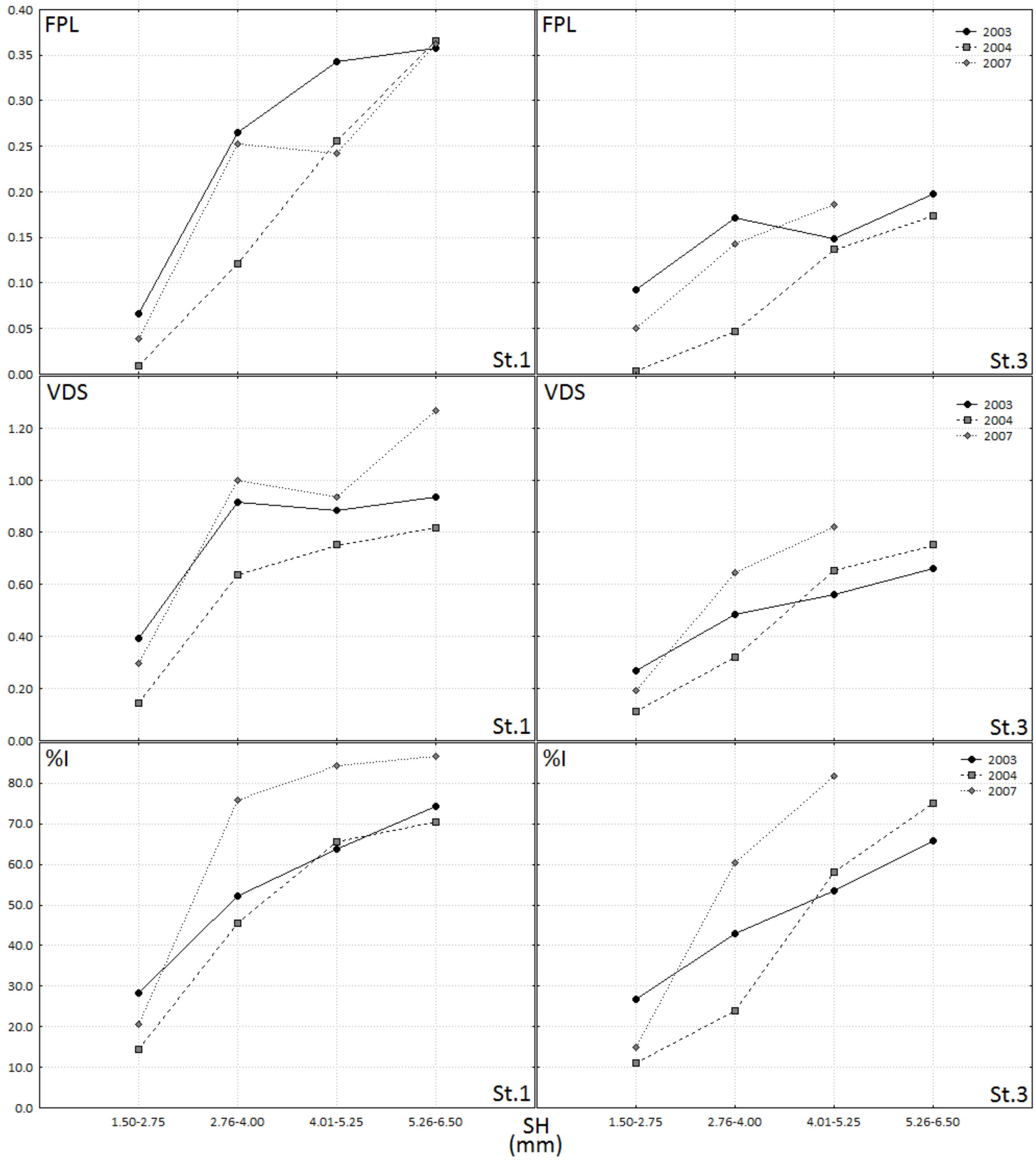

Figure 5.3 *Hydrobia ulvae*. Variation of female penis length (FPL), vas deferens sequence (VDS) and percentage of affected females (%I) with shell height (SH) in specimens collected at St. 1 and 3. For each site, three sampling occasions were included: 2003, 2004 and 2007. The number of specimens analysed for *imposex* (n) per SH class (mm) and per year is indicated on the top table.

A steep increase in all parameters was registered in mud snails from St. 1 that presented SH between 1.5-4.0 mm, followed by a slowdown tendency as animals increase further in size, which is expected since *imposex* tends to develop more rapidly as females reach sexual maturity. At St. 3 the same general trend was observed though with some oscillations. Hence, the size of *H. ulvae* selected for monitoring programmes has a major influence on the results, i.e. smaller females will cause an underestimation of FPL, VDSI and %I, whilst variation in males' size causes oscillations in RPLI, regardless of TBT pollution levels.

Narcotization of mud snails facilitates animal handling and reduces penis measurement variance, since this organ ceases to contract or relax when the animal is anesthetized. However, this procedure caused an increase of the penis length in females [$FPL = 1.35FPL_b - 0.02$; n = 185, $r^2 = 0.922$; $F = 2156.10$, $p < 0.001$] and in males [$MPL = 1.21MPL_b + 0.35$; n = 105, $r^2 = 0.647$; $F = 188.65$, $p < 0.001$] conducing to a significant difference in FPL and MPL between treatments (Wilcoxon test result for females PL: $W = 853.000$, $p < 0.001$; and for males PL: $W = 5565.000$, $p < 0.001$, respectively). For this reason the comparison of *imposex* parameters based on this organ requires that measurements should always be performed under well standardized narcotization conditions.

H. ulvae imposex levels observed in Ria de Aveiro from 2003 to 2007, the number of animals analysed per site and the respective mean SH are presented in Table 5.1. Data obtained by Barroso et al. (2000) from May to July 1998 are also included in this table for comparison with the current data. FPL, RPLI, VDSI and %I varied respectively between: 0.1-0.4 mm, 6.3-20.0%, 0.4-1.0 and 37.5-72.5% in 2003; 0.1-1.3 mm, 4.1-33.8%, 0.6-1.0 and 50.0-85.0% in 2004; 0.1-0.4 mm, 3.6-10.3%, 0.7-1.1 and 63.4-92.5% in 2007. Sterile females were never found. It was not possible to obtain animals with uniform sizes between the different surveys neither we could observe animals as large as those found in 1998 by Barroso et al. (2000), (Table 5.1; Figure 5.4). From 2003 to 2007, there was a reduction in males SH at St. 1-3, 6 and in females SH at St. 1-3 and 6-8, whilst at St. 10 both genders were larger in 2004 than in 2003 (Table 5.1).

Table 5.1 *Hydrobia ulvae*. Male and female mean shell heights (SH) and the number of specimens analysed for *imposex* (n) are presented per sampling site (St. code) and year with indication of mean male and female penis length (MPL and FPL), relative penis length index (RPLI), vas deferens sequence index (VDSI) and percentage of *imposex* affected females (%I). ♂SH, ♀SH, MPL, FPL and VDSI from 2003 to 2007 were statistically compared (indicated by brackets) and when *post-hoc* tests for multi-comparisons (Dunn's method) were significant, the level is indicated by asterisks. Data for 1998 were published by Barroso et al. (2000).

		St. code									
	Year	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
♂SH (n) (mm)	1998	6.2 (31)	5.5 (30)	6.4 (31)	5.6 (28)	5.9 (28)	6.5 (27)	6.0 (33)	6.6 (26)	5.8 (27)	6.2 (26)
	2003	5.6 (44)	5.1 (20)	5.4 (40)	5.3 (20)	5.2 (20)	5.1 (20)	5.0 (15)	5.0 (20)	5.2 (20)	5.3 (20)
	2004	5.6 (41) *	5.6 (20) *	5.5 (33) *	5.6 (26)	5.4 (21)	4.0 (9) * *	6.1 (20) *	4.9 (20)	-	6.2 (20) ***
	2007	5.3 (29) *	4.5 (28) *	3.7 (19) *	5.2 (21)	5.4 (21)	4.3 (20)	4.5 (13) *	5.3 (20) *	3.0 (1)	-
♀SH (n) (mm)	1998	6.0 (33)	5.4 (31)	6.5 (33)	5.7 (29)	5.8 (28)	6.6 (28)	6.1 (33)	5.9 (27)	5.7 (27)	6.2 (28)
	2003	5.5 (48)	5.0 (40)	5.5 (59)	5.3 (40)	5.0 (40)	5.1 (40)	5.1 (45)	5.1 (40)	5.3 (40)	5.0 (40)
	2004	5.4 (52) *	6.4 (33) * *	5.7 (41) *	5.2 (33)	5.2 (40)	4.5 (20) * *	6.2 (40) * *	5.1 (40) *	-	6.3 (41) ***
	2007	5.2 (37)	4.6 (36) *	3.7 (51) *	5.4 (41)	5.4 (41)	4.3 (27)	4.4 (40) *	5.6 (42) *	3.4 (3)	-
MPL (mm)	1998	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2003	2.9	2.0	2.3	1.9	1.6	1.9	1.7	1.6	2.1	2.0
	2004	3.5 *	3.7 * *	2.3 *	2.9 * *	2.9 * *	2.2 *	4.3 * *	3.1 * *	-	3.1 ***
	2007	2.8 *	3.1 *	2.7	3.7 *	3.6	2.7	3.3 *	3.3	2.8	-
FPL (mm)	1998	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	2003	0.4	0.4	0.2	0.3	0.3	0.2	0.3	0.1	0.3	0.3
	2004	0.3	1.3 *	0.2	0.3	0.1	0.1	0.4	0.2 *	-	0.3
	2007	0.3	0.2 *	0.2	0.2	0.4 *	0.1	0.2	0.2	0.1	-
RPLI (%)	1998	15.0	20.0	14.0	11.0	18.0	4.0	6.0	5.0	10.0	5.0
	2003	12.6	17.2	8.0	13.6	20.0	10.7	17.9	6.3	16.2	12.9
	2004	9.0	33.8	7.4	9.4	4.1	6.3	8.9	5.7	-	11.1
	2007	10.3	7.1	5.9	4.5	10.3	5.4	7.0	7.0	3.6	-
VDSI	1998	0.9	1.0	1.0	0.4	1.1	0.4	0.4	0.2	0.8	0.3
	2003	0.9	1.0	0.6	0.7	0.9	0.6	0.9	0.4	1.0	0.6
	2004	0.8	0.8	0.7	0.9	0.6	0.6	1.0	0.6 *	-	0.8
	2007	1.1	0.9	0.7	0.7	1.1 *	0.7	1.0	0.9	0.7	-
%I	1998	60.0	62.0	68.0	36.0	75.0	50.0	45.0	44.0	88.0	50.0
	2003	64.6	72.5	59.3	45.0	52.5	52.5	57.8	37.5	72.5	50.0
	2004	69.2	75.8	73.2	57.6	57.5	55.0	85.0	50.0	-	65.8
	2007	86.5	86.1	64.7	63.4	78.1	70.4	92.5	81.0	72.7	-

*: $p < 0.05$; ***: $p < 0.001$. - : animals not found. n.a.: not available data.

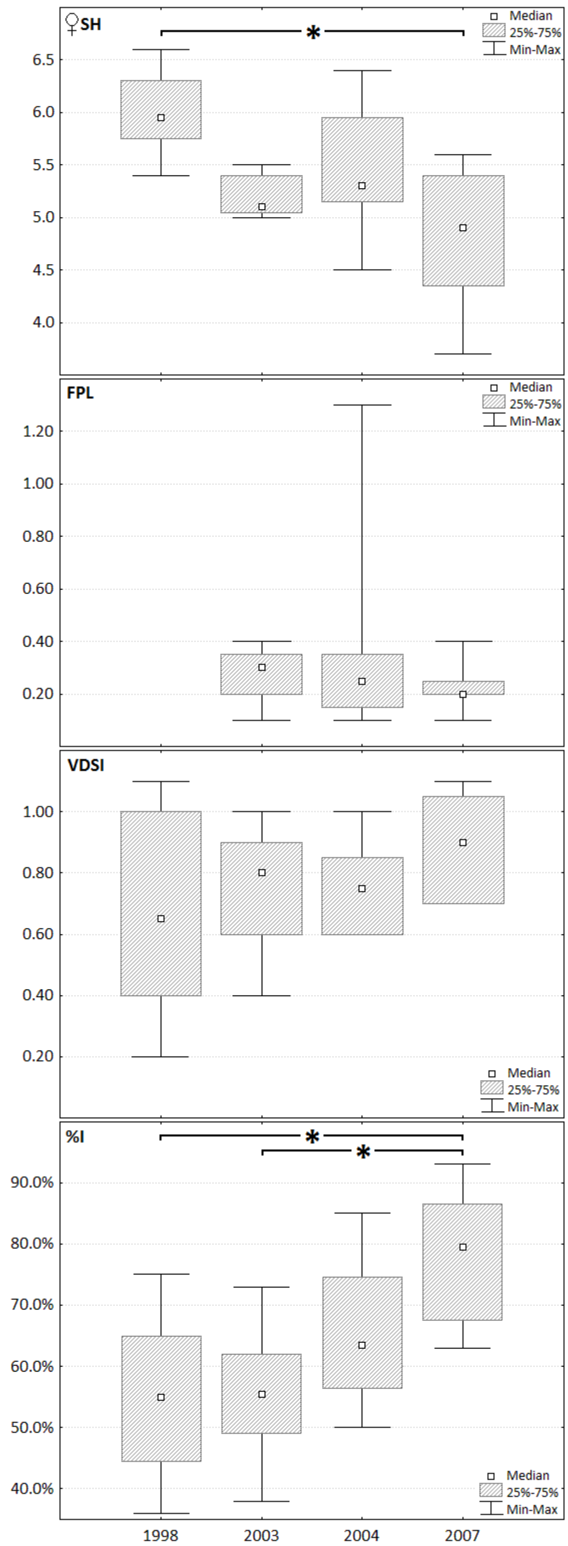

Figure 5.4 *Hydrobia ulvae*. Global temporal trend of females' shell height (♀SH) and *imposex* levels (FPL, VDSI and %I) exhibited by specimens collected at 8 common sites in Ria de Aveiro (NW Portugal): for statistical analysis St. 1-8 were pooled and the median was calculated per year – 1998 (Barroso et al., 2000), 2003, 2004 and 2007 (current study). The significance of the Dunn's test for multi-comparisons between years is indicated on the respective plot (*: $p < 0.05$).

RPLI is an *imposex* parameter that roughly balances the sizes of males and females at each site (Gibbs et al., 1987; Stroben et al., 1992b) but, as stated above, we found that the relationship between penis and male sizes varies throughout time at the same location, which may introduce an additional bias that is hard to control. Hence, this parameter was excluded from temporal comparisons performed in the current study.

The temporal *imposex* evolution analysis performed for each site from 2003 to 2007 revealed that there were no significant differences in FPL and VDS for most of the sampling sites, except the cases which are described below. A significant decrease in FPL was observed at St. 2 and an increase in FPL and VDS was statistically confirmed at St. 8. However, FPL at St. 2 in 2007 might be underestimated, as the mean females' size at that site was significantly lower in 2007. Likewise, the rising of FPL and VDSI in 2007 at St. 8 can simply result from the SH augment from 2004 to 2007 at this site. The %I was not compared statistically under a per site analysis as there is only one reading per local; nevertheless, %I raised progressively from 2003 to 2007 at every sampling site (Table 5.1). Regarding the entire study area analysis no significant differences were observed in FPL from 2003 to 2007 (Friedman result: $s = 1.583$, $p = 0.531$) nor in VDSI from 1998 to 2007 ($s = 3.986$, $p = 0.263$). However, %I varied significantly during the study period ($s = 16.050$, $p = 0.001$) and post-hoc Dunn's tests revealed significant increases between 1998/2007 (Dunn's test: $s = 3.292$, $p < 0.05$) and 2003/2007 ($s = 3.292$, $p < 0.05$), (Figure 5.4).

Parasitized mud snails were all infected by trematodes and occurred at 10%, 56% and 100.0% of the sampling sites in 2003, 2004 and 2007, respectively (Table 5.2). The percentage of parasitized males per site varied between 0-49% in 2004 and 0-50% in 2007, whilst none was found in the 2003 survey. Infected females per site varied between 0-7% in 2003, 0-60% in 2004 and 3-80% in 2007. It is curious to note that all the parasitized females exhibited *imposex* and that parasitism increased over recent years as it happened with %I. As pointed out by Schulte-Oehlmann et al. (1997) the investigations of Krill (1935) and Rothschild (1938) testify the occurrence of small non-functioning penes in *H. ulvae* females before TBT pollution existed, a phenomenon closely related to the infestation with trematode parasites. Nevertheless, our results disclose no significant correlation between *imposex* levels and the prevalence of parasitism across sites in any survey. Anyway, as already stated, we carefully examined the specimens in order to prevent that mud snails with parasites could have passed unnoticed and we excluded these animals from the *imposex* analysis to prevent any bias caused by this factor.

Table 5.2 *Hydrobia ulvae*. The total number (n) of specimens observed in the present study (2003, 2004 and 2007 surveys) and the corresponding percentage of parasitized specimens (P%) are indicated per year and sampling site (St.) for: males (♂), females affected with *imposex* (♀ VDSI ≥ 1) and unaffected females (♀ VDSI = 0).

		♂		♀ VDSI ≥ 1		♀ VDSI = 0	
St.	Year	n	P%	n	P%	n	P%
1	2003	44	0.0	28	7.1	22	0.0
	2004	41	0.0	30	0.0	22	0.0
	2007	29	0.0	41	19.5	4	0.0
2	2003	20	0.0	29	0.0	11	0.0
	2004	20	0.0	25	0.0	8	0.0
	2007	31	9.7	34	8.8	5	0.0
3	2003	40	0.0	34	0.0	25	0.0
	2004	34	2.9	34	2.9	8	0.0
	2007	19	0.0	36	5.6	17	0.0
4	2003	20	0.0	18	0.0	22	0.0
	2004	51	49.0	47	59.6	14	0.0
	2007	30	30.0	45	42.2	15	0.0
5	2003	20	0.0	21	0.0	19	0.0
	2004	22	4.5	23	0.0	17	0.0
	2007	29	27.6	38	15.8	9	0.0
6	2003	20	0.0	21	0.0	19	0.0
	2004	9	0.0	11	0.0	9	0.0
	2007	21	4.8	20	5.0	8	0.0
7	2003	15	0.0	26	0.0	19	0.0
	2004	20	0.0	41	17.1	6	0.0
	2007	13	0.0	42	4.8	3	0.0
8	2003	20	0.0	15	0.0	25	0.0
	2004	20	0.0	20	0.0	20	0.0
	2007	21	4.8	35	2.9	8	0.0
9	2003	20	0.0	29	0.0	11	0.0
	2004	0	0.0	0	0.0	0	0.0
	2007	2	50.0	10	80.0	1	0.0
10	2003	20	0.0	20	0.0	20	0.0
	2004	20	0.0	37	27.0	14	0.0
	2007	0	0.0	0	0.0	0	0.0

5.4 DISCUSSION

The current study shows that *imposex* levels in *H. ulvae* did not decrease in Ria de Aveiro between 1998 and 2007 despite the implementation of the EU Regulation No.782/2003. Instead, for the whole study area there was a global significant increase in the percentage of females affected by *imposex* and a slight increase of VDSI. The mud snails' *imposex* evolution contrasts with what has happened with other indicator species in the same area for the identical period. In fact, a slight decrease of *imposex* levels in *Nassarius reticulatus* between 2003 and 2005 (Sousa et al., 2007) and a highly significant decrease between 2003 and 2008 (A. Sousa, unpublished data) were observed in Ria de Aveiro. On the other hand, a significant reduction of *imposex* intensity in *Nucella lapillus* was observed after 2003 and till 2007 (Galante-Oliveira et al., 2009). It is thus clear that different bioindicators may display different *imposex* temporal evolution trends. Hence, a careful discussion about the possible reasons behind these opposing trends is presented below.

Firstly, it is important to check the correctness of the *imposex* temporal evolution analysis conducted in the current work with mud snails. One obvious pre-requisite is that sampling campaigns from 1998 to 2007 employed identical methods. This was verified with only two exceptions. One regards the decrease in the size of specimens used in the successive surveys (Figure 5.4); however, as this induces an artificial lessening of the *imposex* levels throughout time, *imposex* rising in the study area could not be caused by this factor. The other exception regards the different times of the year when surveys were conducted, but this probably did not have any effect since Silva (2002) observed no significant monthly variation of adult mud snails *imposex* levels at St. 3 between December 1999 and December 2000. One important aspect to be taken into account in *imposex* monitoring programmes using *H. ulvae* is the well known juvenile (SH < 2 mm) dispersal by floating (Armonies, 1992), which occurs in Ria de Aveiro (Meireles and Queiroga, 2004). This may cause some mixing of mud snails from different TBT polluted places. However, this dispersion behaviour at Ria de Aveiro seems to be confined to a very short period in the youngest stage (Meireles and Queiroga, 2004), probably with negligible effect on *imposex* expression in older life. Besides, we minimize the possible bias caused by juveniles' mobility by using adults in the monitoring programmes and, most importantly, by assessing the evolution of *imposex* in the whole study area instead of focusing the analysis solely on isolated locations; either the “whole study area” or “individual site” analysis provided the same conclusion stated above: *imposex* in *H. ulvae* did not decrease over the last years, in a contrary manner to what has been recorded in the other indicator species.

Another methodological aspect in biomonitoring programmes that it is important to mention is the species longevity. This is particularly important when pollution is declining as a consequence of legislative action, since *imposex* is largely irreversible and its reduction can only be registered when older (and most affected) specimens are substituted by younger ones. However, *H. ulvae* presents a short longevity (1 to 5 years; Graham, 1988; Sola, 1996) and, for Ria de Aveiro, Silva (2002) reported longevity of around 2 years, which is very useful for monitoring short-term changes of TBT pollution. The longevity of mud snails is much lower than the reported for *Nucella lapillus* (5 to > 10 years; Crothers, 1985) and than the estimated for *Nassarius reticulatus* in the Ria de Aveiro (about 11 years; Barroso et al., 2005a), and so this is certainly not the cause for the discrepancy noted in the *imposex* evolution in the three species.

We believe from the above discussion that the distinct *imposex* temporal trend found in *H. ulvae* does not result from any methodological bias but rather indicates that mud snails were exposed to a different TBT pollution scenario than the other bioindicators. In fact, the different responses found between the above gastropod species in Ria de Aveiro may simply reflect different biology / ecology traits involving, for instance, distinct routes of TBT uptake and/or bioaccumulation. *H. ulvae* probably accumulates TBT preferentially *via* sediment due to its habitat and diet, as they live on sandy to muddy substrates (Cardoso et al., 2002) and feed on diatoms and bacteria together with sediment particles, and also green algae as *Ulva* and *Enteromorpha* (Newell, 1962 in Barnes, 1974; Jensen and Siegismund, 1980; Nicol, 1935 in Fretter and

Graham, 1994). It is known that *H. ulvae* processes large amounts of sediment during the feeding period: to obtain food, specimens ingest and graze contaminated particles that are then maintained in the gut during the low tide till defecation when the sediment gets re-wetted (Barnes, 2006; Pascal et al., 2008). This way, TBT uptake from the sediment and its bioaccumulation is predicted to be higher in mud snails. On the contrary, *N. lapillus* accumulates TBT preferentially *via* water and food (mainly mussels and barnacles which, by their turn, accumulate TBT from the water column) and so the levels of *imposex* in the dog-whelk predominantly indicate the level of pollution in the water (Bryan et al., 1986; Stroben et al., 1992a; Bryan et al., 1993). Similarly to *H. ulvae*, *N. reticulatus* is a sediment-dweller but adults feed on carrion and do not ingest substrate particles. This nassarid is a scavenger (Tallmark, 1980) in which food was described as contributing more than half of the TBT body burden (Stroben et al., 1992a), minimizing the effect of the direct uptake by the contact with the animal internal / external body surface.

