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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência, Tecnologia e Gestão do Mar, realizada sob a orientação científica do Professor Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação da Professora Doutora Ana Gago-Martínez, Professora do Departamento de Química Analítica e Alimentaria, da Universidade de Vigo, Espanha e do Doutor Pedro Reis Costa, Investigador Auxiliar do Instituto Português do Mar e Atmosfera.

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Dedico este trabalho aos meus pais Henriqueta e Rodrigo, e a minha irmã Magda.

o júri

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palavras-chave

Blooms de algas tóxicas (HABs); Biotoxinas marinhas; Alterações climáticas; Segurança alimentar; Ácido ocadaico (OA); Dinofisistoxinas (DTX); β-N-methylamino-l-alanine (BMAA); Saxitoxinas (STX); Toxicidade; *Prorocentrum lima; Gymnodinium catenatum; Ruditapes decussatus*; *Ruditapes philippinarum;* Amêijoas Nativas e Invasoras; *Mytilus galloprovincialis;* Mexilhões; Stress oxidativo; Genotoxicidade; Ensaio de reparação por excisão de bases (REB); Ensaio cometa.

Resumo

Os bivalves desempenham um papel fundamental nos ecossistemas marinhos costeiros, limitando a produtividade primária através de um forte mecanismo de controlo descendente (top-down) sobre as comunidades fitoplanctónicas e constituindo uma importante fonte de alimento para níveis tróficos superiores, incluindo o Homem. No entanto, os bivalves são afetados negativamente por diversos fatores externos, sejam estes bióticos ou abióticos, naturais ou antropogénicos. A exposição a estes fatores, juntamente com a exploração intensiva, leva a que seja necessária a implementação de uma gestão adequada, quer das populações selvagens, quer da produção em viveiros. Para melhorar esta gestão e alcançar o difícil equilíbro entre os interesses ecológicos e socioeconómicos é então necessário entender como os bivalves lidam com estes fatores. Na presente tese foram estudados os efeitos provocados por (i) *blooms* de algas tóxicas (HABs do inglês *harmful algal blooms*) e (ii) alterações das condições climáticas da água do mar, nomeadamente o aumento da temperatura (W) e acidificação (A). Os HABs são fenómenos que estão a aumentar em frequência, intensidade, persistência e abrangência geográfica, mas cujos impactos ecológicos e efeitos fisiológicos em bivalves são ainda ambíguos. Concomitantemente com a pressão causada pelo aumento da procura de bivalves como alimento para as populações humanas, fortes indícios apontam para uma relação entre a ocorrência de HABs e os parâmetros de alterações climáticas, o que pode afectar quer a indústria, quer as populações de bivalves selvagens. Assim, os objetivos deste trabalho são: 1) Determinar se as espécies nativas e invasoras lidam com a exposição a HABs de maneira diferente, dando particular atenção às respostas específicas de cada espécie e às fragilidades do ecossistema em relação à presença de espécies invasoras e ocorrência dos referidos *blooms*; 2) Avaliar como espécies de bivalves de elevado valor comercial lidam com a exposição simultânea a alterações climáticas e HABs, focando o efeito destas modificações ambientais na toxicocinética, respostas fisiológicas e genotoxicidade exibida pelos bivalves; 3) Avaliar a bioacessibilidade das biotoxinas marinhas, focando a obtenção de informação relevante para estudos de avaliação de risco.

Foram realizados trabalhos complementares de campo e laboratório, de modo a avaliar os padrões de toxicocinética de biotoxinas marinhas em espécies nativas e invasoras. Em laboratório, foram expostos bivalves a algas tóxicas em condições alteradas, simulando a ocorrência de HABs sob cenários previstos de alterações climáticas, de modo a avaliar toxicocinética, respostas fisiológicas e genotoxicidade. Exemplares naturalmente contaminados foram utilizados para investigar o papel dos bivalves como vectores de biotoxinas marinhas para humanos, através da simulação da digestão *in vitro*.