The distribution of *N. lapillus*, *N. reticulatus* and *H. ulvae* in Ria de Aveiro has to be also considered as different areas may have distinct TBT decontamination histories. The muricid and the nassarid occur in the lower estuarine zone (Barroso et al., 2000), where the main port terminals are located (Figure 5.1) but the area is frequently dredged and has faster currents, promoting region cleaning (Lee et al., 2006; Lopes and Dias, 2007). *H. ulvae* extends farthest towards the upper reaches of the channels (Barroso et al., 2000), shallow zones where some marinas are located and where muddy sediment retention / deposition and freshwater influence are higher (Lopes and Dias, 2007). Thus, knowing that: (i) finer estuarine mud has higher affinity for TBT (Vogt et al., 2007), (ii) TBT adsorption to the sediment is greatest in freshwater and decreases at low to intermediate salinities; and (iii) TBT desorption from the sediment is promoted by the reduction of its input in water (Langston and Pope, 1995), it is possible that sediment decontamination is slower in the upper reaches of the estuary. It is well-known that TBT persists adsorbed to anoxic sediment with a half-life of years (Langston and Pope, 1995; de Mora, 1996; Saeki et al., 2007), while its decay in water is much faster with half-lives of days to weeks (Lee, 1989). Hence, the persistence of *imposex* in mud snails over the last years may indicate that sediment continued to bear high levels of TBT which stills a problem in this compartment, in contrast with a rapid amelioration of the problem in water.

The European Union anticipated the adoption of the IMO AFS Convention and so it can serve as a test to evaluate the effectiveness of the legislation in reducing TBT pollution at the global scale. We can foresee that the response of the different prosobranch species around the world may diverge according to the compartment that is being monitored (sediment *vs.* water), and by no way we can assure that female prosobranch masculinization will be completely eradicated in the near future as it is well known that TBT may persist in the sediment for many years.

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Chapter 6

Factors affecting RPSI in *imposex* monitoring studies using *Nucella lapillus* (L.) as bioindicator

Galante-Oliveira et al. (2010) *Journal of Environmental Monitoring* (in press).

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ABSTRACT

Nucella lapillus (L.) is a marine gastropod mollusc widely used as a bioindicator of TBT pollution in the North Atlantic coastlines. The species reproductive cycle and the male penis length seasonal / spatial variation were studied at a single site at Aveiro seashore (NW Portugal) between December 2005 and June 2007. The main objective of this work is to assess if the "Relative Penis Size Index" (RPSI) – an important *imposex* assessment index – varies seasonally and spatially in the same sampling site and how this can affect results obtained in *imposex* monitoring programmes. Animals able to reproduce were found every month but a seasonal pattern in *N. lapillus* reproductive cycle was evident. Female gametogenic maturation varied seasonally and a decrease in capsule gland volume and condition factor occurred in late summer / early autumn. The gametogenesis in males did not show a significant seasonal variation as in females but the condition factor, penis length, amount of sperm and prostate volume also

diminished in late summer / early autumn. On the other hand, males that were close to egg capsules clusters had larger penises than those far away from clusters. The temporal and spatial male penis length variation introduces a bias on *imposex* assessment results when using RPSI and the magnitude of the error involved is evaluated for different TBT pollution levels scenarios. We consider that RPSI provides interesting and complementary information that should not be excluded from monitoring programmes, but temporal or spatial comparisons of *imposex* should be based on other more reliable *imposex* indices like the VDSI.

6.1 INTRODUCTION

The dog-whelk, *Nucella lapillus* (L.), is a marine gastropod mollusc in the family Muricidae (Fish and Fish, 1996). Gregarious and common amongst barnacles (*Balanus spp.*) and mussels (*Mytilus spp.*) on which they preferentially feed (Gibbs, 1999), the species is found on wave exposed to sheltered rocky coasts, from the mid shore downwards. It occurs within a salinity range from 18 to 40 psu and between 0 and 20 ^{0}C isotherms throughout the North Atlantic littoral zone: from the Arctic to the south of Portugal in the east, including Iceland and the Faroe Islands, and from the south west of Greenland to the north of Long Island in the west (Crothers, 1985; Tyler-Walters, 2008).

N. lapillus is a gonochoristic species (separated sexes) with internal fertilization. Individuals can reproduce throughout the year but, in some places, reproduction is restricted to few months (Feare, 1970). The development is oviparous – eggs are laid inside protective vase shaped capsules, attached to hard substrata in crevices and under overhangs; each capsule may contain about 600 eggs, ≈94% of which are unfertilized and designated as “nurse eggs” as they act as food for the developing embryos (Crothers, 1985; Fretter and Graham, 1994; Gibbs, 1999). Without a planktonic larval stage, juveniles emerge from egg capsules as miniatures of adult specimens (Gibbs, 1999).

Although of no relevant commercial value, this species has been largely used in scientific research namely as a bioindicator of tributyltin (TBT) pollution (Gibbs, 1999) since females develop *imposex* – the superimposition of male sexual characters onto females (Smith, 1971; Smith, 1981) – as a specific response to TBT environmental concentration. This species is recommended by the OSPAR JAMP Guidelines for Contaminant-Specific Biological Effects (MEPC, 2008) as the main bioindicator of pollution by this compound. The intensity of *imposex* is positively correlated with the TBT pollution level (Gibbs and Bryan, 1986; Gibbs et al., 1987; Barroso and Moreira, 2002) and may be quantified by several indices: the vas deferens sequence index (VDSI), the relative penis size index (RPSI), the percentage of *imposex* affected females (%I) and the percentage of sterilized females (%S). VDSI can be quantified by using VDS classification schemes that in *N. lapillus* comprises 6 stages increasing females' masculinization as defined by Gibbs et al. (1987), and is the main index adopted by OSPAR Commission. In turn, RPSI relates the females' penis size with that of males', for a given population and is obtained by the formula RPSI = (FPL^3/MPL^3) x100, where FPL is the mean female penis length and MPL is the mean male penis length (Gibbs et al., 1987).

The current work provides the first description of *N. lapillus* reproductive cycle in Portugal and aims to evaluate if any seasonal variation in this cycle, or spatial pattern among individuals at the same rocky shore, may influence *imposex* assessment results.

6.2 MATERIAL AND METHODS

6.2.1 Samples collection and preparation

Random samples of eighty *Nucella lapillus* adults were collected monthly at the mid-tide level, from December 2005 to June 2007, at an hydrodynamically exposed site located on the NW Portuguese Atlantic open coast (40^{o}31'05.90''N 8^{o}47'05.11''W) in the region of Aveiro. Those specimens were divided in two groups, regarding the distance to egg capsules clusters (ECCDist): the first 40 animals found within a distance of 50 cm from capsules clusters were collected and identified as the "near egg capsules clusters" (NECC); the first forty snails found at a distance >50 cm from the edge of any cluster constituted the "distant from egg capsules clusters" (DECC). The distance of 50 cm was set since it had been described as enough to distinguish spawning clusters from winter aggregations in a population in an exposed shore in Yorkshire – UK (Feare, 1968) and based on our observations at the sampling site where labelled animals had showed reduced mobility. The sub-surface (10-20 cm) water salinity and temperature were measured at each sampling occasion and the monthly average air temperature at the sampling site region was obtained from the Aveiro's Port Authority.

Once in the laboratory, animals' shell height (SH – length from the apex to the siphonal canal) was measured with vernier callipers to the nearest 0.01 mm. After drying the water excess on filter paper, specimens were weighted before and after shells removal, to the nearest 0.01 g, in order to calculate the allometric condition factor (CF) according to Ricker (1975); $CF = W/SH^b$ where W is the animal weight and b is the allometric growth constant that was previously calculated by regression analysis using a full range of animal's sizes per gender. Animals were then sexed. Whenever available, 15 females and 15 males from each group (NECC / DECC) were analysed and the examination of the reproductive organs was carried out as described in the next section. Afterwards, individuals were fixed in Bouin's solution for 24h and then preserved in 70% ethanol for histological analysis. The complex gonad / digestive gland was embedded in paraffin and sectioned (5-7 µm). Three slides with 4 gonads' sections each were made by specimen, stained with haematoxylin-eosin and mounted in DPX resin (neutral solution of polystyrene and plasticizers in xylene) for light microscopy observation in order to determine individual gametogenesis stage.

6.2.2 Specimens' *imposex* and reproductive system analysis

6.2.2.1 Macroscopic examination

After shells removal and specimens weighing, males and females penis length (MPL and FPL, respectively) were measured (using a stereo microscope with a graduated eyepiece to the nearest 0.14 mm) and the vas deferens sequence (VDS) stage exhibited by females was assessed using the classification scheme proposed by Gibbs et al. (1987).

The macroscopic examination of the gonads and accessory sexual glands were then performed. The volumes of prostate and capsule glands (SexGlVol) were determined by measuring their width and height (under a stereo microscope with a graduated eyepiece to the nearest 0.14 mm) and approaching their shape to a prolate spheroid, i.e. a spheroid in which the polar diameter is greater than the equatorial diameter. Specifically, glands volumes approached an elongated ellipsoid once two of the three radiuses were considered of the same value (the radiuses across the y and the z-axis; see Figure 6.1). Therefore, volumes were calculated by the formula SexGlVol = $^4/_3.\pi.a^2b$ where *a* is the equatorial radius along the y that is equal to the one along the z-axis (= gland width / 2) and *b* is the polar radius along the x-axis (= gland height / 2). This SexGlVol estimation is not available for entire study period because glands dimensions were only measured since April 2006.

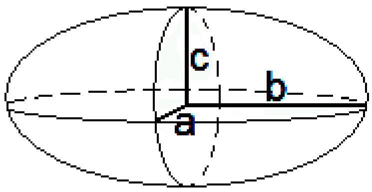

Figure 6.1 Schematic representation of an ellipsoid. a – equatorial radius along the z-axis; b – equatorial radius along the x-axis; and c – polar radius along the y-axis.

The gonads colour and texture were then recorded. Males' ripe sperm volume inside the vesicula seminalis (visible on the columellar side of the visceral whorl) was subjectively classified from 1 ("empty") to 5 ("totally full") for a mere indication of each specimen ripe sperm quantity.

6.2.2.2 Microscopic examination

Microscopic examination of gonad's maturation (MicMat) was classified based on the histological analysis, using a six stages scale – I ("immature"), II ("early recovering"), III ("late recovering"), IV ("ripe"), V ("partially spent") and VI ("spent") –

proposed by Barroso and Moreira (1998) for prosobranch gastropods. Considering that varying proportions of all maturity stages could be observed throughout the year, five follicles from each gonad section were examined (5 follicles x 4 sections x 3 slides = 60 observed follicles) and the individual gametogenesis stage considered was the mode value amongst those registered for the 60 observed follicles.

6.2.3 Statistical data analysis

Imposex assessment indices were calculated per month following the OSPAR Commission Joint Assessment and Monitoring Program (JAMP) guidelines (MEPC, 2008). VDSI monthly values were statistically compared by a Kruskal-Wallis ANOVA since data did not pass the Kolmogorov-Smirnov test for normality. Shell height (SH) between genders was compared using non-parametric statistical tests since data were not normal neither revealed homogeneous variance: the Mann-Whitney U-test was applied to study if SH was generally different between genders, using all individual measurements taken along the studied period; the Kruskal-Wallis ANOVA was used to show if there was any significant pattern in SH between genders per month.

New statistical procedures were implemented in order to study the *Nucella lapillus* reproductive cycle. Basically the linear least squares regression and the ordered logit regression model were applied. Concerning the classic linear least squares regression, there were two problems deserving attention that might arise from the data. One was the non constant errors variance in the linear model. To overcome this problem, a generalized linear model was run using the non constant variance consistent standard errors introduced by White (1980) with some adjustments suggested by Long and Ervin (2000). Comparing to the linear least squares regression, this approach generates new statistically consistent estimates of the parameters estimates variance as well as their t- and *p*-values. The other concern was the non normal errors. Nevertheless, this was not a problem at all once the dataset had a large dimension of observations (above 1000 observations in the all set of analyses). Hence we can make use of the asymptotic theory which ensures that the asymptotic properties of the linear least squares regression estimators guarantee that their distribution is still asymptotically normal, that is for datasets with large number of observations [see, for instance, Johnston and Dinardo (1996)].

The ordered logit regression model was implemented to analyze the gonads microscopic maturation (MicMat). This model can be understood as belonging to the family of the generalized linear models and namely to the models for categorical responses. A seminal book about these models is Maddala (1983) [see also Long (1997) and Agresti (2002) for further details]. As referred above, the variable "MicMat" has six categories: from I ("immature") to VI ("spent") as proposed by Barroso and Moreira (1998). This variable can be considered ordinal in the sense that each one of its values indicates a certain degree of a continuous process of gonads maturation. Obviously, this continuous process cannot be evaluated in a continuous scale so it is a latent, non observable, variable. Therefore, it seems to us that the ordered logit regression model is the more accurate and powerful to make the regression analysis. This can be seen ahead in the Results (section *6.3*).

All these statistical procedures were implemented trough *R* programming (see http://www.r-project.org/). *R* is a powerful open source language and environment for statistical computing. The authors would provide the programming code to those interested in (contact J. A. Santos by josant.santos@gmail.com). The libraries that were used are MASS (for the ordered logit regression model) and CAR (to implement linear hypotheses tests on parameters and to estimate the non constant variance consistent standard errors, according to White (1980) and Long and Ervin (2000).

6.3 RESULTS

The sub-surface water salinity and temperature and the monthly average air temperature during the current study are presented in Figure 6.2. The registered temperatures in both water and air showed a seasonal pattern and, as expected, the fluctuation amplitude was more marked in air then in water.

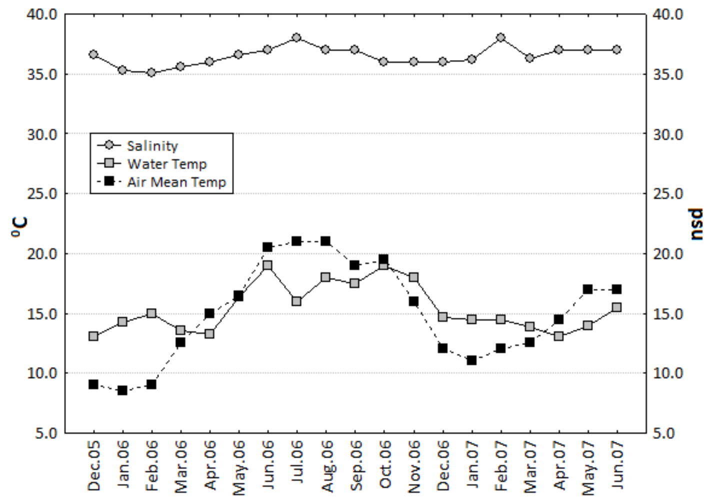

Figure 6.2 Surface water salinity (psu) and temperature (^{0}C) and mean monthly air temperature (^{0}C) at each sampling occasion (from December 2005 to June 2007).

6.3.1 *Imposex* analysis

The vas deferens sequence index (VDSI) presented a minimum value of 1.5 and a maximum of 2.7 during the study period, showing a slight progressive decrease between December 2005 and June 2007 with some minor oscillations (Figure 6.3).

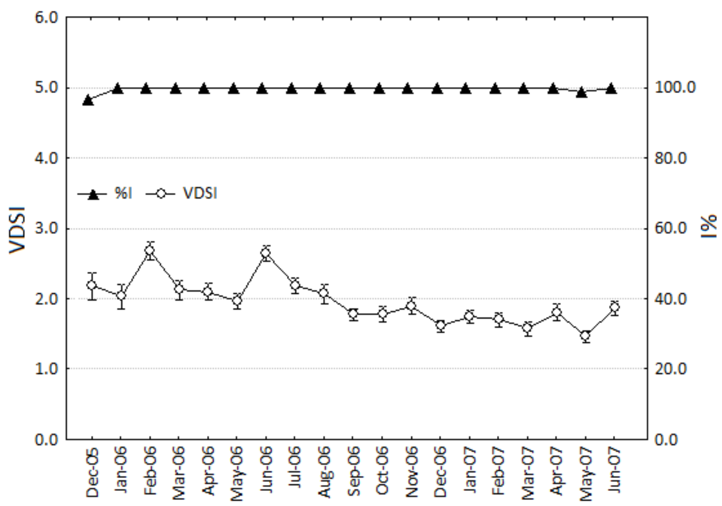

Figure 6.3 *Nucella lapillus*. Vas deferens sequence index (VDSI) exhibited by the females examined from December 2005 to June 2007 and respective standard error. The percentage of *imposex* affected females (%I) per month is also indicated.

Almost all the females were affected by *imposex* at the studied site (%I = 100.0% in 17 of the 19 months; see Figure 6.3) although no sterile females were found (%S = 0.0). The RPSI presented very low values ranging from 0.01 to 0.75% (Figure 6.4) during this study period. It presented a general slight decline over time but with more pronounced oscillations than VDSI and %I (compare Figure 6.3 and Figure 6.4). RPSI is directly dependent on penis length measurements in both genders. Since in males this parameter may be influenced by the reproductive cycle and not by TBT pollution levels (as it is in females), males penis length (MPL) variation was studied in detail to evaluate to what degree it may affect results.

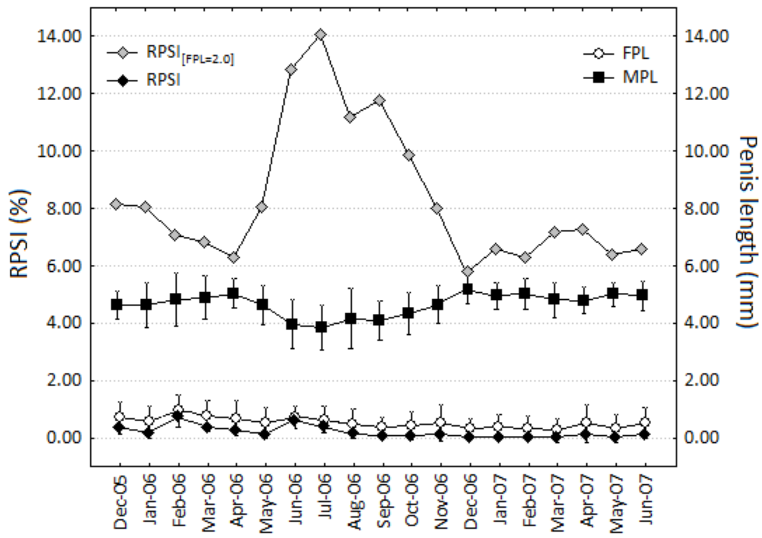

Figure 6.4 *Nucella lapillus*. Monthly mean and respective standard deviation of males and females penis length (MPL and FPL, respectively) from specimens analysed from December 2005 to June 2007. Indication of the relative penis size index (RPSI) values registered per month (calculated from the MPL and FPL plotted). A second RPSI monthly value is presented (RPSI $_{[FPL=2.0]}$) whose calculations were performed using a hypothetical constant FPL of 2.0mm.

6.3.2 Macroscopic examination of the reproductive system

The size of the specimens analysed each month varied throughout the study period (see Figure 6.5). On the other hand, the Mann-Whitney U-test showed that females were in general larger (>SH) than males (s = 1036002; p < 0.001) and the

Kruskal-Wallis ANOVA revealed that SH was significantly higher in females than in males in ≈74% of the samples, i.e. in only 5 of the 19 months the SH in females and males was not significantly different (p > 0.05) considering all animals analysed; see also Figure 6.5).

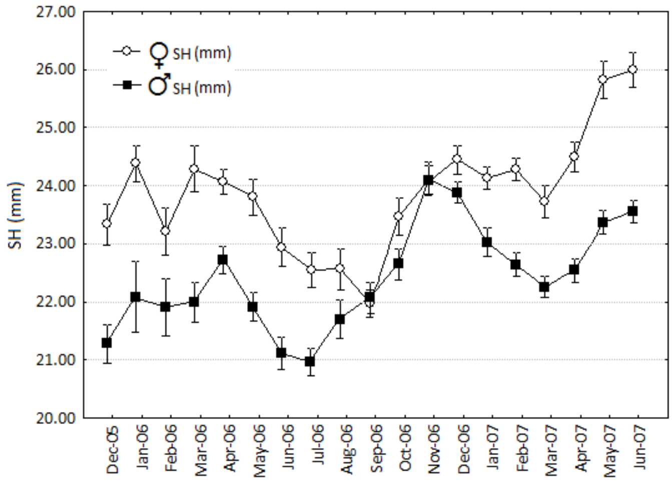

Figure 6.5 *Nucella lapillus*. Females (♀) and males (♂) mean shell height (SH), and respective standard error, of the specimens examined from each month sample, from December 2005 to June 2007.

As the development of the reproductive system may depend on the animal's size (see below), the seasonal or spatial trends of the reproductive cycle can only be properly studied if the effect of SH variation is removed from the analysis. Therefore, we conduce a statistical analysis regarding the morphological variation of sexual organs and accessory glands throughout seasons and distance to egg capsules clusters (ECCDist), taking into account the effect of the individual SH on the results.

Seasonality was represented by air temperature (see Figure 6.2) rather than water temperature for two reasons: (i) water temperature was taken from a single measurement per month whereas air temperature is an average of many measurements; (ii) animals were collected from the mid tide level in an exposed site being largely influenced by air temperature as indicated by Feare (1968) for a similar shore in Yorkshire – UK.

Concerning males penis length (MPL), all the regressors are highly significant (Table 6.1, lines 2-4): larger males (>SH) present longer penis (Table 1, line 2; Figure 6.5 and Figure 6.6); the T_{air} has a negative effect on this variable (Table 6.1, line 3), i.e.

there is seasonality in the MPL achieving higher values during coldest months (compare Figure 6.2 and Figure 6.6); the ECCDist has also a negative effect (Table 6.1, line 4), which means that males located nearer egg capsules clusters (NECC) have longer penis comparing to those that are further apart from egg capsules clusters (Figure 6.6). This multiple regression is globally significant (Table 6.1, line 5) with the model regressors explaining 45.1% of the MPL variation (Table 6.1, line 6).

Table 6.1 Linear Least Squares Regression (corrected for non constant variance consistent standard errors) results for the variable male penis length (MPL). β_0, β_1, β_2, β_3: coefficients; $\mathcal{E}$: associated error; SH: shell height; T_{air}: mean monthly air temperature; ECCDist: egg capsules clusters distance.

$$MPL = \beta_0 + \beta_1 SH + \beta_2 T_{air} + \beta_3 ECCDist + \mathcal{E}$$

	Coefficients	Estimate	F-value	*p*-value	
1	Intercept	1.6839	7.816[a]	$1.3e^{-14}$	***
2	SH	0.1828	411.669	$< 2.2e^{-16}$	***
3	T_{air}	-0.0667	157.595	$< 2.2e^{-16}$	***
4	ECCDist	-0.2466	42.555	$1.0e^{-10}$	***
5	F statistic		302.0	$< 2.2e^{-16}$	***
6	Adjusted R^2		0.4509		

[a] t-value; ***: p<0.001.

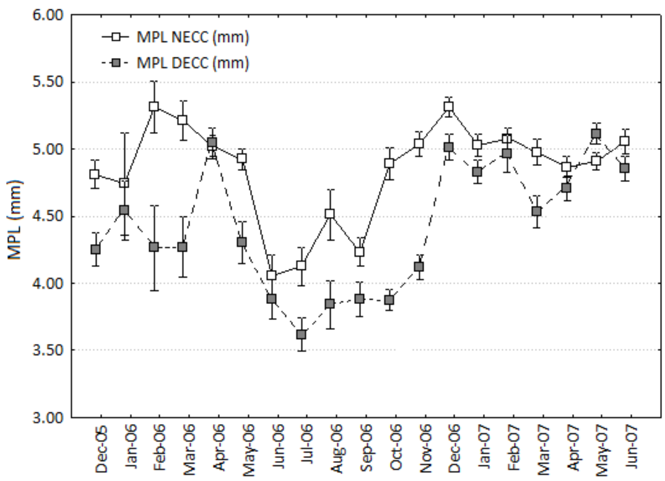

Figure 6.6 *Nucella lapillus*. Mean males' penis length (MPL), and respective standard error, of the animals analysed in each month, from December 2005 to June 2007, and collected near and distant from egg capsules clusters (NECC and DECC, respectively).