O estudo da acumulação de biotoxinas marinhas regulamentadas e não regulamentadas em espécies de amêijoa nativa (*Ruditapes decussatus*) e invasora (*R. philippinarum*) na Ria de Aveiro revelou que a espécie nativa acumula, recorrentemente, concentrações de toxinas mais elevadas, particularmente no que toca a toxinas grupo do ácido ocadaico (OA do inglês Okadaic acid), sendo estas as toxinas mais abundantes e frequentes na costa Portuguesa. A acumulação de menores concentrações por parte da amêijoa invasora pode promover o interesse dos produtores na sua exploração. A avaliação da cinética e da genotoxicidade em amêijoas nativas e invasoras reforçou a existência de comportamentos específicos para cada espécie. A exposição a *Prorocentrum lima,* dinoflagelado produtor de OA e dinofisistoxina-1, aumenta a pressão sobre a espécie nativa, uma vez que esta apresenta as concentrações de toxina mais elevadas, os maiores níveis de dano genético e ainda uma indução mais precoce e intensa dos mecanismos de reparação de dano no ADN. A espécie invasora, por outro lado, está melhor adaptada para lidar com estes desafios.

O estudo dos efeitos de fatores múltiplos combinados revelou que a exposição a W, A e HABs altera a dinâmica de acumulação/eliminação de toxinas paralisantes (PSP) em mexilhões (*Mytilus galloprovincialis*). Os cenários previstos de alterações climáticas e a exposição a HABs sugerem níveis de contaminação mais baixos, mas períodos de interdição de apanha mais prolongados. A exposição simultânea a condições ambientais alteradas e HABs também teve um impacto significativo no sistema antioxidante e na integridade do ADN, resultando numa modulação da resposta antioxidante especifica para cada órgão, aumentando o dano genético e prevenindo/atrasando a sua reparação. O dano encontrado no ADN parece, no entanto, não ser oxidativo. Embora a investigação de fatores que favoreçam a rápida eliminação de toxinas não constasse dos objetivos deste estudo, foi demonstrado que a acidificação pode promover a eliminação de toxinas PSP em mexilhões.

Por último, os estudos de bioacessibilidade revelaram uma redução na quantidade de toxinas do grupo-OA disponível após a digestão *in vitro*, sugerindo uma sobrestimação da exposição a estas biotoxinas e recomendando uma abordagem conservadora na definição dos níveis de segurança e na subsequente avaliação do risco. Este foi o primeiro estudo a determinar a bioacessibilidade das toxinas do grupo-OA e sugere a técnica de digestão *in vitro* como uma ferramenta promissora na obtenção de dados rigorosos no que diz respeito à ingestão de toxinas através de bivalves e a acessibilidade destes compostos no organismo humano. De um modo geral, a complexidade da relação entre HABs, bivalves e os fatores ambientais a serem considerados na gestão de biotoxinas marinhas está a aumentar, e apenas uma contínua e extensiva monitorização das condições ambientais, biológicas e antropogénicas pode permitir um melhor equilíbrio entre os interesses ambientais e socio-económicos.

Keywords

Harmful algal blooms (HABs); Marine biotoxins; Climate change; Seafood safety; Okadaic acid (OA); Dinophysistoxins (DTX); β-N-methylamino-l-alanine (BMAA); Saxitoxins (STX); Toxicity; *Prorocentrum lima; Gymnodinium catenatum; Ruditapes decussatus*; *Ruditapes philippinarum;* Native and Invasive clams; *Mytilus galloprovincialis;* Mussels; Oxidative stress responses; Genotoxicity; BER assay; Comet assay