The results regarding the variable "sexual gland volume" (SexGlVol), are presented in Table 6.2 and Figure 6.7.

Table 6.2 Linear Least Squares Regression (corrected for non constant variance) results for the variable sexual gland volume (SexGlVol). β_0, β_1, β_2, β_3, β_4, β_5, β_6, β_7: coefficients; $\mathcal{E}$: associated error; Sex: gender (♀/♂); SH: shell height; T_{air}: mean monthly air temperature; ECCDist: egg capsules clusters distance.

$$SexGlVol = \beta_0 + \beta_1 Sex + \beta_2 SH + \beta_3 T_{air} + \beta_4 ECCDist + \beta_5 Sex\ SH + \beta_6 Sex\ T_{air} + \beta_7 Sex\ ECCDist + \mathcal{E}$$

		Coefficients	Estimate	F-value	*p*-value	
	1	Intercept	-86.5599	-9.289[a]	$< 2.0e^{-16}$	***
	2	Sex	62.1622	897.109	$2.2e^{-16}$	***
♀	3	SH	7.5224	270.283	$< 2.2e^{-16}$	***
	4	T_{air}	-1.9469	47.844	$7.1e^{-12}$	***
	5	ECCDist	-10.4283	6.614	0.0102	*
♀ *vs* ♂	6	Sex:SH	-5.3133	99.730	$< 2.2e^{-16}$	***
	7	Sex:T_{air}	1.4049	16.078	$6.4e^{-05}$	***
	8	Sex:ECCDist	9.6383	18.582	$1.7e^{-05}$	***
♂	9	SH	2.2090	142.640	$2.2e^{-16}$	***
	10	T_{air}	-0.5420	30.408	$4.2e^{-08}$	***
	11	ECCDist	-0.7900	1.591	0.208	
	12	F statistic		311.5	$< 2.2e^{-16}$	***
	13	Adjusted R^2		0.6125		

[a] t-value; *: $p < 0.05$; ***: $p < 0.001$.

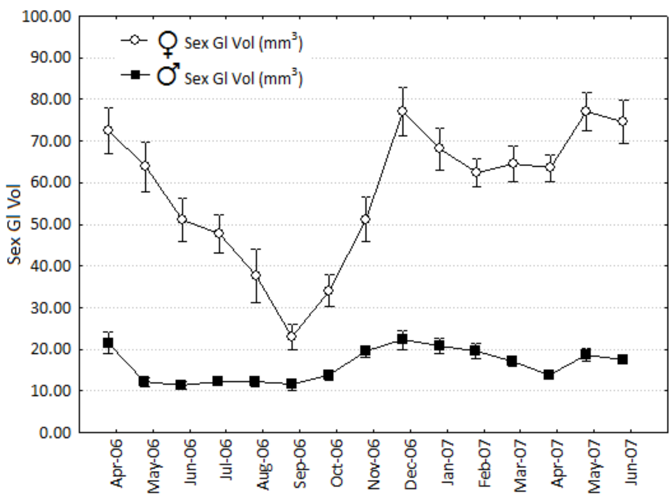

Figure 6.7 *Nucella lapillus*. Mean of the females (♀ - capsule gland) and males (♂ - prostate) sexual gland volume (SexGlVol), and respective standard error, calculated individually for all the animals analysed in each month, from April 2006 to June 2007.

The "Sex" coefficient has no biological meaning and is considered only for modelling purposes (Table 6.2, line 2), since it is an estimation of the response variable out of the range of its values (when all the other regressors take the value zero, which is out of question). All tested regressors had a significant effect on the capsule gland volume concerning females (Table 6.2, line 3-5): larger females (with >SH) present higher SexGlVol (Table 6.2, line 3); "T_{air}" has a negative effect on the variable (Table 6.2, line 4), which means that there is seasonality in the SexGlVol, reaching higher values during the coldest months (compare Figure 6.2 and Figure 6.7); the ECCDist has also a negative effect on this variable (Table 6.2, line 5), that is, NECC females have larger capsules glands. The males' regressors coefficients differ significantly from the females' counterparts (Table 6.2, line 6-8): "SH" and "T_{air}" keep the same sign and are significant as in females (Table 6.2, line 9-10) but their absolute values decrease and the "ECCDist" turns out to be not significant (Table 6.2, line 11). Specifically, larger males present significantly larger prostates (males' SexGlVol) (Table 6.2, line 9) and a significant SexGlVol seasonality occurs, presenting higher values during the months with lower temperatures (Table 6.2, line 10; compare Figure 6.2 and Figure 6.7), but no statistical difference is observed between prostate volumes from animals located NECC and DECC (Table 6.2, line 11). This regression is also globally significant (Table 6.2, line 12) and the model regressors showed to have a good explanatory power (61.3%) that has given by the adjusted R^2 (Table 6.2, line 13).

Regarding the gonads macroscopic aspect, there was no evident seasonality. Ovaries colour was variable throughout the study period, although immature ones (white to creamish and of granular texture; see Feare, 1970) were more frequent during the hottest months (Figure 6.2). Immature testes occurrence (greenish; see Feare, 1970) was much less frequent than the immature ovaries condition. Even so, mature testes were less frequent during the hottest season which also implies a decrease of the male gonad activity during summer. Noticeable was also the substantial reduction of the sperm volume inside the vesicula seminalis in the warmer season, from June to October 2006 (Figure 6.8).

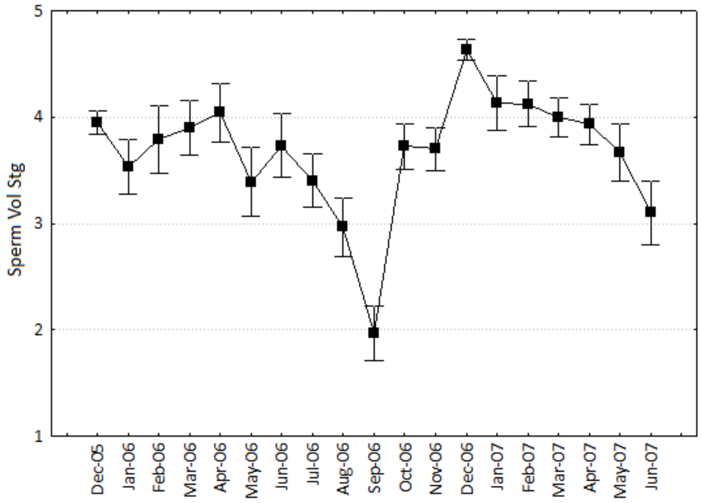

Figure 6.8 *Nucella lapillus*. Mean males' sperm volume stage (Sperm Vol Stg), and respective standard error, of specimens examined in each month, from December 2005 to June 2007 (calculated after the individual macroscopic classification in 5 stages).

6.3.3 Microscopic examination of the gonads

The histological aspect of the different MicMat stages is showed in the photographs of Figure 6.9. The number of animals analysed per MicMat stage is indicated in Figure 6.10 and the mean MicMat stage per month is presented in Figure 6.11 for both genders.

The most frequent gametogenesis stage observed in females was IV while in males was V (Figure 6.10). Results of the ordered logit regression model for this ordinal variable are presented in Table 6.3 and confirm that the gametogenesis stage is generally more advanced in males than in females (Table 6.3, line 1).

Regarding females, all tested regressors had a significant effect on MicMat (Table 6.3, line 2-4): larger animals (>SH) present higher gametogenesis stages (Table 6.3, line 2); the "T_{air}" has a negative effect on the variable (Table 6.3, line 3), i.e., there is seasonality in the gametogenic cycle with the occurrence of lower stages during the hottest months (compare Figure 6.2 and Figure 6.11); more advanced stages of gametogenesis are found distant from egg capsules clusters (DECC), (Table 6.3, line 4; see also Figure 6.10).

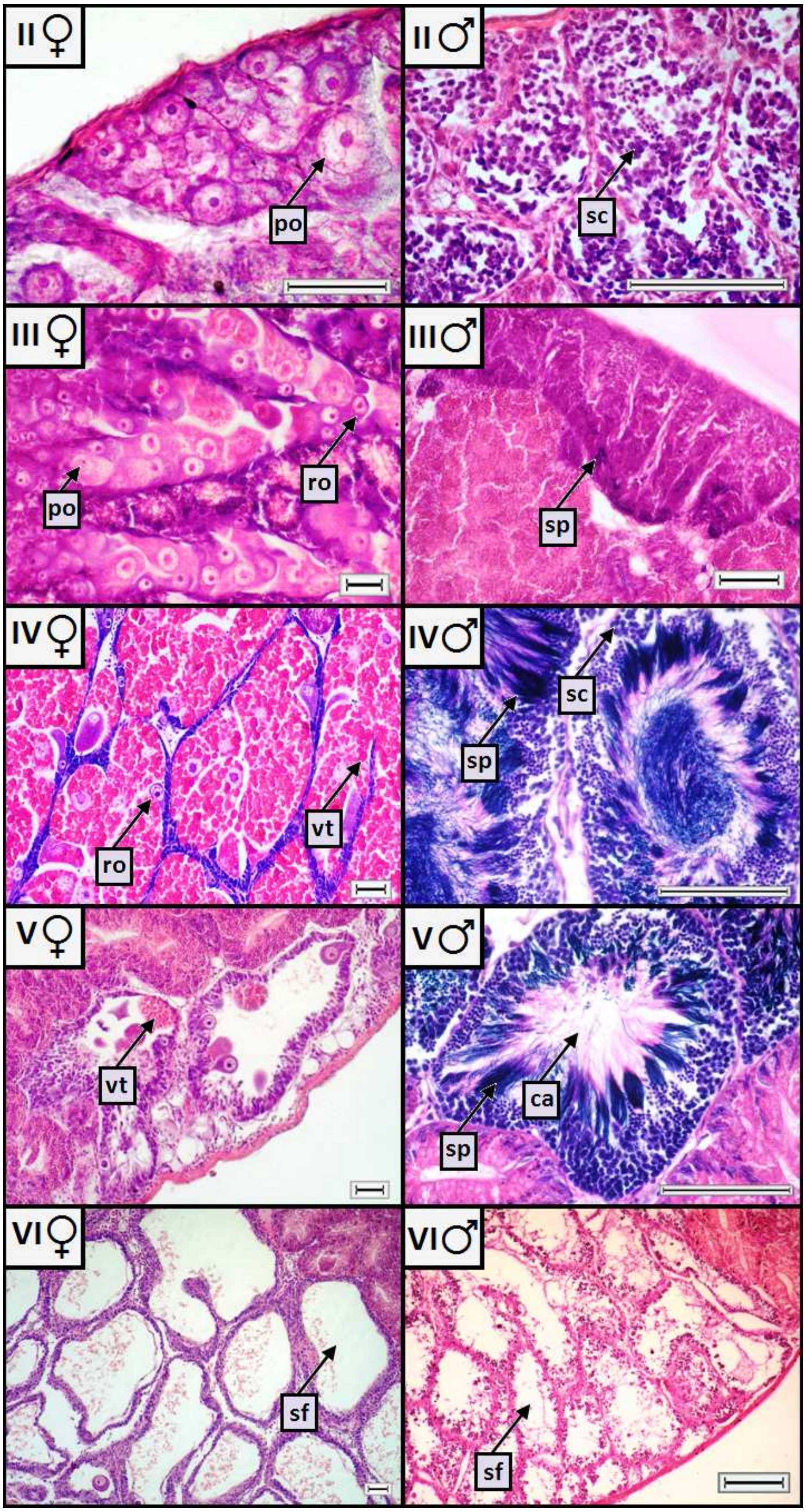

Figure 6.9 *Nucella lapillus*. Gametogenesis stages (indicated on the top-left corner of each picture) of ovaries (left column) and testis (right column); the line on the bottom right hand side of each picture constitutes the respective scale and is always equal to 100 µm. ca: cavity after sperm shed; po: previtellogenic oocyte; ro: ripe oocyte; sc: spermatocyte; sf: spent follicle; sp: spermatozoa; vt: vitelum.

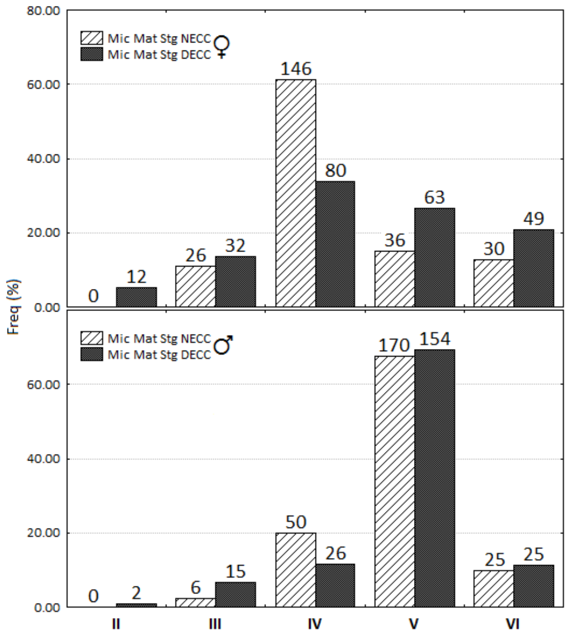

Figure 6.10 *Nucella lapillus*. Microscopic maturation stage (MicMat Stg) frequency (Freq %) of females (♀, top plot) and males (♂, bottom plot) analysed during the present study (every month from December 2005 to June 2007) and collected near and distant from egg capsules clusters (NECC and DECC, respectively). The number of animals involved in the microscopic maturation analysis is indicated on the top of the respective plot and bar.

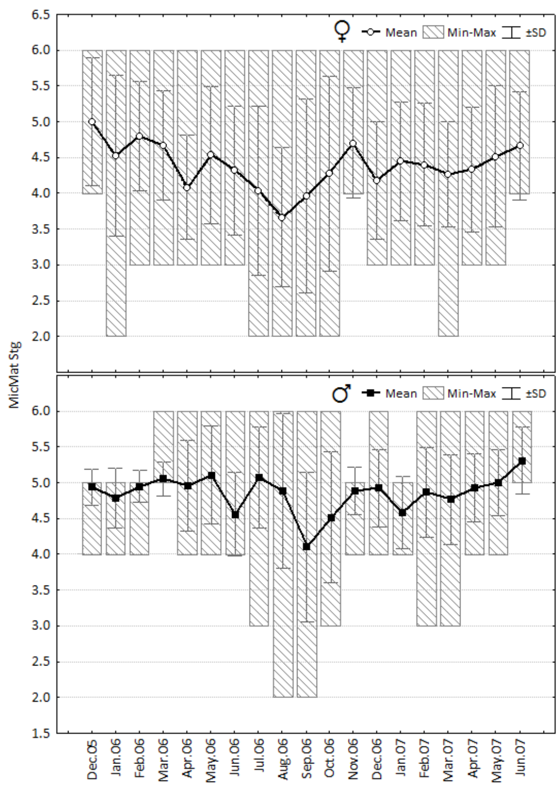

Figure 6.11 *Nucella lapillus*. Females (♀, top plot) and males (♂, bottom plot) microscopic maturation stage (MicMat Stg) mean, standard deviation (SD) and minimum and maximum (Min-Max) values, registered per month (from December 2005 to June 2007).

Table 6.3 Results of the Latent Variable Logit Model (Ordered Logit Model) for the ordinal variable microscopic maturation (MicMat). MicMat*: latent continuous variable of the ordinal MicMat; β_0, β_1, β_2, β_3, β_4, β_5, β_6: coefficients; $\mathcal{E}$: associated error; Sex: gender (♀/♂); SH: shell height; T_{air}: mean monthly air temperature; ECCDist: egg capsules clusters distance.

$$MicMat^* = \beta_0 Sex + \beta_1 SH + \beta_2 T_{air} + \beta_3 ECCDist + \beta_4 Sex\ SH + \beta_5 Sex\ T_{air} + \beta_6 Sex\ ECCDist + \mathcal{E}$$

		Coefficients	Value	t-value	*p*-value	
	1	Sex	2.9654	2.203	0.0276	*
♀	2	SH	0.2433	6.680	$2.4e^{-11}$	***
	3	T_{air}	-0.0856	-3.612	0.000303	***
	4	ECCDist	0.5496	3.045	0.00232	**
♀ *vs* ♂	5	Sex:SH	-0.0945	-1.763	0.0779	
	6	Sex:T_{air}	0.0666	2.094	0.0362	*
	7	Sex:ECCDist	-0.3875	-1.563	0.118	
♂	8	SH	0.1488		0.000193	***
	9	T_{air}	-0.0189		0.374	
	10	ECCDist	0.1621		0.342	
		Intercepts				
	11	II/III	0.8881	0.915	0.360	
	12	III/IV	2.9826	3.152	0.00162	**
	13	IV/V	5.1903	5.410	$6.3e^{-08}$	***
	14	V/VI	7.6071	7.821	$5.3e^{-15}$	***

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

The males' gametogenic cycle differs significantly from the females' regarding the effect of "T_{air}" (Table 6.3, line 5-7). Larger males' also present higher gametogenesis stages (Table 6.3, line 8) but they neither present a significant seasonality pattern in the gametogenic cycle (Table 6.3, line 9) nor differences in relation to their position regarding egg capsules clusters (Table 6.3, line 10; Figure 6.10). The lower part of Table 6.3 (line 11-14) indicates the thresholds (also referred as cutpoints) which states the correspondence between the underlying latent continuous variable "MicMat*" (reflecting the continuous process of the gametogenic cycle) and the observed ordinal variable "MicMat" (resultant from the classification after microscopic observation).

The results of the linear least squares regression with non constant variance consistent standard errors for the variable "alometric condition factor" (CF) are presented in Table 6.4. Similarly to "SexGlVol" regression (see the previous item), the "Sex" coefficient has no biological meaning (Table 6.4, line 2). There is seasonality in the females CF, with better condition achieved during the coldest months (Table 6.4, line 3) but the "ECCDist" proved to be not significant on females (Table 6.4, line 4). The effect of seasonality is not statistically different between genders (Table 6.4, line 5) and males' CF also increases during colder months (Table 6.4, line 7). Regarding the effect of the distance to egg capsules clusters (ECCDist), although there is a statistical difference between genders (Table 6.4, line 6) it is not significant for males as well (Table 6.4, line 8). Even though the regression is globally significant (Table 6.4, line 9), the model regressors' explanatory power was not so good has given by the adjusted R^2 (27.9%) (Table 6.4, line 10).

Table 6.4 Linear Least Squares Regression (corrected for non constant variance consistent standard errors) results for the variable alometric condition factor (CF). β_0, β_1, β_2, β_3, β_4, β_5: coefficients; $\mathcal{E}$: associated error; Sex: gender (♀/♂); T_{air}: mean monthly air temperature; ECCDist: egg capsules clusters distance.

$$CF = \beta_0 + \beta_1 Sex + \beta_2 T_{air} + \beta_3 ECCDist + \beta_4 Sex\, T_{air} + \beta_5 Sex\, ECCDist + \mathcal{E}$$

		Coefficients	Estimate	F-value	*p*-value	
	1	Intercept	0.2971	41.028[a]	< $2.0e^{-16}$	***
	2	Sex	-0.0690	448.785	< $2.2e^{-16}$	***
♀	3	T_{air}	-0.0023	37.190	$1.5e^{-09}$	***
	4	ECCDist	-0.0064	0.003	0.955	
♀ *vs* ♂	5	Sex:T_{air}	0.0009	2.080	0.149	
	6	Sex:ECCDist	0.0109	4.364	0.037	*
♂	7	T_{air}	-0.0014	9.925	0.002	**
	8	ECCDist	0.0045	1.878	0.171	
	9	F statistic		87.6	< $2.2e^{-16}$	***
	10	Adjusted R^2		0.2786		

[a] t-value; * p<0.05; ** p<0.01; *** p<0.001.

6.4 DISCUSSION AND CONCLUSIONS

Hardly any report on *Nucella lapillus* reproductive cycle in the southern range of the species European distribution could be found in the literature. Even the ones describing populations' sexual dynamics at other regions, when available, are old and conclusions are diverse. Various spawning periods were recorded over time: Campbell (1904) found egg capsules in March at Bell Rock – Scotland (in Feare, 1968); Colton

(1916) observed hatching occurrence in August at Mount Desert – Maine, and assuming a capsules development time of four months, spawning must have occurred in April-May (in Moore, 1938); Kostitzine (1934) noted that reproduction was seasonal at Roscoff – France, and synchronised through the population, although giving no specific periods (in Feare, 1970); Pelseneer (1935) cited Peach giving January to April, and Garstang giving the same period and also September, as the main spawning periods in Wick – Scotland and the Southeast coast of Devon – UK, respectively (in Feare, 1968); Moore (1938) found egg capsules throughout the year at Plymouth – UK, but indicated the principal spawning period as the early summer; and Feare (1968, 1970) described breeding in April-May at Robin Hood's Bay, Yorkshire – UK, at the end of the winter aggregation period, and a second spawning period in August. Nevertheless, based on the above descriptions spring and summer are apparently the main breeding period in dog-whelks.

Similarly to what was described by Feare (1970) for a population in the North Yorkshire, varying proportions of gametes at different maturity stages were observed at the same gonad / individual at Aveiro seashore. Nevertheless, a mode was easily depicted in each animal which allowed tracking the monthly gametogenesis evolution in this population from December 2005 to June 2007. We have found every month individuals able to reproduce and recent laid egg capsules (data on egg capsules to be reported elsewhere).

Nevertheless, there is clear evidence that *Nucella lapillus* reproductive cycle has a seasonal pattern at the studied site (Table 6.1 to Table 6.4). In females, higher frequency of MicMat stages associated to spawning (i.e. IV, V, and VI) occurred mainly in the winter and spring (Figure 6.2 and Figure 6.11; Table 6.3) and the statistical regression models also gave evidence that the capsule gland volume is higher (Table 6.2; see also Figure 6.2 and Figure 6.7) and the condition factor is better (Table 6.4) in this period of the year. Comparing to females, we found a higher proportion of mature males ready for breeding all year round. The gametogenesis stage is also significantly more advanced in males than in females (Table 6.3, line 1) denoting that males mature earlier and are therefore able to copulate the females so that ripe oocytes can be fertilised just before they are encased in the capsule gland.

A synchrony of both genders maturation is observed, in part corroborating Kostitzine (1934) results showing that reproduction was seasonal and synchronised through the population at Roscoff – France (in Feare, 1970). In our case, the MicMat maturation in males did not show a significant seasonal variation as in females (although T_{air} coefficient was also negative; Table 6.3, line 9) but the penis and the prostate were significantly larger in the winter / spring (see Table 6.1, line 3; Table 6.2, line 10; and Figure 6.2, Figure 6.6 to Figure 6.7), similarly to what happened to female's capsule gland. The volume of sperm was also highest in this season (Figure 6.8). This suggests that, although males are mature for almost all year, their breeding activity is significantly more intense in the winter / spring, as in females. The breeding activity then decreases, with the temperature rise, in both genders, so that in the end of the summer the capsule gland in females and the prostate, penis and seminal vesicle in males attain their lowest sizes (Table 6.1 and Table 6.2; Figure 6.2, Figure 6.6 to Figure 6.8).

Besides the seasonal variation in the reproductive cycle, we were interested to know if there were also spatial variations in this cycle among individuals collected at the same rocky shore, namely those that are near or distant from egg capsules clusters (NECC or DECC, respectively). Near ECC we found females with significantly larger capsule glands (Table 6.2, line 5) and lower gametogenesis stage (Table 6.3, line 4) with a higher proportion of "ready to breed" females in stage IV (Figure 6.10), which denotes an increased potential for egg laying activity in these spots. In males, the prostate gland volume and the gametogenesis stage showed no differences related to their position regarding ECC. However, males located NECC exhibited longer penis (Table 6.1, line 4; Figure 6.6), giving support in a certain way to what was observed by Feare (1970), i.e. that the fertilization occurs immediately before spawning. The seasonal pattern in the male penis length (MPL) referred above also supports this idea, i.e., longer penis occurs during coldest months when females are more active for spawning.

Nucella lapillus is largely used as a bioindicator of TBT pollution throughout the Atlantic coasts. Our data show a decrease of *imposex* intensity in the studied site from

December 2005 to June 2007 (Figure 6.3 and Figure 6.4), but this decline started at least since August 2005, a time when *N. lapillus* presented a VDSI=1.7 and a RPSI=8.7 (Galante-Oliveira et al., 2009). These results corroborate the findings obtained by other authors for the region of Aveiro (Sousa et al., 2007; Galante-Oliveira et al., 2009), indicating that legislation banning the use of TBT in AF paints in 2003 is producing a recovery of *imposex*. This pollution decline seems to be the actual dominant scenario in Europe (Bray and Langston, 2006) and perhaps will be in the rest of the world following the implementation of the IMO AFS Convention in 2008. In this context, the temporal evolution analysis of *imposex* is of particular relevance and methods should be rigorously validated.

As shown above, RPSI is dependent on the male penis length that varies with season and the animals' position regarding egg capsules clusters and, consequently, temporal comparisons based solely on RPSI may give wrong conclusions. A good example of this is provided in Figure 6.4. At the study site the MPL varied between 3.8 mm in the summer and 5.2 mm in the winter. Considering a hypothetical constant FPL of around 2 mm (as it was observed earlier in the summer of 2005), the RPSI in the two seasons would be respectively 14.6% and 5.7% (see Figure 6.4). This means that a difference of about three fold could be artificially created by the different sampling dates, regardless the level of TBT pollution. Therefore one would wrongly conclude that there was an increase in *imposex* if sampling would have occurred in the winter 2005 and in the summer 2006. This difference would be much greater if FPL would be higher (at places with higher TBT pollution levels). When FPL is very low, as it happened after December 2005 in this sampling site, the RPSI tends to zero and the influence of seasonality is less pronounced, with only a slight increase of RPSI in summer (Figure 6.4). Hence, due to the MPL seasonality, *imposex* monitoring surveys can give different results, and desirably animals should be collected at the same year period in pluriannual monitoring programmes to reduce this problem. MPL seasonal variation was already referred as inducing bias on RPSI levels assessment in narcotized *Nucella lapillus* (Oehlmann et al., 1991; Davies et al., 1997) from north Europe but the current study confirms this conclusion for non-narcotized animals (as recommended by OSPAR) and for the southern range of the species distribution. In addition, a special care has also to be taken by using similar size animals in consecutive monitoring surveys – as larger males present longer penis – and also the position in the rocky shore regarding the distance to egg capsules clusters (ECCDist) – as males near ECC also present longer penis. For instance, in December 2005 males with SH = 21.0 mm had MPL = 4.2 mm DECC and in December 2006 males with SH = 24.0 mm had MPL = 5.0 mm DECC (see Figure 6.5 and Figure 6.6); for a given constant FPL=2.0 mm, RPSI would had been 10.8% in 2005 and 6.4% in 2006. So, even if pollution levels did not suffer any change the RPSI would wrongly indicate a decrease in TBT environmental levels. Moreover, if in December 2006 males would had been collected NECC (MPL = 5.3 mm), the RPSI in 2006 would be 5.4%. So, for monitoring purposes, individuals should not differ significantly in size and should be from about the same location in relation to ECC (or near or far from ECC) to allow truthful comparisons.

Even though bioindicators / biomarkers are extremely useful for environmental monitoring, a careful validation to avoid inaccurate readings is needed. *Imposex* is undoubtedly one of the best biomarkers in pollution monitoring – e.g. *imposex* biomonitoring is mandatory by OSPAR (MEPC, 2008) – and RPSI is an useful index in view of its power to elucidate the relative dimensions of penis in affected females as well as the magnitude of TBT pollution at a given site. This work intends to better validate the interpretation of this index so that one should have in mind that variations of its value may not necessarily reflect a change of the TBT pollution level but rather oscillations related to the sampling season, the animals size or even the distance from ECC, not to mention the problems related to the Dumpton Syndrome (Gibbs, 1993; Huet et al., 1996; Barreiro et al., 1999; Quintela et al., 2002; Gibbs, 2005; Huet et al., 2008).

Considering that the common practice is to use several *imposex* indices in TBT monitoring programmes, among which VDSI render robust and biologically meaningful results, the possible RPSI bias referred above can be detected and controlled.

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Chapter 7

Nucella lapillus (L.) *imposex* levels after legislation prohibiting TBT antifoulants: temporal trends from 2003 to 2008 along the Portuguese coast

Galante-Oliveira et al. (2010) *Marine Ecology-Progress Series* (submitted).

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ABSTRACT

Nucella lapillus imposex levels were assessed along the mainland Portuguese coast in 2006 and 2008 and were compared with available data from 2003 for the same area. As specimens' size has been described as a factor inducing variation in some of the *imposex* assessment indices, thus conducing to less reliable results, new monitoring and data analysis procedures are described and applied to study *imposex* levels evolution from 2003 to 2008. A significant global decline in *imposex* intensity was observed in the study area during the referred period and the Portuguese coast ecological status, under the terms defined by the OSPAR Commission, has notably improved after 2003 confirming the effectiveness of the Regulation (EC) No.782/2003 in reducing TBT pollution. Nevertheless, *N. lapillus*

populations are still extensively affected by *imposex* and fresh TBT inputs were detected in seawater throughout the coast in 2006. These recent inputs are attributed to vessels still carrying TBT antifoulants applied before 2003, as their presence was only forbidden in 2008. Considering that Regulation (EC) No.782/2003 is an anticipation of the IMO global ban entered into force in September 2008, a worldwide-scale decrease in TBT pollution can be expected in the near future.

7.1 INTRODUCTION

For over four decades, tributyltin (TBT) compounds were extensively used as powerful biocides in antifouling (AF) paints applied on submerged structures to prevent bioincrustation (Yebra et al., 2004). However, its extreme toxicity has resulted in numerous and widespread adverse biological effects in non-target organisms, namely *imposex* – the superimposition of male sexual characters, such as vas deferens and/or penis, onto prosobranch female’s (Smith, 1971; Smith, 1981). Extreme cases of complete female functional sterilization, population declines and extinctions were among the reported consequences of *imposex* in many species (Bryan et al., 1986; Gibbs and Bryan, 1986).

In 2001 the International Maritime Organization (IMO) adopted the “International Convention on the Control of Harmful Antifouling Systems on Ships” (AFS Convention), calling for a worldwide prohibition on the application of organotins (OTs) as biocides in AF paints on ships by the effective date of 1 January 2003, and a complete banishment by 1 January 2008 (IMO, 2001). However, this resolution could legally enter into force only 12 months after 25 States representing 25% of the world's merchant shipping tonnage had ratified it. Until then, the legal effect of 1 January 2003 would be suspended. Meanwhile, European Union (EU) countries anticipated the AFS Convention by implementing Regulation (EC) No.782/2003, prohibiting the application or re-application of TBT coatings on Member States’ national mercantile fleets and on ships operating under their authority, from 1 July 2003. The AFS Convention entry into force date was met on 17 September 2007, with the 25th State ratification representing a total of 38% of the world’s merchant shipping tonnage (IMO, 2009). As a result, TBT is globally forbidden from 17 September 2008.

TBT compounds are on the Oslo and Paris (OSPAR) Commission "List of chemicals for priority action" (OSPAR, 2007) and also on the Water Framework Directive 2000/60/EC (2000). Besides the chemical monitoring of TBT environmental concentrations, *imposex* assessment is also a mandatory element of OSPAR Co-ordinated Environmental Monitoring Programme (CEMP; OSPAR, 2008a). *Imposex* development can be followed by the vas deferens sequence (VDS) and its intensity quantified, amongst other parameters, by using VDS classification schemes. For *N. lapillus*, a 6 stages scheme was proposed by Gibbs et al. (1987): from *0* to *6*, in an increasing females' masculinization scale. For a determined sample, females VDS stage mean constitutes the VDS index (VDSI) which is an indication of *imposex* intensity in the sampled population. The other parameters recommended for TBT pollution biological effects monitoring in this species are: mean females' penis length (FPL), relative penis size index (RPSI), percentage of *imposex* affected females (%I) and percentage of sterile females (%S), (Gibbs et al., 1987).

OSPAR Commission also adopted specific guidelines to monitor *imposex* in some gastropod species (see Technical Annex 3 in OSPAR, 2008b) in which a clear indication is made regarding the use of *N. lapillus* as the main bioindicator. Also arising through the OSPAR CEMP, some assessment criteria for *imposex* in different species were developed and Ecological Quality Objectives (EcoQO) set. For *N. lapillus* these assessment criteria define 5 classes (*A*-*E*) through VDSI intervals (from VDSI < 0.3 to VDSI ≥ 5) and the EcoQO for *imposex* in this species corresponds to VDSI values below 2 (the limit between assessment classes *B* and *C*). As it was set for an extremely sensitive indicator, this EcoQO would measure the effectiveness of the international agreements to phase out and prohibit TBT-based AF paints usage, and the recovery progress of the marine environment from TBT presence (OSPAR, 2005).

The current work aims to describe the evolution of *imposex* levels in *Nucella lapillus* populations along the Portuguese mainland coast between 2003 – date when the Regulation (EC) No.782/2003 was implemented – and 2008, in order to evaluate the effectiveness of the European legislation. The other objective is to create an *imposex* levels baseline at the moment when the IMO TBT-based AF paints global ban came into force. As some factors have been described to induce variation in *imposex* assessment indices, which might conduce lo less reliable results, new monitoring and data analysis procedures are described.

7.2 MATERIAL AND METHODS

7.2.1 Sampling

Since *imposex* expression follows sexual maturation (Gibbs, 1999), the minimum size at which *Nucella lapillus* specimens' are sexually mature was studied within the study area. A large sample of animals of all sizes (from juveniles to adults) was collected in April 2006, at a single site located on the NW Portuguese coast (40°31'05.90''N 8°47'05.11''W) near St. 9 (see Figure 7.1). Sampling was carried out right before the main spawning period described for the population at that site (Galante-Oliveira et al., 2010), a moment when all the adults are expected to be sexually mature.

For the assessment of long-term evolution of *N. lapillus imposex* levels in the Portuguese mainland coast, sites previously sampled in 2003 by Galante-Oliveira et al. (2006) were revisited in 2006 and 2008: animals were collected at 16 sites (St. 1-16; Figure 1A) from May to July 2006 and at 12 sites (St. 1-2, 5-6, 8-13 and 15-16; Figure 7.1A) from May to July 2008. Three time-searching periods, of 15 minutes each, were performed at each site: all the specimens (of all sizes) found were collected randomly, by hand, by the same single person in 2006 and 2008.

Additionally, in order to verify if TBT recent inputs still occur in the study area in July 2006, OTs concentrations were determined in water samples collected at 47 sites (St. 1'-47'; Figure 7.1B) on the lowest-tide level. Water sampling covered an extensive area of the Portuguese coast to allow a representative image of OTs water contamination and its gradients and to identify the main pollution sources: sampling sites were spread not only across the Atlantic open coast but also in estuarine systems where the country major harbours are located (see Figure 7.1A and B).

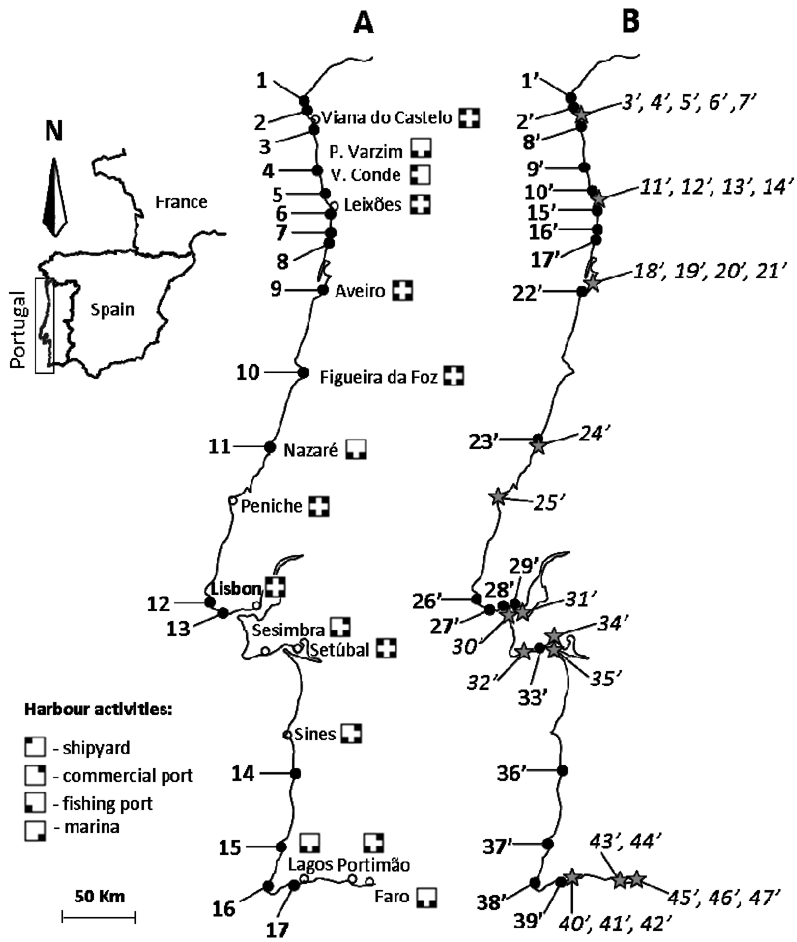

Figure 7.1 *Nucella lapillus*. Map of the NW Portuguese coast indicating: A. main harbour activities and sites (St. 1-16) where animals were collected for *imposex* levels assessment in 2006 and 2008; B. sites (St. 1'-47') where water samples were collected for OTs quantification in 2006. Italic code numbers indicate sites located inside harbours (also evidenced by ★).

7.2.2 Sexual maturation analysis

The minimum shell height (SH) at which animals are sexually mature was determined in specimens collected as referred above (Figure 7.1A). Animals SH (length from the apex to the siphonal canal) was measured with vernier callipers to the nearest 0.1 mm. After shells removal, specimens were sexed and parasitized were discarded. The complex gonad / digestive gland (≈1.0 cm of the animal proximal portion) was individually fixed in Bouin's solution for 24h and then preserved in 70% ethanol for histological analysis. Three slides with 4 gonads' sections each were made by specimen, stained with haematoxylin-eosin and mounted in DPX resin for light microscopy observation, in order to determine individual gametogenesis stage. Gametogenesis classification for prosobranch gastropods was proposed by Barroso and Moreira (1998) and was applied to study *Nucella lapillus* reproductive cycle by Galante-Oliveira et al. (2010) being therefore fully described for this species. Animals at stages I, II and III were grouped and classified as having gonads maturation (Gonads Mat) *0* – "immature"; specimens at gametogenesis stages IV, V and VI were classified as Gonads Mat *1* – "mature". Gonads Mat was plotted as a function of the respective animal SH and the minimum SH at which specimens are sexually mature was graphically determined.

7.2.3 *Imposex* analysis

Imposex levels monitoring was carried out in samples collected in 2006 and 2008 along the Portuguese coast (Figure 7.1A). Specimens' SH was measured with vernier callipers to the nearest 0.1 mm. Animals were separated into SH classes of amplitude 2.50 mm, from a minimum of 5.00 mm to the maximum size collected at each site. After shells removal, specimens were sexed and the parasitized were discarded from the analysis. The penis length (PL) was measured using a stereo microscope with a graduated eyepiece to the nearest 0.14 mm and the females vas deferens sequence (VDS) was classified according to the scheme proposed by Gibbs et al. (1987). For animals with SH ≥ 17.50 mm (minimum SH at which animals are sexually mature; see the section *7.3*) the following parameters were determined for each sample: mean male and female penis length (MPL and FPL, respectively), relative penis size index (RPSI = FPL^3 / MPL^3 x100), vas deferens sequence index (VDSI), percentage of *imposex* affected females (%I) and percentage of sterilized females (%S).

7.2.4 Hexane-extractable tin concentrations in water

Samples of 2 l of sub-surface (15 cm depth) water were collected in two 1 l glass bottles, previously washed in 0.5% hydrochloric acid (HCl). Immediately before each sample collection, bottles were rinsed with local water. Samples were then acidified with 5 ml concentrated HCl per litre. Methods used for extraction (from unfiltered water) and OTs analysis are those described by Bryan et al. (1986) providing a detection limit of about 0.2 ng $Sn.l^{-1}$. However, washing of hexane extracts with 1N sodium hydroxide (NaOH) to separate dibutyltin (DBT) from the TBT fraction was not performed; hence values are reported as hexane extractable tin, as we only aim to depict any OTs recent inputs into the study area in 2006.

7.2.5 Statistical data analysis

The evolution of *imposex* levels in *Nucella lapillus* adults (SH ≥ 17.50 mm) between 2003 and 2008 was performed using two different approaches depending on whether sampling sites were analysed together or separately. In the first approach, the subjects were the 12 sampling sites common to 2003, 2006 and 2008 (St. 1-2, 5-6, 8-13 and 15-16; Figure 7.1A) and the observations were the mean females SH (♀SH), VDSI, mean FPL or %I at each site; the purpose of the analysis was to test if the mean ♀SH, the VDSI, the mean FPL and the %I of all sites together changed significantly (representing the entire study area over the studied period) using the repeated-measures Friedman test followed by the post-hoc Dunn’s test for multi-comparisons. The second approach was applied to evaluate changes in *imposex* intensity at each site from 2003 to 2008. In this case, the subjects were the specimens analysed per site (with SH ≥ 17.50 mm) and the observations were the assessed individual parameters [each male SH (♂SH), each ♀SH, each animal PL and the VDS stage exhibited by each female] i.e., samples were assumed to be independent since specimens collected randomly over time were not the same; a non-parametric Kruskal-Wallis test, followed by the post-hoc Dunn's test for multi-comparisons, was applied in this case. Since St. 3-4, 7 and 14 were only sampled in 2006, the Mann-Whitney U-test was used to compare individual parameters between 2003 (Galante-Oliveira et al., 2006) and 2006 in these cases. *SigmaStat v2.0* software was used to compute these analyses. Since the SH of animals sampled in the different surveys was not constant, and it is known that the PL may also depend on specimen's size, statistical comparisons using the penis based RPSI were not performed.

Additionally, a different statistical procedure to remove the effect of animals' size on *imposex* temporal trends was used: the ordered logit regression model was applied to study *imposex* levels evolution from 2003 to 2008, using all the animals collected at each site (from juveniles to adults and not only the ones with SH ≥ 17.50 mm). The ordered logit regression belongs to the family of the generalized linear models and namely to the models for categorical responses. These models are the best way to deal with special kind of response variables arising from classification, count processes, and so on. A seminal book about these models is Maddala (1983) and further details can be found in Agresti (2002) and Long (1997). As above referred, *imposex* development can be followed by the vas deferens sequence (VDS) and its intensity quantified by using VDS classification schemes. For *N. lapillus*, the variable "VDS" has six categories (Gibbs et al., 1987). "VDS" can be considered an ordinal variable in the sense that each of its values evaluates a certain degree of the continuous process of the female virilization. Obviously, this continuous process can not be evaluated nor measured in a continuous scale so it is a latent, non observable, variable. Therefore, the ordered logit regression seems to be the more adequate, accurate and powerful model to make the statistical analysis of this process evolution. This can be seen ahead in section *7.3*.

This statistical procedure was implemented trough *R* programming (see http://www.r-project.org) using "MASS" library. *R* is a powerful open source language and environment for statistical computing. The authors would provide the programming code to those interested in (josant.santos@gmail.com).

7.3 RESULTS

The minimum SH at which *Nucella lapillus* specimens are sexually mature was graphically determined as being 17.50 mm for the given sampling site (Figure 7.2). As we could not determine this parameter for all sites along the Portuguese coast, we assumed the same value for the entire study area and so *imposex* indices were calculated for specimens of SH ≥ 17.50 mm.

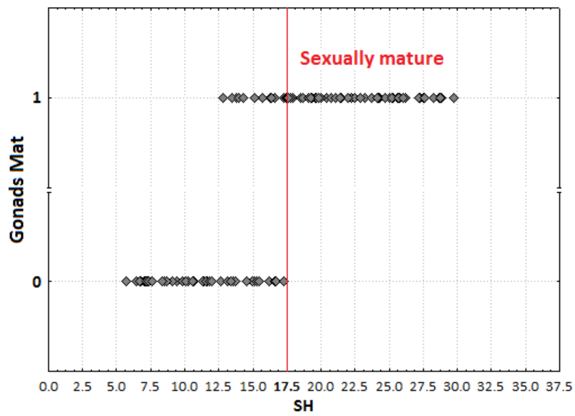

Figure 7.2 *Nucella lapillus*. Gonads microscopic maturation (Gonads Mat) by the respective specimens' shell height (SH) for animals collected at a single site (40°31'05.90''N 8°47'05.11''W) near St. 9 in April 2006. Red line identifies the minimum SH from which 100% of the animals are sexually mature. *0*: immature gonad (MicMat Stg. I, II, III); *1*: mature gonad (MicMat Stg. IV, V, VI).

The number of specimens used per gender, sampling site and year are presented in Table 7.1 together with the respective mean ♂SH and ♀SH. The number of specimens collected at each site during a period of 45 minutes is also indicated in Table 7.1.

No correlation was found between *imposex* levels and *N. lapillus* abundance at each site and no temporal trend was observed in the abundance: the number of specimens collected during 45 minutes increased at St. 6, 9, 11, 13 and 15 from 2006 to 2008; decreased at St. 2, 5 and 10; and was approximately constant at St. 8, 12 and 16.

Table 7.1 *Nucella lapillus*. Male and female mean shell heights (♂SH and ♀SH; expressed in millimetres) and the number of specimens with SH ≥ 17.50 mm analysed for *imposex* (n) are presented per sampling site (St. code and name) and year. ♂SH and ♀SH were statistically compared and the respective significance is indicated by asterisks (*) next to the last year of the tested pair (i.e., if the 2003 value is significantly different from the 2006 one, asterisks are indicated next to the 2006 value). Data for 2003 were published by Galante-Oliveira et al. (2006). For additional data on sites location compare Figure 7.1.