Abstract

Bivalves play a marked role in coastal marine ecosystems, impacting primary productivity with a strong top-down control on phytoplankton communities. In addition, they are an important food source for higher trophic levels, including humans. However, bivalves can be impaired by several external factors, either biotic or abiotic, natural or anthropogenic. Exposure to these stressors, coupled with intensive farming, raised the need for adequate management of the wild populations and farmed production. To improve the difficult balance between ecological and socio-economic interests, it must be investigated how bivalves cope with external stressors. For this reason, the effects caused by the following stressors were studied in the present thesis: (i) harmful algal blooms (HABs) and (ii) climate change drivers in the seawater, such as warming (W) and acidification (A). HABs are increasing in frequency, intensity, persistence and geographic distribution, but are ambiguous regarding their ecological impacts and physiological effects on bivalves. A strong relationship is emerging between HABs and climate change drivers, what may affect both shellfisheries and wild populations. Hence, the objectives of this work are: 1) To determine whether native and invasive species cope with exposure to HABs differently, providing new insights on species-specificities and ecosystem functioning fragilities in the presence of invasive species and HABs; 2) To evaluate how commercially valuable bivalve species cope with simultaneous exposure to several climate change drives and HABs, providing new insights on how environmental changes affects toxicokinetics, physiological and genotoxic bivalves responses, under HABs; 3) To assess, marine biotoxins bioaccessibility, contributing for new information relevant for risk assessment. Different approaches were used to achieve these goals. Complementary field and laboratory works were performed to assess toxicokinetics patterns of marine biotoxins in native *vs.* invasive bivalve species. Bivalves were exposed to toxic blooming algae species under climate change scenarios in laboratory-controlled conditions to assess toxicokinetics as well as physiological and genotoxic responses. Naturally contaminated bivalves were used to investigate their role as vectors of marine biotoxins to humans through *in vitro* digestion methodology.

Relevant new data were obtained regarding the effects of several marine biotoxins in bivalves. Assessing accumulation of regulated and non-regulated biotoxins in native (*Ruditapes decussatus*) and invasive (*R. philippinarum*) clam species from Aveiro Lagoon revealed that higher toxin content, particularly regarding OA-group, the most abundant and frequent toxins in the Portuguese coast, is reached by native species. Accumulation of lower toxin levels by invasive clam may then favour farmers interest for their production. The kinetics and genotoxicity study in native and invasive clams reinforced the existence of species-specific behaviours. Exposure to the dinoflagellate *Prorocentrum lima*,

an OA and dinophysistoxin-1 producer, increases the pressure over native clams, with higher toxin accumulation and genetic damage, as well as early and increased induction of DNA repair activity. Invasive clams, on the other hand, are better adapted to cope with these challenges.

Investigation of the effects of combined exposure to multiple stressors revealed that W, A and HABs alter the accumulation/elimination dynamics of Paralytic Shellfish Poisoning (PSP) toxins in mussels (*Mytilus galloprovincialis*). Lower accumulation levels and slower elimination rates were observed. The predicted climate change scenarios and exposure to HABs may then lead to lower contamination levels but to longer harvesting closures. Simultaneous exposure to altered environmental conditions and HABs also had significant impacts in the antioxidant system and DNA integrity, resulting in an organ-specific modulation of the antioxidant response, increasing genetic damage and preventing/retarding DNA damage repair. However, the DNA damage observed seems to be non-oxidative. While the investigation of factors enhancing the elimination of toxins was not a main objective of this study, it was shown that acidification might promote PSP toxins elimination in mussels.

Finally, the bioaccessibility studies revealed a significant reduction in the OA-group content available after *in vitro* digestion, suggesting an overestimation of exposure to these biotoxins and an over-conservative approach in safety levels definition and risk assessment studies. This was the first study assessing bioaccessibility of OA-group toxins, pointing to *in vitro* digestion as a promising tool to obtain accurate data regarding toxin ingestion from bivalves to the consumer. Overall, the complexity of the relation between HABs, bivalves and the environmental factors to consider in marine biotoxins management is increasing, and only continuous and extensive monitoring of environmental, biological and anthropogenic conditions may allow for a healthier balance between environmental and socio-economic interests.