St. code and name	Coordinates (EUR 50)		♂SH (n)			♀SH (n)			n (45')	
			2003	2006	2008	2003	2006	2008	2006	2008
1. Vila Praia de Âncora	41°48.93N	8°51.94W	21.29 (23)	22.07 (47)	23.49 (48)	22.67 (28)	23.88 (66)	23.75 (57)	518	395
2. Praia Norte	41°41.85N	8°41.13W	19.43 (19)	20.81 (27)*	19.68 (18)	20.86 (32)	21.71 (41)	20.94 (27)	190	163
3. Praia da Amorosa	41°38.72N	8°49.31W	20.80 (11)	21.29 (29)		20.50 (13)	22.63 (47)**		82	
4. Póvoa do Varzim	41°23.18N	8°46.40W	22.51 (19)	21.37 (45)		23.07 (31)	22.66 (47)		160	
5. Praia de Leça	41°12.21N	8°42.82W	22.41 (31)	21.79 (36)	21.03 (34)	23.16 (25)	22.63 (41)	22.72 (47)	219	167
6. Praia da Foz	41°09.78N	8°41.10W	20.92 (25)	21.88 (40)	21.30 (41)	21.06 (25)	23.50 (41)*	23.75 (61)	100	267
7. Aguda	41°03.09N	8°39.18W	21.26 (30)	23.74 (50)*		21.24 (19)	26.23 (78)***		288	
8. Espinho	41°00.44N	8°38.71W	21.04 (20)	22.76 (44)	22.24 (47)	22.10 (24)	25.24 (69)*	23.96 (56)	579	580
9. Aveiro	40°38.71N	8°44.82W	23.26 (24)	25.14 (61)	26.19 (75)	24.79 (21)	25.23 (55)	30.30 (67)*	316	367
10. Fig. Foz	40°10.18N	8°53.26W	19.68 (18)	20.96 (33)	19.77 (18)	19.68 (30)	23.27 (39)*	21.67 (39)*	312	188
11. Nazaré	39°36.26N	9°04.49W	18.99 (13)	18.29 (13)	19.65 (10)*	19.52 (27)	19.20 (21)	20.70 (58)*	393	560
12. Praia do Guincho	38°43.74N	9°28.46W	19.34 (19)	19.81 (20)	19.31 (21)	20.21 (32)	20.21 (33)	20.80 (38)	333	320
13. Praia das Avencas	38°41.21N	9°21.27W	20.95 (21)	20.02 (16)	21.09 (19)	21.27 (22)	19.71 (35)*	20.99 (26)*	30	64
14. Vila nova de Mil Fontes	37°43.30N	8°47.25W	21.10 (20)	19.79 (19)**	-	22.36 (25)	22.44 (14)	-	19	-
15. Zambujeira do Mar	37°33.20N	8°47.44W	18.94 (14)	19.93 (20)	20.54 (24)	19.68 (29)	21.41 (17)*	20.72 (38)	35	122
16. Praia do Amado	37°15.22N	8°38.45W	21.64 (17)	18.93 (5)*	20.32 (26)	23.18 (17)	20.18 (17)*	21.70 (36)	59	49

- : animals not found; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

The temporal evolution of VDSI at each site and concentrations of hexane extractable tin in water in 2006 are represented in Figure 7.3 while the MPL, FPL, RPSI, VDSI and %I values are shown in Table 7.2. Data for 2003 (Galante-Oliveira et al., 2006) were reorganized and *imposex* indices recalculated using only the animals of SH ≥ 17.50 mm.

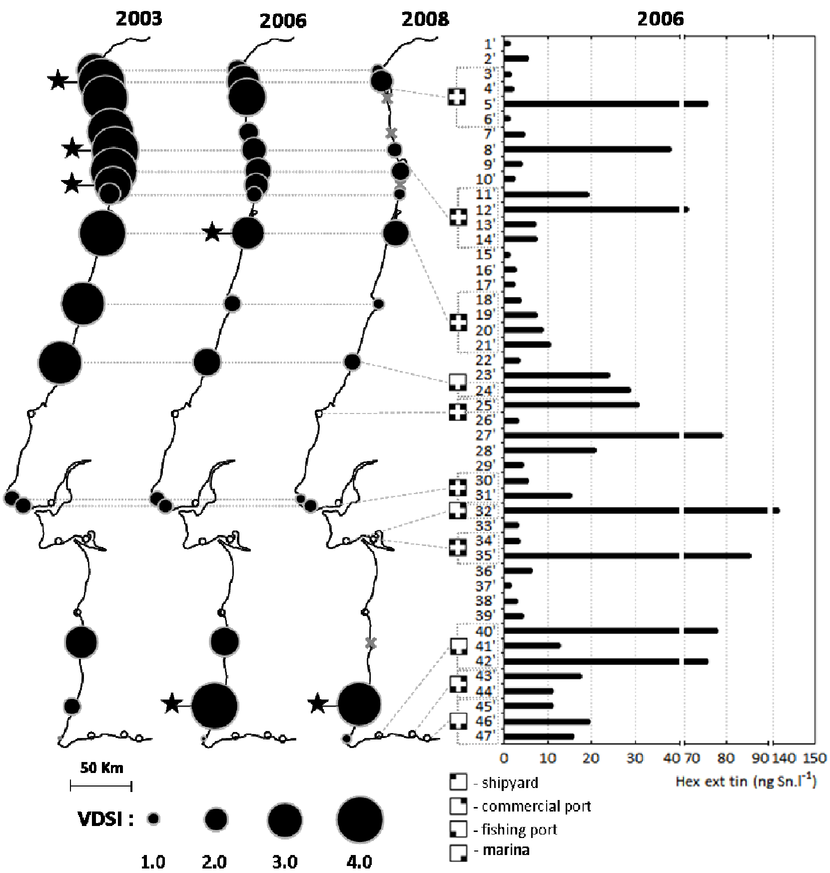

Figure 7.3 *Nucella lapillus*. Vas deferens sequence index (VDSI) quantified in females collected at sites along the NW Portuguese mainland coast in 2003 (Galante-Oliveira et al., 2006), 2006 and 2008. On the left plot are indicated the hexane extractable tin (Hex ext tin) concentrations in water samples collected along the coast in 2006 (expressed in ng Sn.l^{-1}). Sites located inside port terminals are signalized by the respective local harbour activities symbol (as previously showed in Figure 7.1). For additional data on sites location compare Figure 7.1. ★: Sites where sterile females were recorded.

A general decrease in *imposex* indices (apart from %I) is evident in the entire coast, with the exception of St. 15 and 16. Sterile females were recorded at St. 9 (5.5%) and 15 (11.8%) in 2006 and at St. 15 (26.3%) in 2008. Recent inputs of organic tin were detected in the whole study area in 2006 with hexane extractable tin concentrations in water ranging from 0.7 to 140.8 ng Sn.l^{-1}.

Table 7.2 *Nucella lapillus*. Mean male and female penis length (MPL and FPL), relative penis size index (RPSI), vas deferens sequence index (VDSI) and percentage of *imposex* affected females (%I) indicated per sampling site (St.) and year. MPL, FPL, RPSI and VDSI of specimens with SH ≥ 17.50 mm were statistically compared and the respective significance is indicated by asterisks (*) next to the last year of the tested pair (i.e., if the 2003 value is significantly different from the 2006 one, asterisks are indicated next to the 2006 value). Data for 2003 were published by Galante-Oliveira et al. (2006). For additional data on sites location compare Figure 7.1.

St.	MPL (mm)			FPL (mm)			RPSI (%)			VDSI			%I		
	2003	**2006**	**2008**	**2003**	**2006**	**2008**	**2003**	**2006**	**2008**	**2003**	**2006**	**2008**	**2003**	**2006**	**2008**
1.	3.47	4.52*	4.46	0.99	0.43*	0.08*	2.33	0.09	0.00	2.93	1.71*	1.04*	100.0	100.0	91.2
2.	2.97	3.16	3.24	2.02	0.65*	0.43	31.43	0.87	0.23	4.00	2.78*	1.93*	100.0	100.0	100.0
3.	3.31	3.95**		1.97	0.99***		20.95	1.57		3.92	3.23*		100.0	100.0	
4.	3.33	3.94***		1.67	0.36***		12.72	0.08		3.94	1.66***		100.0	100.0	
5.	3.44	4.55*	4.40	2.04	0.69*	0.18*	20.95	0.35	0.01	4.04	2.10*	1.28*	100.0	100.0	95.7
6.	3.28	4.69*	4.49	1.71	0.78*	0.32*	14.14	0.45	0.04	4.00	2.20*	1.59	100.0	100.0	100.0
7.	3.15	4.84***		1.13	0.55***		4.70	0.14		3.16	2.05***		100.0	100.0	
8.	3.40	4.48*	3.86*	0.60	0.21*	0.10	0.55	0.01	0.00	1.83	1.38	1.02*	91.7	100.0	83.9
9.	2.93	3.79*	4.67*	2.40	0.97*	0.77	54.72	1.67	0.44	4.00	2.82*	2.27	100.0	100.0	100.0
10.	3.47	4.33*	3.88	1.31	0.25*	0.09	5.43	0.02	0.00	3.73	1.46*	0.95	100.0	100.0	82.1
11.	3.41	4.11*	3.88	1.56	0.82*	0.25*	9.65	0.80	0.03	3.70	2.38*	1.48*	100.0	100.0	96.6
12.	3.42	4.07*	4.52	0.36	0.10	0.02	0.12	0.00	0.00	1.38	1.27	0.84	84.4	100.0	78.9
13.	2.63	2.56	4.78*	0.28	0.09	0.10	0.12	0.00	0.00	1.41	1.23	1.15	77.3	100.0	96.2
14.	3.37	3.53	-	1.05	0.65**	-	3.05	0.63	-	2.80	2.50	-	100.0	100.0	-
15.	3.80	4.24	4.55	0.19	2.05*	1.35	0.01	11.26	2.60	1.48	4.06*	3.79	86.2	100.0	100.0
16.	3.80	3.95	5.27*	0.00	0.00	0.02	0.00	0.00	0.00	0.18	0.41	0.78	17.6	41.2	75.0

- : animals not found; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Sampling sites were grouped regarding its location relatively to harbours: (1) sites located less then 1 mile from the main port infrastructures (St. 2-3, 5-6, 9-11 and 13); (2) sites around small boats mooring facilities (St. 1, 4, 7-8 and 14-15); and (3) sites at pristine areas (St. 12 and 16). VDSI registered in 2006 and 2008 was compared between these three groups of sites by the one-way ANOVA, after confirming the data normality and homoscedasticity. There is a significant difference in *imposex* intensity between groups (s = 3.6754, p < 0.05) and the post-hoc Fisher's LSD test revealed that the difference is between the first group (sites near harbours) and the other two (p < 0.05).

When the 12 sampling sites – common to 2003, 2006 and 2008 (St. 1-2, 5-6, 8-13 and 15-16; Figure 7.1A) – are analysed together, no significant difference in ♀SH is observed from 2003 to 2008 (Friedman's test: s = 3.106, p = 0.212) and a global decline in both VDSI (s = 12.167, p = 0.002) and FPL (s = 11.617, p = 0.003) is detected (Figure 7.4). Dunn's multi-comparisons tests between different years show that there was a significant reduction of VDSI and FPL levels from 2003 to 2008 and also of FPL from 2003 to 2006 (Figure 7.4). Nevertheless, no significant difference in %I is observed from 2003 to 2008 (s = 6.097, p = 0.05) and a median >95% was still registered in 2008, showing that the phenomenon remains widespread in the study area (Figure 7.4).

When sampling sites are analysed separately, several differences in both genders SH are registered (Table 7.1). Mean ♂SH variation at St. 7 can explain the difference also registered in MPL at that site (Table 7.2): males collected in 2006 were larger than the ones in 2003 and MPL is also significantly higher in 2006. However, other MPL significant variations between years are not simply justified by differences in ♂SH.

Regarding mean ♀SH (Table 7.1), with the exception of St. 13 and 16 (between 2003 and 2006) and of St. 10 (between 2006 and 2008) where animals collected in the last campaign were smaller (in 2006 and 2008, respectively), all significant differences were towards increasing animals' size. In turn, with the exception of St. 15 where significant increases were registered in both FPL and VDSI between 2003 and 2006, all significant differences in *imposex* indices were towards a reduction (Table 7.2). Briefly, VDSI > 2 was registered at 68.75% of the sampled sites in 2003, at 56.25% in 2006 and at 16.67% in 2008 (see Table 7.2). VDS stages relative frequencies are plotted in Figure 7.5 for an overview of their individual evolution over time.

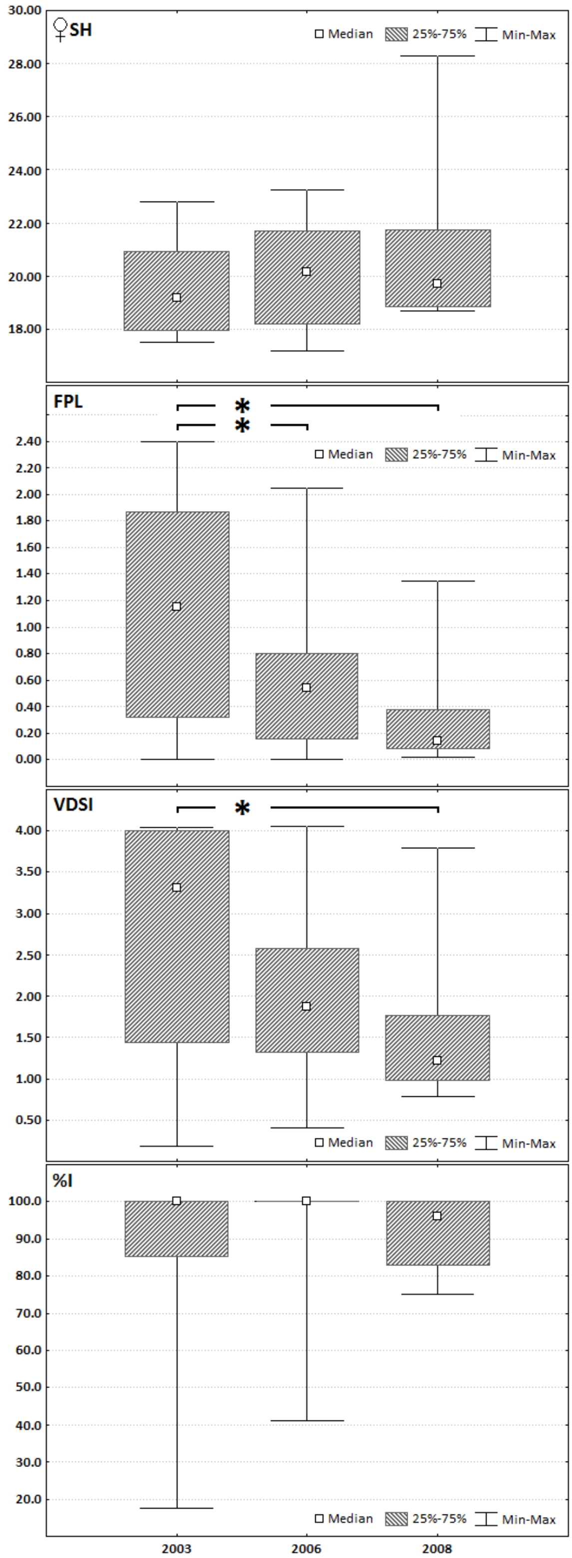

Figure 7.4 *Nucella lapillus*. Global temporal trend of females' shell height (♀SH) and *imposex* levels (FPL, VDSI and %I) exhibited by specimens collected at 12 common sites along the NW Portuguese coast: for statistical analysis St. 1-2, 5-6, 8-13 and 15-16 were pooled and the median was calculated per year – 2003 (Galante-Oliveira et al., 2006), 2006 and 2008. The significance of the Dunn's test for multi-comparisons between years is indicated on the respective plot (*: $p < 0.05$).

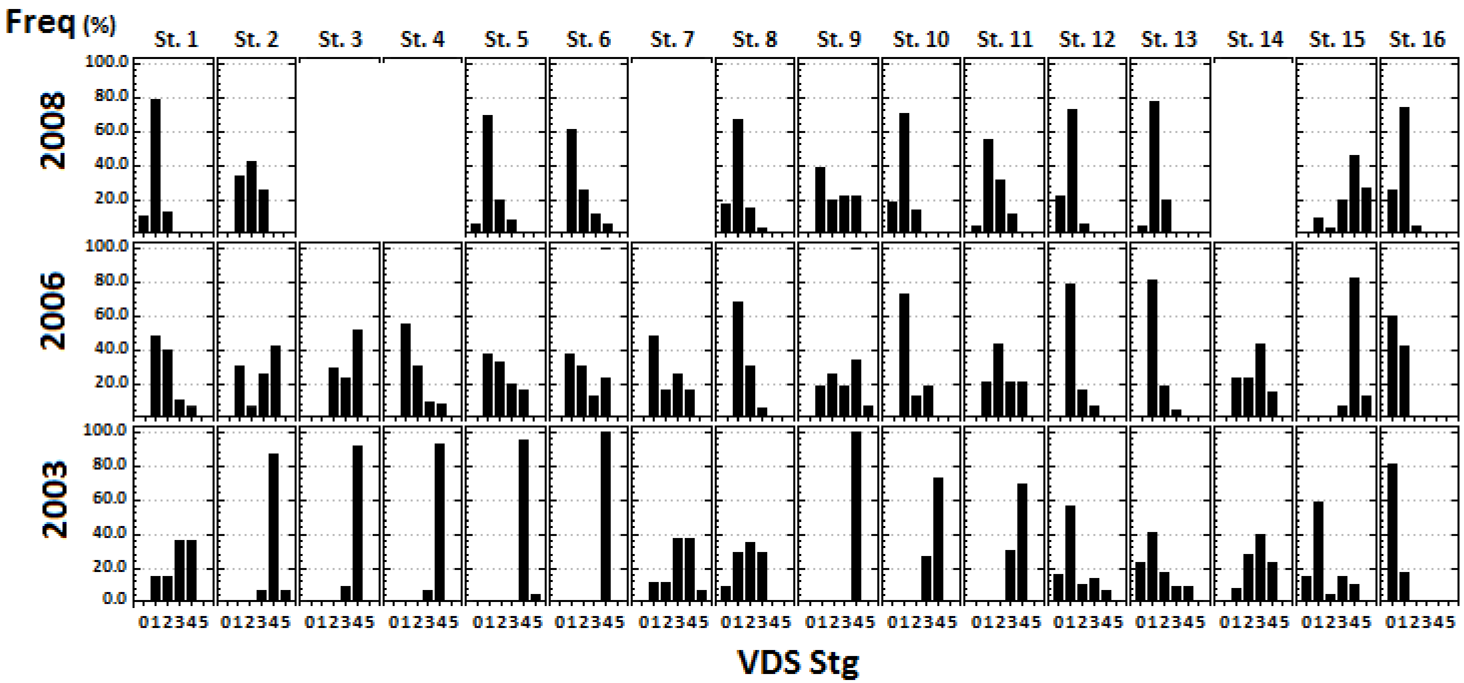

Figure 7.5 *Nucella lapillus*. Relative frequency (Freq) of each VDS stage exhibited by females collected in 2003 (Galante-Oliveira et al., 2006), 2006 and 2008 at St. 1-16. For total number of specimens (n) at each site and year compare Table 7.1 and for additional information on sites location see Figure 7.1.

The ordered logit regression model was implemented to remove the effect of animals' size on the evaluation of *imposex* temporal trends. Absolute frequencies of VDS stages exhibited by all females (of all sizes) analysed from each sampling site are presented in Table 7.3 and the model estimations results are summarised in Table 7.4.

Table 7.3 *Nucella lapillus*. Absolute frequencies of all the animals analysed at each sampling site (St.) in all the monitoring campaigns being compared – 2003 (Galante-Oliveira et al., 2006), 2006 and 2008 – indicated by vas deferens sequence (VDS) stage. Due to the very low number of observations for the model application, values shaded in gray were grouped into a single class. For additional data on sites location compare Figure 7.1.

St.	VDS					
	0	**1**	**2**	**3**	**4**	**5**
1.	38	104	43	16	13	0
2.	16	33	21	23	47	2 (1.41%)
3.	0	0	13	11	37	0
4.	9	30	17	6	32	0
5.	12	53	22	10	30	1 (0.78%)
6.	3 (2.26%)	55	27	11	37	0
7.	0	40	15	26	18	1 (1.00%)
8.	50	123	50	12	0	0
9.	13	43	45	30	57	3 (1.57%)
10.	27	93	16	15	23	0
11.	34	66	45	22	33	0
12.	61	94	13	6	2 (1.14%)	0
13.	6	61	15	3 (3.45%)	2 (2.30%)	0
14.	4	5	11	16	8	0
15.	5	20	3	21	44	12
16.	85	48	2 (1.48%)	0	0	0
1-2, 5-6, 8-13, 15-16	350	878	359	229	384	19 (0.84%)

Table 7.4 *Nucella lapillus*. Comparisons of vas deferens sequence (VDS) levels between 2003 (Galante-Oliveira et al., 2006), 2006 and 2008, after the application of the ordered logit regression model at each site (St.). The estimated probability of VDS > 2 [*P* (VDS > 2)] is also indicated, expressed in percentage (%). Each *P* (VDS > 2) is the result of the model application for a determined shell height (SH) mean value at each site (this value was calculated for animals from 17.50 mm to the maximum SH recorded at that the respective site). For additional data on sites location compare Figure 7.1.

$$VDS^* = \beta_0 SH + \beta_1 D_{2006} + \beta_2 D_{2008} + \mathcal{E}$$

St.	Coefficients (*p*-value)				*P* (VDS>2)			SH
	SH	2003/2006	2003/2008	2006/2008	% 2003	% 2006	% 2008	(mm)
1.	[a]	[a]	[a]					
2.	0.316 (7×10^{-9})	-3.614 (6×10^{-6})	-5.239 (3×10^{-10})	-1.625 (7×10^{-5})	98.85	69.89	78.32	21.23
3.	0.310 (0.019)	-3.463 (0.003)			98.80	72.07		22.17
4.	0.267 (5×10^{-6})	-5.752 (2×10^{-9})			98.06	13.82		22.82
5.	[a]	[a]	[a]					
6.	0.234 (1×10^{-4})	-19.995 ($<10^{-10}$)	-21.289 ($<10^{-10}$)	-1.294 (0.001)	100.00	29.00	10.07	22.78
7.	0.137 (0.003)	-2.335 (2×10^{-5})			84.50	34.55		25.26
8.	0.133 (2×10^{-10})	-1.990 (1×10^{-5})	-2.213 (3×10^{-6})	-0.223 (0.415)	27.21	4.86	3.92	24.25
9.	0.111 (4×10^{-8})	-4.341 (4×10^{-5})	-5.019 (2×10^{-6})	-0.678 (0.027)	99.04	57.41	40.63	24.06
10.	[a]	[a]	[a]					
11.	0.367 (2×10^{-16})	-2.450 (1×10^{-5})	-5.475 ($<10^{-11}$)	-3.025 (≈0)	95.31	63.68	7.84	20.10
12.	[a]	[a]	[a]					
13.	[a]	[a]	[a]					
14.	0.326 (2×10^{-4})	-0.718 (0.243)			68.32	51.26		22.39
15.	0.168 (0.017)	3.847 (2×10^{-9})	3.765 (2×10^{-10})	-0.082 (0.860)	26.91	94.52	94.08	20.50
16.	[b]	[b]	[b]					
1-2, 5-6, 8-13, 15-16	0.170 ($<10^{-7}$)	-1.693 ($<10^{-7}$)	-2.404 ($<10^{-7}$)	-0.711 (4×10^{-14})	68.84	28.89	16.63	22.64

[a] : not computed due to the algorithm non convergence;
[b] : model estimation not performed due to the insufficient VDS classes (2).

The algorithm was not able to converge in St. 1, 5, 10 and 12-13 (Table 7.4). Shell height (SH) proved to be always positively and highly significantly associated with the VDS level, meaning that this variable should always be incorporated on VDS stage modelling (see SH coefficients and respective *p*-values estimated for all the sampling sites in Table 7.4). At St. 2, 6, 9 and 11 there is a significant decrease of VDS between 2003/2006, 2003/2008 and 2006/2008 (Table 7.4). Similarly, there is a significant decrease of VDS at St. 3-4 and 7 from 2003 to 2006 (the only years that were sampled as these sites were not revisited in 2008) (Table 7.4). At St. 8 there is also a significant decrease of the VDS level between 2003/2006 and 2003/2008 although there is no statistical difference between 2006/2008 (*p*-value = 0.415; Table 7.4). Animals at St. 14 were only collected in 2003 and 2006 and there is no significant difference of VDS levels between these years at this site. At St. 15 there was a highly significant increase in VDS between 2003/2006 and 2003/2008, but no significant difference between 2006/2008 (*p*-value = 0.860; Table 7.4).

Probabilities of females presenting VDS > 2 were also estimated by the model, for animals of a given SH, at each sampling site and year (Table 7.4). The SH value for which the probability was estimated corresponds to the mean SH of adult animals at each site (Table 7.4). We choose females VDS > 2 as they shift the VDSI to values higher than 2, which is the limit between OSPAR classes *B* and *C* (details will be published elsewhere). The probability of occurring adult females with VDS > 2 varied between 27-100% in 2003, 5-95% in 2006 and 4-94% in 2008 (Table 7.4). This probability decreased consistently from 2003 to 2008, with the exception of St. 2 and 15. Finally, making the model algorithm much more powerful, all the observations coming from all the sites common to 2003, 2006 and 2008 were pooled (St. 1-2, 5-6, 8-13 and 15-16; Table 7.4). A highly significant decrease of the VDS level is estimated along the years being compared (“2003/2006”, “2003/2008” and “2006/2008”; see Table 7.4). Globally, the probability of females presenting VDS > 2 for a mean SH of 22.64 mm in the study area decreased from 68.8% in 2003 to 28.9% in 2006 and to 16.6% in 2008 (Table 7.4).

7.4 DISCUSSION

7.4.1 *Imposex* evolution from 2003 to 2008

Nucella lapillus imposex levels in 2006 and 2008 were higher at sites near harbours, a tendency earlier reported in 2000 (Barroso and Moreira, 2002) and 2003 (Galante-Oliveira et al., 2006), corroborating previous studies that identify larger vessels AF systems as the main TBT environmental source in the Portuguese coast (Barroso et al., 2000; Barroso et al., 2002; Sousa et al., 2005; Galante-Oliveira et al., 2006; Rato et al., 2006; Sousa et al., 2007; Rato et al., 2008; Rato et al., 2009; Sousa et al., 2009a; Sousa et al., 2009b). Although *imposex* levels in the study area did not change significantly between 2000 and 2003 (Galante-Oliveira et al., 2006), a general decline of *imposex* intensity along Portuguese coast is finally registered in the present study from 2003 to 2008 (Figure 7.4), suggesting that the EU Regulation No.782/2003 was effective in reducing pollution. This measure prohibited OT AF painting but did not banned completely the use of OTs AF systems since vessels could still bear TBT in their hulls until 17 September 2008 (date when the IMO ban entry into force). This may partly explain why OTs were still ubiquitous in seawater throughout the Portuguese coast in 2006 and concentrations continued to be higher at sites located inside port terminals or in its vicinity (Figure 7.3). OTs mean concentrations in sub-surface waters in 2006 varied from 0.7 to 140.8 ng $Sn.l^{-1}$ which major fraction is known to be butyltins, as other OT species are almost negligible in the Portuguese coast (Sousa et al., 2007; Sousa et al., 2009a; Sousa et al., 2009b). However, vessels may not be the unique source for these recent TBT inputs. Some authors still consider that after the IMO ban entry into force TBT inputs will be maintained by other sources beyond AF paints namely: (i) the release from dockyard facilities as a result of the old coatings removal in dry dock (Kotrikla, 2009); (ii) the remobilization from sediments to the water column (Langston and Pope, 1995; Ruiz et al., 2008); (iii) some illegal use; and (iv) other sources such as preservative or disinfecting agents (Sousa et al., 2009b).

Imposex reductions from 2003 to 2008 are noticeable mainly in the north and central coasts of Portugal (see Figure 7.3, Figure 7.5 and Table 7.2), regions where the most important harbours are located and so where a higher number of sites were sampled, comparing with the south. Thus, the statistically suggested overall decline in *imposex* levels is probably influenced by this higher number of samples in the north / centre of Portugal. In fact, a dramatic raise in VDSI and %S was registered at St. 15 from 2003 to 2006, values that remained high in 2008; besides, a higher frequency of successively advanced VDS stages was also recorded at St. 16 over time (Figure 7.5), although not significant in terms of VDSI increase (Table 7.2). In the south, an almost threefold increase in ships and respective gross tonnage was registered at Sines harbour (north to St. 15) between 2003 and 2008, and a twofold increase at Portimão harbour (southeast to St. 16) from 2003 to 2006 (see Figure 7.6 and Table 7.5).

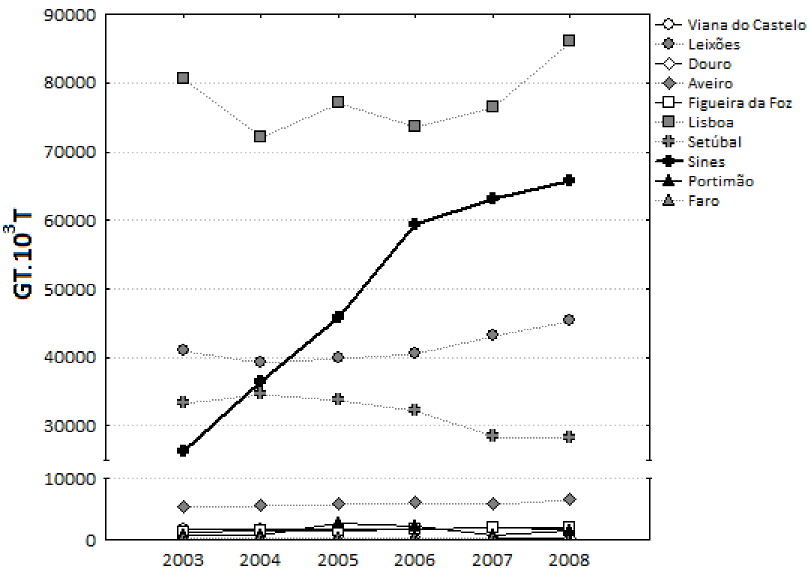

Figure 7.6 Gross tonnage expressed in tons (GT.10^{3}T) of commercial ships entering and leaving the Portuguese main harbours: temporal evolution from 2003 to 2008 indicated by port. Data published by *Instituto Nacional de Estatística* (INE) and obtained from the institution website (online available publications at www.ine.pt).

Table 7.5 Commercial ship traffic in and from Portuguese main harbours between 2003 and 2008. Indication of the total number of ships (No.) incoming and outgoing each harbour and the respective gross tonnage expressed in tons ($GT.10^3T$). Data published by *Instituto Nacional de Estatística* (INE) and obtained from the institution website (online available publications at www.ine.pt).

Harbour	**2003**		**2004**		**2005**		**2006**		**2007**		**2008**	
	No.	**$GT.10^3T$**	**No.**	**$GT.10^3T$**	**No.**	**$GT.10^3T$**	**No.**	**$GT.10^3T$**	**No.**	**$GT.10^3T$**	**No.**	**$GT.10^3T$**
Viana do Castelo	522	1 732	416	1 609	376	1 713	421	1 850	456	1 982	369	1 718
Leixões	5 381	41 030	5 221	39 187	5 471	39 978	5 301	40 679	5 352	43 136	5 189	45 357
Douro	162	218	162	235	90	129	156	228	128	184	129	186
Aveiro	2 005	5 445	2 076	5 713	2 100	5 826	2 040	6 101	1 901	6 004	1 932	6 525
Figueira da Foz	537	1 191	588	1 437	594	1 469	640	1 619	726	1 917	776	2 051
Lisboa	7 052	80 589	6 544	71 984	6 696	77 036	6 667	73 619	6 565	76 522	6 501	86 156
Setúbal	3 215	33 288	3 325	34 660	3 003	33 870	2 947	32 218	2 845	28 507	2 744	28 281
Sines	1 502	26 310	1 837	36 504	2 386	45 833	2 701	59 418	2 822	63 221	2 884	65 785
Portimão	66	802	100	694	147	2 755	129	2 205	79	704	93	1 574
Faro	98	283	66	181	66	188	45	133	47	132	22	67

This fact, associated to an increase in ship traffic in routes accessing these ports, could eventually be the cause for the increase of *imposex* levels at St. 15 and 16. Nevertheless, St. 15 remains under study once such increases in both *imposex* intensity and females' sterility are considered extreme even considering an increased naval traffic in the region. St. 14 is closer to Sines harbour but, on the other hand, is located inside the Mira estuary without any large maritime infrastructure and is somehow protected from the influence of Sines port considering the high mixture between fresh water from Mira river and seawater, justifying *imposex* intensity maintenance from 2003 to 2006.

7.4.2 Effect of specimens' size in VDSI assessment

The reliability of using *N. lapillus* for monitoring TBT pollution requires control of factors known to cause some bias in *imposex* assessment. For instance, penis based indices like RPSI (directly dependant on FPL and MPL measurements) are difficult to manage since PL is affected by animals' size, sexual maturation and reproductive cycle seasonality (Galante-Oliveira et al., 2009; Galante-Oliveira et al., 2010). The current study shows that the VDS stage (and consequently the VDSI) is also dependant on animals' size (SH), (see "SH" coefficient and respective *p*-value column in Table 7.4). This can be problematic since *imposex* assessment at a given site is generally carried out using adult animals that rarely present uniform sizes. One procedure to overcome this problem, when performing temporal trend analysis, is to avoid making *imposex* comparisons when significant differences in SH are detected between two or more sampling dates. Many times the SH differences render not significant due to low number of animals in the samples, which by their turn are inappropriate as well. A common procedure is to conduct the temporal analysis but be aware of possible bias caused by differences in shell size (see Table 7.1 and Figure 7.4). Seeking for a better approach, in the current work we used an innovative statistical method to analyse VDS data: we consider the SH as a regressor in latent VDS modelling in order to control its effect on temporal comparisons. Looking at St. 8 as an example, through the commonly used data analysis, and considering only mature females (SH ≥ 17.50 mm),

♀SH increased significantly from 2003 to 2006 and no difference was observed in VDSI between those years using a non-parametric Kruskal-Wallis test. Knowing that SH is positively correlated with the VDS stage, if ♀SH had not been higher in 2006, a significant decrease in VDSI from 2003 to 2006 could have been registered as it was when VDS stage was estimated by the latent VDS (VDS*) model (see the St. 8 “2003-2006” coefficient and respective *p*-value in Table 7.4).

7.4.3 Evolution of the Portuguese coast Ecological Quality (EcoQ)

As initially referred, OSPAR Commission developed Environmental Assessment Criteria for *imposex*, taking into account the objectives of the OSPAR Hazardous Substances Strategy and the existing Ecotoxicological Assessment Criteria (EAC) for TBT in water (upper EAC = 0.04 ng $Sn.l^{-1}$), sediment and biota. For *N. lapillus* assessment criteria classes are: Class *A* corresponding to VDSI **C** [0, 0.3[; Class *B* to VDSI **C** [0.3, 2.0[; Class *C* to VDSI **C** [2.0, 4.0[; Class *D* to VDSI **C** [4.0, 5.0]; Class *E* to VDSI > 5.0.

In retrospect, in 2003 (Galante-Oliveira et al., 2006) *N. lapillus* exhibited VDSI ≥ 2.0 at about 70% of the surveyed sites, falling into Classes *C-D* and indicating exposure to TBT concentrations higher than the EAC. The present study shows an evident decline in TBT pollution in the following years: VDSI ≥ 2.0 was just reported at 56% of the sampled sites in 2006 and in 2008 this percentage decreased to 17%.

VDS latent modelling also allows environmental risk evaluation by estimating the probability of a VDS stage above the EcoQO for *imposex* in *N. lapillus*, thus we consider that specimens’ with VDS stage >2 may indicate somehow the insufficient Ecological Quality (EcoQ) of the study area. Regarding the ordered logit regression model estimations, for the whole study area, the probability of VDSI > 2 in a putative adult of SH = 22.64 mm decreased from 68.8% in 2003 to 16.6% in 2008, corroborating the previous conclusion that a general amelioration of the ecological quality occurred in the sampling sites surveyed in the Portuguese coast.

7.5 CONCLUSIONS

The current study shows that *Nucella lapillus* populations sampled along the Portuguese mainland coast are still extensively affected by *imposex* and that, even three years after the European ban on TBT, fresh inputs continued to occur. Nevertheless, *imposex* levels evolution indicate a decline in TBT pollution in the north and central coasts from 2003 to 2008.

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Chapter 8

Potential recolonization of former hotspots of tributyltin (TBT) pollution by *Nucella lapillus* (L.)

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ABSTRACT

N. lapillus is a marine gastropod widely distributed along the North Atlantic rocky shores. During the 80s, some populations were extinct near hotspots of tributyltin (TBT) pollution – ports, shipyards and marinas – once females were sterilized due to the accumulation of high levels of TBT. The pollution by these compounds is now declining as a result of legislation restricting organotins (OTs) antifoulants usage and therefore it is possible that the species start to recolonize sites where presumably it became extinct. This work speculates about possible strategies of recolonization of former hotspots based on the available knowledge of the species biology and on the new research carried out regarding the species ecotypes that occur in the Portuguese coast. One possible strategy could be specimens crawling through continuous habitats from the open shore (exposed ecotype) into far in distance estuarine areas (sheltered ecotype) near hotspots of pollution where extinctions occurred. The weakness of this hypothesis seems to be the adults reduced mobility. Having in mind that early life stages are essential sources underlying any new populations' establishment and that *N. lapillus* life cycle has no pelagic

larval phase, the hypothesis of recolonization by juveniles' dispersion was also considered. Some factors that could affect both strategies of recolonization were studied. First, the ecotypes available to recolonize the most sheltered regions inside estuaries were investigated and it was found that shell shape along the Portuguese coast is different between sites in relation to wave action exposure – shell shape ratios (SH/AH) ranged from 1.55 at the most exposed locations and 1.83 at the most sheltered ones; nevertheless, the 2n = 26 karyotype, typical from exposed shores, was the only one registered among the studied populations. Additionally, it was investigated under laboratory conditions if juveniles are resistant to decreasing salinities, a fact that might compromise recolonization of the less saline estuarine areas. Results show that these early life stages are indeed less resistant to low salinities. Even though, a floating behaviour at water surface was observed and, once confirmed with data collected from field studies, might be considered a specific asset regarding colonization of inner estuarine areas.

8.1 INTRODUCTION

Nucella lapillus (L.) is a prosobranch gastropod widely distributed along the North Atlantic rocky shores (Crothers, 1985a; Tyler-Walters, 2008), which populations have suffered a drastic decline in specimens' number from the early 80's due to tributyltin (TBT) pollution (Bennett, 1996). Organotins (OTs), especially TBT compounds, were intensively used as biocides in antifouling (AF) paints on ships hulls to prevent bioincrustation, but their harmful effects on non-target organisms were only found several years after their initial application, when the use of TBT AF systems had already been generalized. Deleterious effects as a consequence of TBT intense usage were then described, namely *imposex* – the superimposition of male sexual characters onto prosobranch females' reproductive tract (Smith, 1971; Smith, 1981).

During the 80s, extreme cases of complete female functional sterilization, populations declines and extinctions were amongst the reported consequences of high TBT pollution levels (Gibbs and Bryan, 1986, 1987; Gibbs et al., 1987; Gibbs et al., 1988), especially in sheltered inlets where harbours, shipyards and marinas are frequently located (Crothers, 1989; Colson and Hughes, 2004).

Legislation restricting TBT-based AF paints had been applied but a global ban on its use had only entered into force on September 2008 (for a review on the legislation see

Sonak et al., 2009). Some signs of *N. lapillus* recovery from *imposex* have already been described as a result of the successive legislative measures banning TBT-based antifoulants application, as for example around the British Islands (Crothers, 2003; Colson and Hughes, 2004; Oliveira et al., 2009a) but, although some hypotheses have been put forward, those resettled specimens sources remain mere suggestions of how recolonization might have occurred.

Nevertheless, despite these encouraging reports, TBT pollution is still a matter of concern in many regions, namely along the Portuguese coast where local extinctions near maritime infrastructures have also been mentioned as the possible reason for *Nucella lapillus* absence inside sheltered estuarine areas where main ports are frequently located (Barroso and Moreira, 1998; Barroso et al., 2000; Barroso and Moreira, 2002; Barroso et al., 2002; Galante-Oliveira et al., 2006; Sousa et al., 2007; Galante-Oliveira et al., 2009; Galante-Oliveira et al., 2010b).

N. lapillus specimens can be found from extremely exposed places to the most sheltered inlets, fact considered as the main reason why a high variability in shell appearance is observed (Crothers, 1985b). Regarding colour, shells can be from white to brown but also grey, brown shading to black, mauve grading to pink and yellow shading to orange; rarely true orange, pink or black; and occasionally showing a brown spiral banding (Crothers, 1985a; Tyler-Walters, 2008). Besides colour, shell shape plasticity has been extensively studied as being selected by both wave exposure and predation pressure, namely concerning size and shape (see review by Crothers, 1985b). Two specific morphs or ecotypes are defined: the sheltered and the exposed (Rolán et al., 2004).

Briefly, at sheltered shores *N. lapillus* grow faster and have longer shells than wave exposed populations (Crothers, 1985a). At sheltered places the heavy crabs' predation pressure is probably responsible for the selection of stronger and thickened shells, relatively small bodies (Currey and Hughes, 1982), smaller apertures (Etter, 1989) and sometimes toothed lips (Cowell and Crothers, 1970). In contrast, individuals at exposed shores have normally weaker shells, wider apertures and larger bodies, in particular larger feet to increase pedal adhesion to rocks surface under rough wave conditions (Currey and Hughes, 1982). A progression from the elongated (sheltered) to the squat (exposed) ecotype is found across increasing wave-exposure gradients and, therefore, a range of intermediate forms can also be observed (Crothers, 1985b, a; Etter, 1989). The analysis of *N. lapillus* shell shape was used by Crothers (1985b) as a way to classify wave-exposure of a given shore. The author found a linear relation between *N. lapillus* shell shape, expressed by the ratio shell height / aperture height (the author's definition is L/Ap where L is the total length and Ap is aperture height), and the classical Ballantine biologically-defined exposure scale (Ballantine, 1961) and used it to classify wave-exposure at Pembrokeshire – SW Wales (Crothers, 1985b).

In 1961, Ballantine had defined a scale to measure the degree of the SW Wales rocky shores exposure to wave action based on the observation that "different species growing on rocky shores require different degrees of protection from certain aspects of the physical environment, of which wave action is often the most important" (Ballantine, 1961). Thus, using this biologically-defined scale, an ecologist could look at the local fauna and flora communities and directly classify wave-exposure in 8 different stages (from *1* – "extremely exposed" to *8* – "extremely sheltered"). Some years later, this scale was modified for northern shores of Norway by Dalby et al. (1978): it remained focused on local communities but was extended to a 10 point scale (from *0* – "ultimately exposed" to *9* – "ultimately sheltered").

In turn, instead of looking at the entire biological community, Crothers developed a way to relate a single variable (*N. lapillus* shell shape) with wave-exposure by regression analysis of the shell shape ratio and the exposure degree given by the Ballantine's scale, maintaining its quantification ability (for a review on the topic see Crothers, 1985b). The method was then applied throughout both eastern and western North Atlantic coasts, where this species is widely distributed, although some adjustments to the initial Pembrokeshire regression were made due to few obvious, and some rather obvious, exceptions of *N. lapillus* shell shape variation pattern at different regions (see Crothers, 1983, 1985b).

Beyond the species phenotypic variation, differences in chromosome numbers are also present between sheltered and exposed morphs (Bantock and Cockayne, 1975). *N. lapillus* is the only muricid in which karyotype variations by intraspecific Robertsonian (Rb) chromosomal translocations are described (Bantock and Cockayne, 1975; Pascoe and Dixon, 1994; Pascoe et al., 1996; Pascoe et al., 2004). This chromosomal rearrangement, which name is owed to the process relator Robertson (1916), is a type of non-reciprocal translocation involving two non-homologous chromosomes.

The chromosomal feature commonly found to undergo such translocations is the acrocentric centromere, partitioning the chromosome into a large arm containing the vast majority of genes, and a short arm with a much smaller proportion of genetic content (Schulz-Schaeffer, 1980). During an Rb translocation, the participating chromosomes break at their centromeres and the long arms fuse to form a single chromosome with a single centromere. Short arms also join to form a reciprocal product, which typically contains non-essential genes and is usually lost within a few cell divisions. Therefore, Rb translocation also results in a chromosome number reduction (Schulz-Schaeffer, 1980).

Differences in *N. lapillus* karyotype were first described at populations around Roscoff – Brittany, being associated with shell thickness variations among sites at that region (Staiger, 1957 in Crothers, 1985a). Since then, a link between chromosome number and the degree of wave-exposure was proposed and widely reported (Bantock and Cockayne, 1975; Crothers, 1985a; Kirby et al., 1994; Pascoe and Dixon, 1994; Pascoe et al., 1996; Kirby, 2004; Pascoe et al., 2004; Rolán et al., 2004). Over much of its wide geographic distribution, *N. lapillus* has a karyotype consisting of 2n = 26, but in a restricted part of its range chromosome counts of 2n = 36 are common (Bantock and Cockayne, 1975; Pascoe and Dixon, 1994). As the Rb translocation results in a chromosome number reduction (Schulz-Schaeffer, 1980), it is accepted that the 2n = 26 form probably arose from another with higher chromosome number by reduction (Crothers, 1985a). The 2n = 26 morph was reported to occur on exposed shores, typified by high levels of wave action, while dog-whelks with higher chromosome numbers were observed in restricted areas of sea coasts where wave-exposure is attenuated: the 2n = 36 morph occurred almost exclusively in sheltered bays and harbours where hydrodynamics is reduced to a minimum (Kirby et al., 1994; Pascoe and Dixon, 1994; Pascoe et al., 2004).

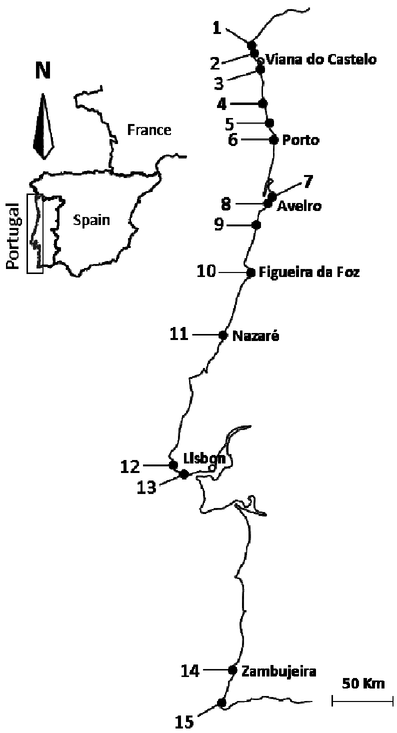
Considering the species apparently low dispersal capacity, since it has a relatively long generation time, the reproductive cycle does not include a planktonic larval phase and adults have reduced mobility (Feare, 1970; Crothers, 1985a; Etter, 1989; Gibbs, 1993, 1999), this work aims to discuss *N. lapillus* potential strategies to recolonize sites where populations were putatively extinct due to females' sterilization by high TBT concentrations. On one hand, specimens' shells shape and chromosome numbers are analysed along the Portuguese mainland coast in order to define which ecotypes are available to recolonize the most sheltered places where the main harbours are usually located. In fact, there are evidences that animals of the appropriate shell shape would move along from adjoining shores (Crothers, 1989) and that even populations from exposed shores possess the genetic capacity, when subjected to the appropriate selection pressure, to produce the typical sheltered morph (Gibbs, 1993) and so recolonize those areas.

Although, as *N. lapillus* normal dispersal is limited to its ability to crawl and, in addition, there is no pelagic phase in its life cycle (Currey and Hughes, 1982), is it possible for specimens from open shore populations, far in distance from estuarine and less saline areas, to achieve the most protected regions as, for instance, estuaries inner parts?

It is almost common sense that juveniles are essential sources underlying any new populations' establishment and so, in order to answer the above mentioned question, the juveniles' role in the recolonization process of estuarine innermost areas was also investigated. Using Ria de Aveiro as a case study of an estuarine system in the NW Portuguese mainland coast, the period of egg capsules maturation and juveniles hatching was studied to find out if there is any specific strategy of increase early life stages abundance, allowing episodes of successful migration and/or dispersion. Furthermore, as estuaries are transitory environments namely regarding salinity, the juveniles' resistance to decreasing salinities was also studied, under laboratory conditions, aiming at establishing the limiting salinity, that is, below which this species early life stages hampers the establishment of new populations in this estuarine system.

8.2 MATERIAL AND METHODS

8.2.1 Shell shape analysis



From May to July 2006, *N. lapillus* specimens of all available sizes in each population were randomly collected during 45 minutes (min.) by a single person at 15 sites (St. 1-15; Figure 8.1) along the Portuguese mainland coast. Of these, 2 sites are located inside Ria de Aveiro estuarine system (St. 7 and 8) and the remained are on the open coast, though with different wave exposure degrees (Figure 8.1).

Figure 8.1 *Nucella lapillus*. Portuguese mainland coastline map indicating sites (St. 1-15) were specimens were collected in 2006.

Once in the laboratory, specimens' shell and aperture heights (SH and AH, respectively; see Figure 8.2) were measured with vernier callipers to the nearest 0.1 mm.

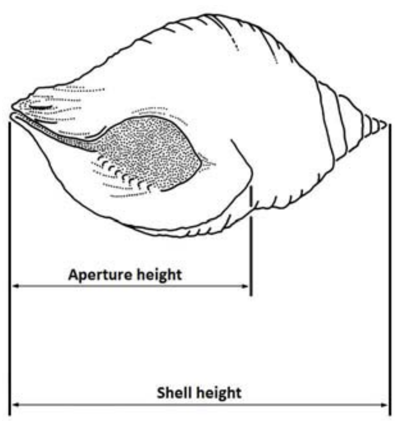


Figure 8.2 *Nucella lapillus*. Linear shell measurements used for comparing shell morphology: aperture and shell heights (AH and SH, respectively). Adapted from Son and Hughes (2000).

These individual parameters were plotted and their allometric relation established per site by the regression AH = a SH^b. In order to standardize comparisons between populations, the mean SH for animals with SH ≥ 17.50 mm (SH at sexual maturity; see Galante-Oliveira et al., 2010c) was calculated. With this mean SH obtained for each site, and using the respective regression equation, an AH value was calculated. It was then possible to find one shell shape ratio value (SH/AH) for each of the sampled populations, in order to compare local wave-exposure following Crothers (1985b) method.

8.2.2 Chromosome counts

Ten adults collected randomly from each sampling site were maintained in aerated sea water from the respective site (St.1-15; Figure 8.1) for chromosome number analysis. The methods used for the preparation of mitotic metaphase spreads, slide-making and karyotyping are those described by Dixon and Pascoe (1993) with some adjustments. Adults were exposed to a 50 $mg.l^{-1}$ Colchicine solution in sea water during 10h. Ended this period, shells were removed and animals' sexed. Six animals per site, 3 per gender, were used to obtain metaphase spreads. Gills were collected and submitted to a hypotonic treatment for 45 min. in 0.075M Potassium Chloride (KCl) in distilled water. Afterwards, tissues were fixed by immersion in Carnoy's solution without chloroform (3:1 Absolute Ethanol, Acetic Acid) for 20 min. This procedure was repeated 3 times, changing the solution between the 20 min. periods. After the last period, samples were kept in the solution, in the dark and at 0-4 ^{0}C for at least 16h or until slide-making procedure. Slides were prepared from a small peripheral portion of the gill, which cells were suspended in a 60% Acetic Acid in distilled water for 1.5 min. using a Pasteur pipette up-and-down movement. Suspension was then placed on a clean microscope slide by releasing drops from 25 cm height, allowing the cells to dissociate and spreading chromosomes. Slides were transferred to a hot-plate (previously kept at 45 ^{0}C) for partial drying during 20 seconds. The suspension that did not evaporate was aspirated from each drop center and released again on a new slide. The procedure was repeated until suspension depletion and complete evaporation.

Finally, and for conventional microscopic observation, slides were stained for 22 min. in Giemsa solution (10 ml Giemsa 5% in 100 ml of Sorensen buffer), rinsed twice in distilled water and mounted in DPX resin. Slides were observed and, whenever available, the chromosome number in 30 spreads per specimen was recorded. Better-quality spreads were photographed for posterior production of the species karyotype.

8.2.3 Egg capsules development

Nucella lapillus egg capsules maturation was studied at St. 9 (Figure 8.1) from January to April 2006. An area of 25 m^2 was established (covering the whole intertidal zone) and marked by a removable 3D axis-system made of rope. Within the established axis system, 10 egg capsules clusters (ECC) were properly referenced (from A to J), monitored and photographed in approximately a fortnightly basis (T_0 = 28 January to T_4 = 7 April). At each moment, ECC size (approximate number of egg capsules) and external aspect were registered.

Based on our own previous observations, egg capsules were classified as “recent”, “middle age” or “old” according to their colour, volume / shape and internal texture (visible across the capsule wall). “Recent” laid capsules are usually of a light and uniform yellow, also indicating its homogeneous egg content, not very bulky and with a well defined vase shape (thinner and elongated in the upper half, near the apex). “Middle age” capsules are of variable colour (from an intense yellow, going through a pink / purple stage and ending on a light brown hue), with an heterogeneous interior of developing embryos / juveniles that are now visible through the capsule wall transparency, normally large, rounded and sometimes with a darker or even opened apex, ready for the juveniles hatching. “Old” capsules are of a brownish dark yellow, or even brown coloured, empty and usually squashed by the lack of content inside (after juveniles hatching).

8.2.4 Juveniles' sensibility to decreasing salinities

In order to test *Nucella lapillus* juveniles' resistance to salinity decreases, middle-age capsules with a darker / opened apex (ready for juveniles' hatching) were collected at St. 9 (Figure 8.1) in June 2006. Capsules were transported to the laboratory in local sea water and maintained aerated at 18±1 ^{0}C until juveniles' caesarean, right before exposure. For testing solutions preparation, two sea water litres were also collected in a previously acid washed Schott bottle. Likewise, all the material needed for animals' handling and exposure was acid washed: firstly treated in a 10% Hydrochloric Acid (HCl) solution in distilled water for 24h, followed by a 24h period in distilled water and 3 last rinses in running distilled water before being dried in the oven at 60 ^{0}C. In addition, and also to prevent testing solutions infections / contaminations, the 2l sea water were autoclaved right after arrival from sampling. Once cooled, 10 solutions of different salinities ranging from 35 to 9 psu (inclusively) were prepared by diluting original 35 psu in distilled water obtaining 31, 27, 25, 23, 19, 15, 13, 11 and 9 psu solutions with constant pH.

Juveniles' were then removed from capsules by caesarean section just before being exposed in sterile 24-well plates. Ten replicates of 6 animals were exposed: juveniles were individually placed in 2.5 ml of testing solution per plate-well. The solution was replaced after 48h and mortality was recorded after 1, 24, 48, 72 and 96h of exposure. Furthermore, each animal was observed for 2 minutes and some behavioural aspects were also recorded. The percentage of mortality was calculated per replicate and then statistically compared. Differences between replicates in the same salinity were studied applying the one-way ANOVA, after the data normalization by arcsine transformation and the respective normality and homoscedasticity confirmation. The replicates mortality mean by treatment was then calculated and the effect on juveniles' survival was statistically tested by a factorial ANOVA (considering the salinity, exposure time and salinity *vs.* exposure time effects on juveniles' mortality) followed by post-hoc tests for multi-comparisons by the Dunnett's method. All statistical procedures were performed using *Statistica v6* software.

8.3 RESULTS AND DISCUSSION

8.3.1 Ecotypes along the Portuguese coast

Results on *Nucella lapillus* shells' allometry are presented in Table 8.1. The mean shell height (SH) of specimens' sexually mature at each sampled population varied between 18.67 mm at St. 11 and 23.98 mm at St. 8. Generally, with the exception of females at St. 13, allometric regression r^2 values were highly significant and generally higher than 0.7, confirming the relation between shell and aperture heights (AH = a SH^b) and allowing AH calculation through the respective equation (Table 8.1).

Males and females shell shape ratio (SH/AH) are also presented in Table 8.1. Males SH/AH varied from 1.55 at St. 11 to 1.86 at St. 2, in agreement with the females' range from 1.55 to 1.80 registered at the same sites, respectively. After confirming data normality and homoscedasticity, we tested (repeated measures t-student) differences between shell shape ratio between genders and found that in males it is significantly higher ($t = 6.104$; $p < 0.001$). Nevertheless, that difference is consistent across sites and thus a single SH/AH value for the whole sample (the mean SH/AH of both genders) was calculated per site and used hereafter. Shell shape ratios registered along the Portuguese shoreline were always higher than the ones described by Crothers (1985b) for all populations surveys in his studies in the west and east Atlantic littorals. Even so, we cannot conclude that the Portuguese mainland shore is less exposed because many factors differ between coasts and populations under comparison when they are very distant from each other. Crothers regression between SH/AH and exposure described for Portuguese populations (Crothers, 1985a) revealed an r value much lower than the desirable in regression analysis ($r = 0.266$) not allowing the calculation of the Ballantine exposure degree in order to compare different shores exposure.

Table 8.1 *Nucella lapillus*. Shells' allometry regression equations per gender, and respective r^2, for populations sampled at sites (St. 1-15) along the Portuguese coast. The mean shell height (SH) at each population was calculated using animals' randomly collected at each site for 45 minutes with SH ≥ 17.50 mm. This value was applied in the respective regression equation to calculate the mean aperture height (AH) per gender at each population. Shell shape ratio (SH/AH) was then obtained. Results on chromosome number counts at 3 males and 3 females per site are also given with the indication of: total number of mitotic metaphase spreads visible (Visible spreads), number of spreads karyotyped (Karyotyped spreads) per site and the respective diploid chromosome number (2n) observed at each karyotyped spread.

Site code and name	Coordinates (EUR 50)		SH	Males				Females				Visible spreads	Karyot. spreads	2n
				$AH = aSH^b$	r^2	AH	SH/AH	$AH = aSH^b$	r^2	AH	SH/AH			
1. Vila Praia de Âncora	41°48.93N	8°51.94W	20.72	$AH = 0.9810\ SH^{0.8421}$	0.9720	12.60	**1.65**	$AH = 0.8603\ SH^{0.8902}$	0.9833	12.78	**1.62**	323	178	26
2. Praia Norte	41°41.85N	8°41.13W	20.12	$AH = 0.3536\ SH^{1.1389}$	0.8013	10.79	**1.86**	$AH = 0.7215\ SH^{0.9136}$	0.7940	11.20	**1.80**	47	29	26
3. Praia da Amorosa	41°38.72N	8°49.31W	22.06	$AH = 0.8859\ SH^{0.8571}$	0.6658	12.56	**1.76**	$AH = 0.9048\ SH^{0.8600}$	0.7759	12.94	**1.70**	64	31	26
4. Póvoa de Varzim	41°23.18N	8°46.40W	22.18	$AH = 0.8935\ SH^{0.8559}$	0.9552	12.68	**1.75**	$AH = 0.9161\ SH^{0.8532}$	0.9634	12.89	**1.72**	160	75	26
5. Praia de Leça	41°12.21N	8°42.82W	22.07	$AH = 1.4337\ SH^{0.7071}$	0.7417	12.78	**1.73**	$AH = 1.6993\ SH^{0.6610}$	0.7441	13.14	**1.68**	90	33	26
6. Praia da Foz	41°09.78N	8°41.10W	22.52	$AH = 1.5995\ SH^{0.6756}$	0.8214	13.12	**1.72**	$AH = 1.9617\ SH^{0.6219}$	0.8095	13.61	**1.65**	84	38	26
7. Aveiro (Forte da Barra)	40°38.56N	8°43.59W	23.04	$AH = 0.9946\ SH^{0.8346}$	0.9781	13.64	**1.69**	$AH = 1.0077\ SH^{0.8344}$	0.9834	13.81	**1.67**	n.a.	n.a.	26
8. Aveiro (Marégrafo)	40°38.71N	8°44.82W	23.98	$AH = 1.1184\ SH^{0.7970}$	0.9551	14.07	**1.70**	$AH = 1.2261\ SH^{0.7743}$	0.9347	14.35	**1.67**	31	19	26
9. Aveiro (Areão)	40°31.05N	8°47.05W	21.54	$AH = 0.9577\ SH^{0.8640}$	0.9628	13.59	**1.59**	$AH = 0.8541\ SH^{0.9076}$	0.9816	13.85	**1.55**	120	45	26
10. Fig. Foz	40°10.18N	8°53.26W	20.72	$AH = 0.9894\ SH^{0.8402}$	0.9562	12.63	**1.64**	$AH = 1.0214\ SH^{0.8322}$	0.9632	12.73	**1.63**	73	27	26
11. Nazaré	39°36.26N	9°04.49W	18.67	$AH = 0.8150\ SH^{0.9192}$	0.8976	12.01	**1.55**	$AH = 0.9201\ SH^{0.8795}$	0.9760	12.07	**1.55**	48	17	26
12. Praia do Guincho	38°43.74N	9°28.46W	19.52	$AH = 0.7715\ SH^{0.9266}$	0.9366	12.11	**1.61**	$AH = 0.6501\ SH^{0.9886}$	0.9586	12.27	**1.59**	28	12	26
13. Praia das Avencas	38°41.21N	9°21.27W	19.72	$AH = 1.0969\ SH^{0.7730}$	0.7377	10.99	**1.79**	$AH = 2.9131\ SH^{0.4563}$	0.3302	11.36	**1.74**	12	4	26
14. Zambujeira do Mar	37°33.20N	8°47.44W	20.00	$AH = 0.8715\ SH^{0.8878}$	0.9488	12.45	**1.61**	$AH = 1.1007\ SH^{0.8189}$	0.9296	12.80	**1.56**	34	19	26
15. Praia do Amado	37°15.22N	8°38.45W	19.95	$AH = 0.7741\ SH^{0.9305}$	0.9416	12.54	**1.59**	$AH = 0.8102\ SH^{0.9146}$	0.9506	12.52	**1.59**	40	20	26

n.a.: not available data

However, a mean SH/AH variation of 0.28 between the maximum and minimum recorded in the current study indicates the occurrence of different wave-exposure degrees in the study area and by looking the physiographic properties of the sampling sites we can confirm that higher SH/AH are registered at the most sheltered locations (St. 2-8 and 13) and the lower values at the most exposed ones (St. 1, 9-12 and 14-15). For instance, considering three sites that constitute an increasing wave-exposure gradient from inside Ria de Aveiro estuarine system to the open coast (St. 7 > 8 > 9), a decrease in the mean SH/AH is observed from 1.68 at St. 7 and 8 (sheltered areas) to 1.57 at St. 9 (exposed).

Even though SH/AH amplitude registered along the Portuguese coast is higher then the one described around UK coasts (see review by Crothers, 1985a) and, therefore, more variation between populations between sheltered and exposed sites would be expected, there was no difference in chromosome numbers between individuals either at the same or among sites (Table 8.1). All specimens analysed for chromosome numbers revealed 2n = 26 (Table 8.1), the normal diploid form of *N. lapillus* at exposed shores (Bantock and Cockayne, 1975; Dixon and Pascoe, 1993; Pascoe et al., 2004), situation also described at our neighbour Galician coast (Rolán et al., 2004). The species karyotype was prepared and is showed in Figure 8.3.

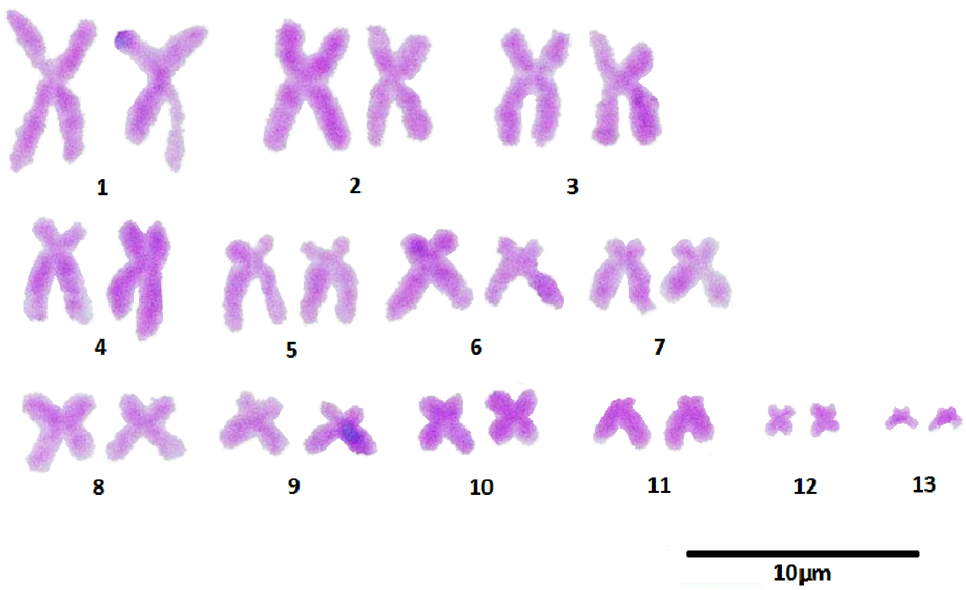


Figure 8.3 *Nucella lapillus*. Karyotype of 2n = 26 from an adult collected at St. 4 in 2006. The 13 chromosomes' pairs are organized by the respective size decreasing order.

8.3.2 Egg capsules clusters evolution

Nucella lapillus egg capsules were always observed during the current study in all visited sites (St. 1-15; Figure 8.1). St. 9 was visited every month during 2006 and egg capsules were observed all year round. For this study we monitored 10 egg capsules clusters (EEC) evolution at St. 9, from January to April 2006 in order to depict average time of egg development, from spawning to juvenile hatching (see Figure 8.4).

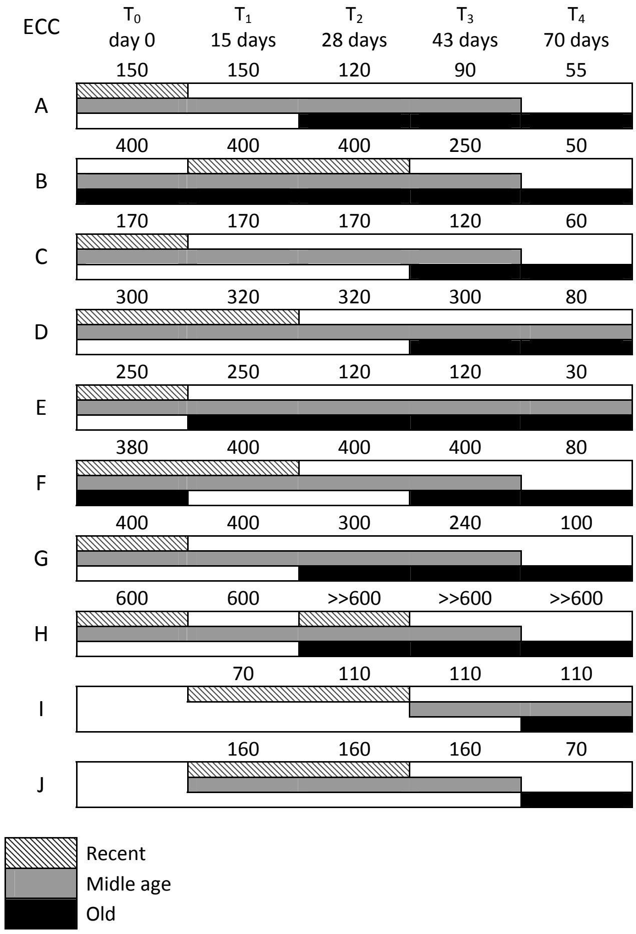


Figure 8.4 *Nucella lapillus*. Schematic development of 10 (A-J) egg capsules clusters (ECC) from January to April 2006 on a fortnightly basis (the number of days passed from T_0 is indicated). Indication of the presence of recent, middle age and old capsules at each observation occasion (T_0 to T_4). Over each cluster development scheme is indicated the approximate total number of capsules present at the picture moment.

Within this period of 70 days (about 2.5 months), it was possible to follow the complete development of recent spawned egg capsules (see Figure 8.4, ECC I and J). The presence of old egg capsules at T_0 with the occurrence of recent laid egg capsules at T_1 (in ECC B and F) and T_3 (in ECC H) may reveal that, at this site (St. 9; Figure 8.1), the species spawning tendency is to occur where clusters were previously formed. Even though, a decrease in the total number of capsules at T_3 and T_4 was registered at the majority of the monitored clusters along time. It is important to remember that this site is located at one of the most heavily exposed sites (mean SH/AH = 1.57), subjected to strong hydrodynamics, and so old capsules (already hatched and thus empty) are removed from rocks by waves' action, which was easily noted both *in situ* and by the observation of pictures taken at successive occasions.

8.3.3 Juveniles' survival at different salinities

After percentages normalization by arcsine transformation, data normality and homoscedasticity were statistically confirmed. No significant differences were found between replicates of each of the tested salinities.

The mean mortality by treatment along the 96h exposure is presented in Figure 8.5. Differences in juveniles' mortality were significant (ANOVA global result: F = 2449.760; p < 0.001) between salinities (F = 399.682; p < 0.001), along time at each salinity (F = 55.145; p < 0.001) and a combined effect of the treatment and the exposure time was statistically confirmed (F = 8.220; p < 0.001).

For an easier overview on the results, significance of post-hoc tests for multi-comparisons is indicated in Figure 8.5. Globally, a recently hatched *N. lapillus* juvenile can not survive after exposures of 1h to salinities ≤ 9 psu or 72h to salinities ≤ 11 psu.

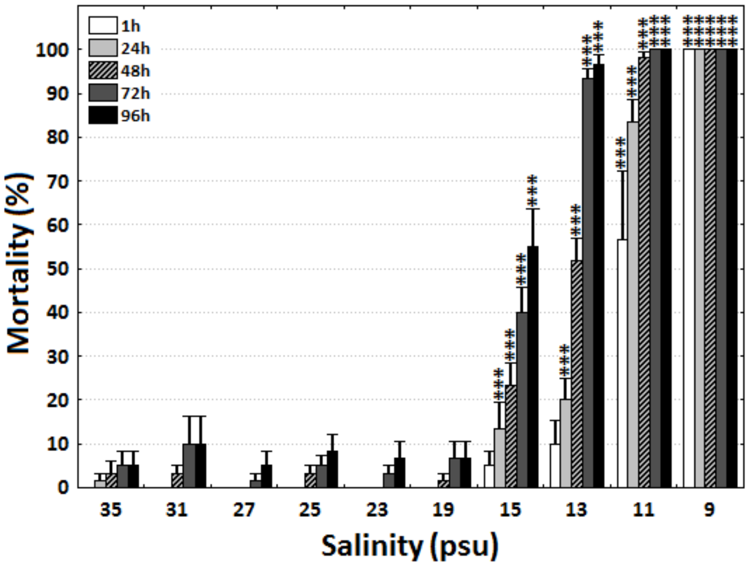


Figure 8.5 *Nucella lapillus*. Juveniles' mortality during a 96h exposure to solutions of decreasing salinities (from 35 to 9 psu). The mean mortality (expressed in percentage) and the respective stardard error are plotted as a function of the juveniles' exposure to different salinities over time (from 1h to 96h). Results of post-hoc tests for multi-comparisons (by the Dunnett's method) of mortality at different salinities with the control (seawater – 35 psu) are indicated by asterisks (***: p < 0.001) on the top of the respective bar.

About 80% of the surviving juveniles were rafting at the solution surface when observation was carried out. This behaviour was immediately initiated by the animal even after its sink (either by a soft pincer touch or a little swirl of the solution when the plate has been positioned for observation). Additional observations were also registered: (i) was always seen the presence of a translucent mucus around rafting juveniles' feet; (ii) small air bubbles within mucus were sometimes observed and seemed to help keeping animals' rafting at surface; (iii) the process usually started by juveniles' crawling through the plate-wells walls, achieving the surface and only then inverting their position. Once confirmed in the field, it can be hypothesized that this rafting behaviour might be crucial for the species dispersal as described for example for *Hydrobia ulva* (Armonies, 1992; Armonies and Hartke, 1995; Meireles and Queiroga, 2004). For *N. lapillus* this strategy would gain other dimension since this species reproductive cycle does not include a planktonic larval phase and adults have reduced mobility (Feare, 1970; Crothers, 1985a; Etter, 1989; Gibbs, 1993, 1999).

8.4 SPECIFIC CONSIDERATIONS ON THE INITIAL ASSUMPTIONS

Several reports on *Nucella lapillus* recoveries from *imposex* have emerged in the literature since TBT pollution has been declining, especially near harbours and areas were naval traffic is more intense (Evans et al., 1996; Harding et al., 1997; Miller et al., 1999; Birchenough et al., 2002; Crothers, 2003; Colson and Hughes, 2004; Huet et al., 2004; Morton, 2009; Oliveira et al., 2009). Although high in number, most of these reports are simply descriptions of populations' recoveries in terms of *imposex* levels reduction and only few of them disclose populations' reestablishments at sites where extinctions were assured (Gibbs, 1993; Crothers, 2003; Colson and Hughes, 2004; Huet et al., 2004; Morton, 2009). Likewise, some studies confirming TBT pollution and *imposex* levels decline along the Portuguese coast have been published (Sousa et al., 2007; Rato et al., 2009; Sousa et al., 2009) and namely using *Nucella lapillus* as a bioindicator (Galante-Oliveira et al., 2009; Galante-Oliveira et al. 2010a). It is therefore appropriate to consider how sheltered areas, most of them inside estuaries where TBT sources have been described as responsible for *N. lapillus* extinctions (Barroso et al., 2000; Barroso and Moreira, 2002), might be naturally recolonized. Several hypotheses have been proposed and discussed based on the obtained results. Nevertheless other considerations should be made.

After episodes of strong wave-action, specimens' dislodgment can lead to some places colonization (Gibbs, 1993). However, adults' movement occurs only by crawling through continuous habitats (Crothers, 1985a; Etter, 1989; Crothers, 2003) and for short distances (Currey and Hughes, 1982; Burrows and Hughes, 1990) and so places colonized by dislodged adults' must be nearby their original area (Gibbs, 1993). Accordingly, Hughes (1972 in Currey and Hughes, 1982) tagged *N. lapillus* in a Nova Scotia population and observed that specimens' remained 12 months within 4 m of the site where they were released; Crothers (2003) has also described a period of 13 years to *N. lapillus* recolonize a distance of 30 m in the Watermouth Cove natural harbour – SW England. In addition, animals' are often buried and/or reburied during storms and it is doubtful whether many of these colonists would survive after being displaced by strong waves (Gibbs, 1993). Regarding juveniles, they hatch already as well-formed crawl-aways, and so are also described as having low mobility. Feare (1970), while studying the ecology of an exposed population at Robin Hood's Bay – Yorkshire, referred that newly hatch juveniles are unlikely to be washed down the shore once they protect themselves from water movement on empty cases of barnacles, avoiding bare rock. Only two of the sites sampled in the current study (St. 7 and 8; Figure 8.1) are located inside sheltered areas, corroborating observations along the Portuguese coast in the last decade that *N. lapillus* actual distribution is mainly restricted to the open coast and most of them far in distance from estuarine sheltered places. Although possible, recolonization of sheltered areas by dislodged specimens and/or crawling is questionable in our study area both because of the distance and of the specimens' limited mobility.

Another possibility that cannot be ruled out is the introduction of individuals from distant populations, transported on floating material and/or by vessels in areas of intense maritime traffic (Colson and Hughes, 2004). Animals' left accidentally in a rocky groin by a vessel, even being from an exposed shore and exhibiting the respective ecotype, possess the genetic capacity to produce soon (in the next generation – F1) at least some of the characteristics needed to survive in a sheltered place (Gibbs, 1993). Even though, the salinity regime inside estuaries is another limiting factor for the most inner areas recolonization. *N. lapillus* adults can survive at salinities of 10 psu although they cannot breed (Fisher, 1931 in Crothers, 1985a). Furthermore, non feeding animals were reported when subjected to brackish conditions (Crothers, 1985a). Hence, it is easily understandable that, as populations of this non-dispersing species can only survive where adults can eat and breed successfully, the species had never occurred under this salinity level (10 psu).

Regarding juveniles, hardly any report was found in the literature addressing their resistance to low salinities. Our results corroborate the above referred for adults i.e. new *N. lapillus* populations can not be established under 10-11 psu and juveniles contribution for colonization will also be limited by salinity, as about 50% of them dye when kept 96h to a salinity of 15 psu. Even though, juveniles' rafting was observed and confirmed under laboratory conditions which, if confirmed in the field, might be an asset for the species due to its limited dispersal potential. This hypothetical scenario would explain many questions asked over time as, for example: (i) How is it possible that this species is observed on both sides of the Atlantic Ocean? (Colton, 1922); and (ii) How is it possible such rapid recoveries and no marked genetic consequences of extinction / recolonization events? (Colson and Hughes, 2004); (iii) How can isolated rocky groins, such as those found in Aveiro´s coastline, be colonised by dogwhelks?.

Recolonization of sheltered areas by rafting juveniles' is here hypothesised. The present work shows laboratory evidences that *Nucella lapillus* juveniles' can float at surface and so, if they exhibit the same behaviour under field conditions, the recolonization of areas within estuaries by this mechanism is probably the answer to those questions. The following question is how they resist lower salinities. As initial life stages are generally more sensitive, it was predicted that they would be even less tolerant than adults. Even considering the juveniles low resistance to decreasing salinities (≤ 15 psu), taking Ria de Aveiro as a case study, values for long time under 15 are only expected during early Spring (in March; see Génio et al., 2008). It seems obvious that, during this period, juveniles' dispersal by rafting will not be effective for the sheltered sites recolonization. Nevertheless, as eggs capsules maturation at St. 9 was confirmed to be around 2.5 months (current study data) and Galante-Oliveira et al. (2010c) referred the species main spawning period at the same site is in the late Spring / early Summer (from May to July), juveniles may hatch mainly from July to September, when salinities below 15 psu are only expected at upstream limits of the channels.

Although reasonable, this hypothesis has to be evaluated in the field, work to be continued after this thesis conclusion.

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Part III

Final Remarks

Chapter 9

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9.1 THE "TBT POLLUTION": THE END OF A TALE

The "TBT tale" began long ago and has been suggested as one of the "late lessons from the last century early warnings" that can help modern society to achieve a better balance between the innovations benefits and their unknown hazards (Santillo and Langston, 2001). This story onset and development are extensively documented in the literature and the *State of the Art* of the present thesis presents a comprehensive review on the topic (see Chapter 1). Nevertheless, any classically structured story has also an end to be told and, as this one will certainly be used as an example to avoid ignoring similar early warnings in the future, it was as important to report its beginning as it will be to tell its end.

The current work pretends to be a contribution to describe the "TBT tale" end. The closure of an important chapter of that story is present within this thesis pages since the most strict measures taken to eradicate tributyltin (TBT) compounds and their damaging effects from aquatic ecosystems are contained in this study period: (i) it was initiated at the moment when TBT-based antifouling (AF) paints were ruled out from ships operating under European Member States control by the EU Council Regulation

No.782/2003 and (ii) it ends right after the TBT global ban by the IMO "International Convention on the Control of Harmful Antifouling Systems on Ships" (AFS Convention) entry into force (see this dissertation aims and rationale in section *2.1*).

Despite the need to verify this measures effectiveness in reducing TBT pollution, these compounds monitoring is also required once they are considered priority hazardous substances in EU legislation (Directives 2000/60/EC; 2008/56/EC and 2008/105/EC) and, in addition, are included in the OSPAR list of chemicals for priority action (OSPAR, 2007). Since the OSPAR Convention is the current legislative instrument regulating environmental protection in the North-East Atlantic, the implementation of the European Environmental Quality Standards (EQS) on the presence of hazardous substances in the sea and the baseline monitoring of environmental conditions across this geographic area are among the OSPAR Commission responsibilities (Directives 2008/56/EC and 2008/105/EC).

Imposex assessment is mandatory for monitoring TBT pollution biological effects within the OSPAR area and is now kept under regular scrutiny as an indication of TBT environmental concentrations (OSPAR, 2008c). Even so, errors and omissions in the data should be identified and corrected, and data assessment methods progressively updated, tested and improved, to evaluate the efficacy of the already applied legislation and also to ensure that the assessment system is fully prepared to provide an up-to-date evidence base for future policy making (OSPAR, 2008b).

The application of rigorous methods to assess *imposex* levels is essential to obtain reliable conclusions on the follow up of TBT pollution trends and the current work shows that some of the *imposex* assessment parameters are influenced by critical variables that may weaken results reliability.

9.2 DETERMINANT FACTORS FOR THE USE OF *Nucella lapillus IMPOSEX* IN TBT POLLUTION MONITORING

Imposex analysis shall follow the OSPAR JAMP guidelines, through which *Nucella lapillus* is the first recommended bioindicator (OSPAR, 2008a). Among the different available *imposex* indices for this species – relative penis size index (RPSI), vas deference sequence index (VDSI), percentage of *imposex* affected females (%I) and percentage of sterilized females (%S) – the two former ones have been largely used to track evolution of TBT pollution and so the current work seek for a proper validation on the use of RPSI and VDSI in *N. lapillus* for monitoring programmes.

Regarding RPSI, it is showed that this index is strongly influenced by the specimens' size (Chapter 6 and 7). In fact, it was observed that larger specimens' present longer penis. Therefore, under the same TBT pollution level [i.e. keeping the females penis length (FPL) constant in the RPSI calculation formula], the analysis of larger males would result in a lower RPSI value and consequently indicate an incorrect decrease in TBT environmental concentrations (Chapter 6). The reverse effect would be expected if, keeping the male penis length (MPL) constant, larger females were analysed. Nevertheless, RPSI had also been developed to minimize the effect of variations in specimens' size between samples collected in different sites or in successive surveys. Even though, such shell height (SH) variations are not always registered at the population level (i.e. with the same trend and magnitude in both genders) and differences in males and females SH in opposite directions are frequently observed at a same site (see an example in Chapter 7: at St. 14 from 2003 to 2006, males SH decreased in contrast to the females SH increase). Thus, regarding SH oscillations, it is very difficult to simultaneously control MPL and FPL and the simplest way to deal with this problem is to test the differences in SH between samples to be compared and take this aspect into consideration when interpreting the results.

Other factors had been referred as affecting MPL in *N. lapillus* and, consequently, the RPSI consistency (Minchin and Davies, 1999b, a). *N. lapillus* cryopreservation, specimens' narcotization and time during which animals' are handled before penis measurements were already described as inducing variations in MPL (Minchin and Davies, 1999b, a). Since such reports were available in the literature, and once OSPAR JAMP guidelines expressly indicate that *N. lapillus* should be examined alive and without narcotization to "retain consistency with many previous studies" (OSPAR, 2008a), the effect of these factors on *imposex* results were not analysed in the current work. But besides the specimens' size, it was studied if *N. lapillus* MPL is also affected by the individual reproductive condition and the males' position in the shore (Chapter 6). Regarding MPL seasonality, other authors had already referred that "intensive unpublished analyses of many stations have shown that, besides an individual variability, penis size of males varies during the year even in continuously-breeding populations" (Oehlmann et al., 1991). Although valid, the information given by Oehlmann et al. (1991) refer to narcotized specimens and so this subject is not very relevant to be taken into account for OSPAR monitoring programmes that recommend that MPL should be measured in non narcotized animals. Besides, these authors studied populations from Brittany – Northern France, an area where the Dumpton Syndrome was widely described (Huet et al., 1996a; Huet et al., 1996b; Huet et al., 2004; Huet et al., 2008) affecting MPL. Finally, *N. lapillus* populations present geographical variability (as showed in Chapter 8) and the MPL seasonal variation reported to Northern France may not be applicable to the Portuguese coast (the species southern distribution limit in Europe). For these reasons, *N. lapillus* reproductive cycle and the MPL seasonal variation were also investigated at Aveiro coast for non narcotized animals (Chapter 6) and it could be confirmed that MPL does present a marked variation throughout the year in this population as well, presenting lowest values in the summer. Consequently, for a certain TBT environmental level, a difference in RPSI can be artificially created by different sampling dates. This phenomenon is hard to control in *imposex* surveys since the described seasonal pattern may present slight variations from year to year. However, even knowing that is not possible to eliminate this effect, MPL seasonal variation can be minimized by collecting animals at the same year period in pluriannual monitoring programmes (Chapter 6).

The effect of the specimens position in the rocky shore (regarding the distance to egg capsules clusters) on the RPSI was never reported before in the literature and is pointed out for the first time in present thesis. It was shown that this may influence RPSI results once males near egg capsules clusters present longer penis than the ones positioned far from breeding spots (Chapter 6). Hence, for monitoring purposes, individuals should be sampled at the same location in relation to egg capsules clusters (near or distant) to allow truthful comparisons between surveys. Another possibility is to keep specimens separated by groups during collection, *imposex* analysis and till the end of the results computation, in order to compare the values obtained when using animals from different locations.

Regarding VDSI, it was demonstrated that the VDS level is positively and highly significantly associated with the SH (see Chapter 7). As VDSI increases with animals' size and it is extremely difficult to collect animals exactly of the same SH in different occasions, VDSI modelling based on regression analyses using SH as regressor, to remove the effect of this variable, should be employed.

VDSI modelling for the assessment of temporal trends in *N. lapillus imposex* levels had already been studied (ICES, 2007). However, some problems arose from the statistical procedure applied when several time-series were modelled simultaneously, mainly because VDS classes are ordinal and no sense of distance between classes is identified (Gibbs et al., 1987; ICES, 2007). In addition, the procedure developed by Fryer and Gubbins (ICES, 2007) does not take into account the influence of animals' size in *imposex* intensity and, as proved in the current work, *imposex* indices (VDSI included) are dependant on SH (Chapter 5 and 7). The innovative statistical procedure developed within this thesis analyses VDS data in a latent VDS modelling using specimens' SH as a regressor in order to: (i) contour the fact that VDS is not a continuous variable and (ii) control the effect of the specimens' size on *imposex* levels temporal comparisons (Chapter 7).

Despite all the indices used to assess *N. lapillus imposex* levels give complementary information, it seems that VDSI is the most important to follow TBT-specific biological effects, as already prompted by OSPAR (OSPAR, 2008a). In fact, VDSI gives information on the population reproductive capability; it is not subject to seasonal nor spatial variation, as the RPSI; allows the distinction between sites from low to moderate TBT pollution levels (Chapter 4, 6 and 7), which is not possible with the RPSI (values close to zero; see Chapter 4, 6 and 7) nor with %I (≈100% at all sites in a decreasing pollution scenario, in which RPSI values are already close to zero; see Chapter 4, 6 and 7).

Nevertheless, the use of RPSI is not totally discouraged, but a careful validation to avoid inaccurate readings is needed, regardless of being an index recommended by OSPAR (OSPAR, 2008a). In fact, RPSI is valuable for tracking TBT pollution in view of its power to elucidate the relative dimensions of penis in affected females as well as the magnitude of TBT pollution at a given site. However, we should have in mind that variations of its value may not necessarily reflect a change of the TBT pollution level but rather oscillations related to the sampling season, the distance to egg capsules clusters and also to the animals' size (Chapter 6 and 7). These situations were not taken into account in 2003 monitoring campaign (Chapter 3) being possibly the reason why RPSI values did not show any consistent change, increasing at some sites and decreasing at others, contrarily to the VDSI that exhibited a slowing down tendency (Chapter 3).

9.3 DETERMINANT FACTORS FOR THE USE OF *Hydrobia ulvae IMPOSEX* IN TBT POLLUTION MONITORING

As reported above for *N. lapillus*, one major concern when analysing *imposex* in *Hyrobia ulvae* was to control the effect of "confounding" factors in *imposex* assessment indices in order to provide reliable conclusions. It was verified that the MPL, FPL, VDS and %I are also strongly influenced by specimens' size in this species (Chapter 5) influencing monitoring results when using this bioindicator. Explicitly, smaller females will cause an underestimation of FPL, VDSI and %I, whilst smaller males cause an overestimation of the relative penis length index (RPLI) in *H. ulvae*, regardless of TBT pollution levels (Chapter 5), similarly to what was verified and is above mentioned for *N. lapillus* (Chapter 6 and 7). Moreover, as *imposex* analysis in this species have been carried out under specimens' narcotization, it was shown that such procedure causes a significant increase of both MPL and FPL, and so *H. ulvae* should always be observed under well standardized narcotization conditions (Chapter 5).

9.4 TEMPORAL TRENDS IN TBT POLLUTION ALONG THE PORTUGUESE COAST

The study area was still heavily impacted by TBT pollution in 2003, when Regulation (EC) No.782/2003 was implemented (Chapter 3). *Nucella lapillus imposex* levels and TBT females' body burdens were highest at sites located in the proximity of the main harbours and dockyards, confirming these as the main sources of TBT in the Portuguese shoreline, corroborating results obtained by other authors (Barroso and Moreira, 2002; Santos et al., 2002; Sousa et al., 2005; Rato et al., 2006; Sousa et al., 2007; Rato et al., 2008; Rato et al., 2009; Sousa et al., 2009).

In the same period, *N. lapillus imposex* levels in populations of the Portuguese coast were lower than those reported in NW Spain (Ruiz et al., 1998; Barreiro et al., 2004; Ruiz et al., 2008) and NW France (Huet et al., 2004). Even so, the Portuguese values remained higher than the ones observed in the Northern coast of Ireland (Mallon and Manga, 2007) and SW England (Birchenough et al., 2002) where TBT levels had declined in the vicinity of recreational harbours as a result of TBT AF paints restrictions. Nevertheless, regardless of those improvements, TBT was also “slow to abate” near spots of prevalent commercial shipping in SW England (Bray and Langston, 2006). Thus, it was obvious that a continued and more extensive monitoring would be needed to confirm temporal trends in pollution levels.

From 2003 to 2008 (Chapter 7), a significant global decline in *imposex* intensity was verified in the study area together with a remarkable improvement of the Portuguese coast ecological status after 2003, under the terms defined by the OSPAR Commission (see Chapter 1 section *1.2.1.3*). Although a slight decreasing tendency seemed to had occurred in VDSI between 2000 and 2003 in the Portuguese coast, the recovery was much more evident and faster after 2003, confirming the effectiveness of Regulation (EC) No.782/2003. The explanation for the TBT pollution slowdown tendency recorded between 2000 and 2003 could be related to: (i) some companies may have started to phase out the commercialization of OT-AF paints due to the upcoming European ban; and/or (ii) some ship-owners may have decided not to reapply TBT coatings, predicting its global ban on short-term.

Despite these encouraging results, OTs analyses in *N. lapillus* females' tissues and in water indicate that TBT inputs were still occurring in 2006. This situation was expected since vessels from outside Europe not operating under the authority of an EU Member State could still apply TBT coatings and, in addition, the EU vessels could still circulate with OT-based AF paints until September 2008.

Some authors have also been mentioning that other sources of TBT than AF paints will probably keep OTs inputs after the AFS Convention entry into force by (i) some illegal use; (ii) the release from dockyard facilities as a result of old coatings removal (Kotrikla, 2009); (iii) other sources such as disinfecting agents (Sousa et al., 2009); and (iv) the remobilization from sediments to the water column (Langston and Pope, 1995; Ruiz et al., 2008).

The affinity of TBT for aquatic sediments and the long term fate of this major persistent reservoir are widely recognised and remain current concerns (Ruiz et al., 2008). These are legitimate worries mainly in estuaries where TBT inputs occurred more intensively due to the major sources presence. That situation seems to have been confirmed in Ria de Aveiro using *H. ulvae* as a bioindicator of TBT levels in sediments (Chapter 5) since no *imposex* decrease was registered between 1998 and 2007 in this estuarine system, despite the implementation of the EU Regulation No.782/2003 (Chapter 5). Furthermore, increases in the phenomenon levels were recorded, both in the percentage of affected females and the expression intensity, contrasting with what was described for the same period and area using other bioindicators namely *Nassarius reticulatus* (Sousa et al., 2007) and *N. lapillus* (Chapter 4). In fact, using *N. lapillus* as a bioindicator, a significant decrease of TBT pollution in Ria de Aveiro was detected, improvement that was evident after 2003 and most notable from 2005 to 2007 (Chapter 4). This amelioration was attributed to the implementation of the EU Council Regulation No.782/2003 revealing the efficacy of this measure in reducing water TBT levels.

Therefore, the results obtained in Chapter 4 and 5 of this thesis highlight the importance of choosing the bioindicator regarding the ecosystem compartment being monitored, otherwise contradictory conclusions can be drawn. The concept of bioaccumulation of any noxious substance suggests, by itself, the process dependency on the organism exposure and/or uptake type and rate. Hence, it is not difficult to accept that TBT uptake / accumulation occurs by different ways – directly from water or sediment and by food, or after mobilization from contaminated sediments (Fent, 1996; Meador, 2000) – and that those distinct routes may have a different relative weight depending on the indicator species biology / ecology (Chapter 4 and 5). Therefore, as a sediment-dweller which feeding habit includes sediment ingestion, *H. ulvae imposex* expression is mainly determined by TBT sediment content and so the species should be used as the indicator of TBT pollution persistence in this compartment.

Apart from the expected TBT persistence in sediments around hotspots, the Regulation (EC) No.782/2003 proved to be effective in reducing TBT concentrations, at least in the water compartment (Chapter 4, 6 and 7). Since this European ban can be seen as an anticipation of the IMO global ban entered into force in September 2008, a worldwide-scale decrease of TBT pollution levels in water is predicted.

Even knowing that the banishment of OT-AF paints is global, it will possibly have a massive expression in developing countries where no legal restrictions were implemented before and where high OTs concentrations have been described not long ago (for a review see Bray and Langston, 2006).

Under this scenario, it is also expected that *N. lapillus* starts to recolonize estuarine inner areas near hotspots where it became apparently extinct due to the high levels of TBT pollution in the past. New recruits may possibly come by crawling through continuous habitats from the open shore into far in distance estuarine areas. The weakness of this hypothesis sounds to be the adults reduced mobility. Having in mind that early life stages are essential sources underlying any new populations' establishment and that *N. lapillus* life cycle has no pelagic larval phase, we also consider the hypothesis of recolonisation by juveniles dispersed through water floating. The rafting behaviour of these early life stages at water surface was observed under laboratory conditions in the current study and, if confirmed with data obtained from field studies, could be a specific asset regarding colonization of inner estuarine parts. One possible problem in both recolonisation strategies could be the different ecotypes between “open shore” and “sheltered shore” populations. In fact, shell shape ratios analyses showed differences between sites along the Portuguese coast, regarding wave exposure, but there is apparently no genotypic variation among *N. lapillus* populations in the region regarding the karyotype, as all studied populations presented 2n=26 chromosomes (typical from exposed shores), (Chapter 8). Since a phenotypic variation was verified an adaptation of the new recruits from the exposed to the sheltered conditions is required (namely regarding hydrodynamics pressure). Nevertheless, reports on similar cases are available in the literature showing that this adaptation, beyond possible, is extremely fast and can even happens as soon as the next generation (Gibbs, 1993; Crothers, 1998, 2003).

It is therefore not excluded the hypothesis that populations may upsurge in the near future in harbours areas through recolonization by specimens from more exposed shores (Chapter 8) and that TBT pollution decrease may also be accompanied by the populations recovery.

Thus the “TBT Tale” is also a proof that Mankind is able to recognize their own mistakes and correct them since it is imperative to protect the environment towards Life maintenance on this planet.

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