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List of abbreviations

5´-epi-DA - Domoic Acid Epimer
ALS/PDC - Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex
ASA - Amylase Specific Activity
ASP - Amnesic Shellfish Poisoning
Asp - Aspartic Acid
ATP - Adenosine Triphosphate.
AZA - Azaspiracid
BECs - Bioactive Extracellular Compounds
BMAA - β-N-methylamino-l-alanine
C1 - N-sulfocarbamoyl-gonyautoxin-2
C2 - N-sulfocarbamoyl-gonyautoxin-3
C3 - N-sulfocarbamoyl-gonyautoxin-1
C4 - N-sulfocarbamoyl-gonyautoxin-4
CAT - Catalase
CYP 450 – Cytochrome P450
DA - Domoic Acid
dcGTX1 – Decarbamoylgonyautoxin-1
dcGTX4 – Decarbamoylgonyautoxin-4
dcGTX2 – Decarbamoylgonyautoxin-2
dcGTX3 – Decarbamoylgonyautoxin-3
dcNeo - Decarbamoylneosaxitoxin
dcSTX - Decarbamoylsaxitoxin
DNA - Deoxyribonucleic Acid
DSP - Diarrheic Shellfish Poisoning
DTX1 - Dinophysistoxin 1
DTX2 - Dinophysistoxin 2
DTX3 - Dinophysistoxins 3
DW - Dry Weight
EC - European Council
EEC - European Economic Community
EFSA - European Food Safety Authority
FAO - Food and Agriculture Organization of the United Nations
Fer - Ferritin
GCs - Hydroxybenzoate Saxitoxin Toxins
Glu - Glutamate
GPx - Glutathione Peroxidase
GR - Glutathione Reductase
GSH - Reduced Glutathione
GST - Glutathione-S-Transferase
GTX1 – Gonyautoxin-1
GTX4 – Gonyautoxin-4
GTX2 – Gonyautoxin-2
GTX3 – Gonyautoxin-3
GTX5 – Gonyautoxin-5
GTX6 – Gonyautoxin-6
HABs - Harmful Algal Blooms
HSP 90 - Heat Shock Protein 90
INE - Instituto Nacional de Estatística /Statistics Portugal
KAI - Kainic Acid
LC-MS Grade - Liquid Chromatography-Mass Spectrometry Grade
LC-MS/MS - Liquid Chromatography-Mass Spectrometry
LOD - Detection Limit
LPO - Lipid Peroxidation
MBA - Mouse Bioassay
MDA - Malondialdehyde
MU - Mouse Units
NeoSTX - Neosaxitoxin
NO - Nitric Oxide
OA - Okadaic Acid
PO - Phenoloxidase
POPs - Persistent Organic Pollutants
PSP - Paralytic Shellfish Poisoning
PTXs - Pectenotoxins
ROS - Reactive Oxygen Species
RP-HPLC - Reversed-Phase High-Performance Liquid Chromatography
SOD - Superoxide Dismutase
STX - Saxitoxin
STXs - Saxitoxin Toxins Group
TEF - Toxicity Equivalent Factor
THC - Increased Total Hemocyte Count
TTX - Tetrodotoxin
UPLC-MS/MS - Ultra Performance Liquid Chromatography-Tandem Mass Spectrometer
YTXs – Yessotoxins

Chapter 1

General Introduction

General Introduction

1. Bivalve aquaculture

1.1. Development and economic importance

The harvesting and consumption of bivalves evolved side by side with the human species. Pointed as an evolutionary driver, the inclusion of bivalves in human diet is one of the reasons leading to the establishment of sedentary communities, and also had a critical role in the human brain evolution (Smaal et al., 2019).

Despite being believed that Chinese and Egyptian cultures were the first to introduce bivalves into an "aquaculture" environment, in Europe records of this activity goes back to the XIII century (Grant and Strand, 2018; IPIMAR, 2008). Nowadays, shellfish are one of the most demanded seafood protein sources, not only for being the embodiment of the "fresh seafood", as most times these animals are sold alive and even in some cases eaten alive, but also due to their health benefits and food value. Not only the food resource point of view has contributed for bivalve production increase, several other direct and indirect factors, from pearls and mineral extraction, to ecosystem repair and mitigation actions, contributed for the bivalve aquaculture development. The easy manipulation and the low position of shellfish in the food chain, allowing its production without the need to add external food source, also enhanced the development of bivalve aquaculture (Grant and Strand, 2018).

The demand for bivalves and aquaculture products reached a peak during the XX century (Fig. 1.1), when the annual growth rate reached 10 %. Despite the slight reduction in the growth rates in the first two decades of the XXI century, the worldwide bivalve production is still growing and in 2016 reached 1.1 million tonnes of molluscs, which corresponds roughly to 29.2×10^9 US dollars (equivalent approximately to 24.7×10^9 Euros; FAO, 2018). These values do not correspond, however, to the full impact of bivalve aquaculture in the local economies. Several secondary products and services deriving from bivalve production also contribute to the increase of income associated with their production, namely shucking and packaging houses, transport, manufacture of prepared products, retail sales. Moreover, contributions to public revenues through

licensing and lease fees should be considered (Schug and Wellman, 2009; Wijsman et al., 2018).

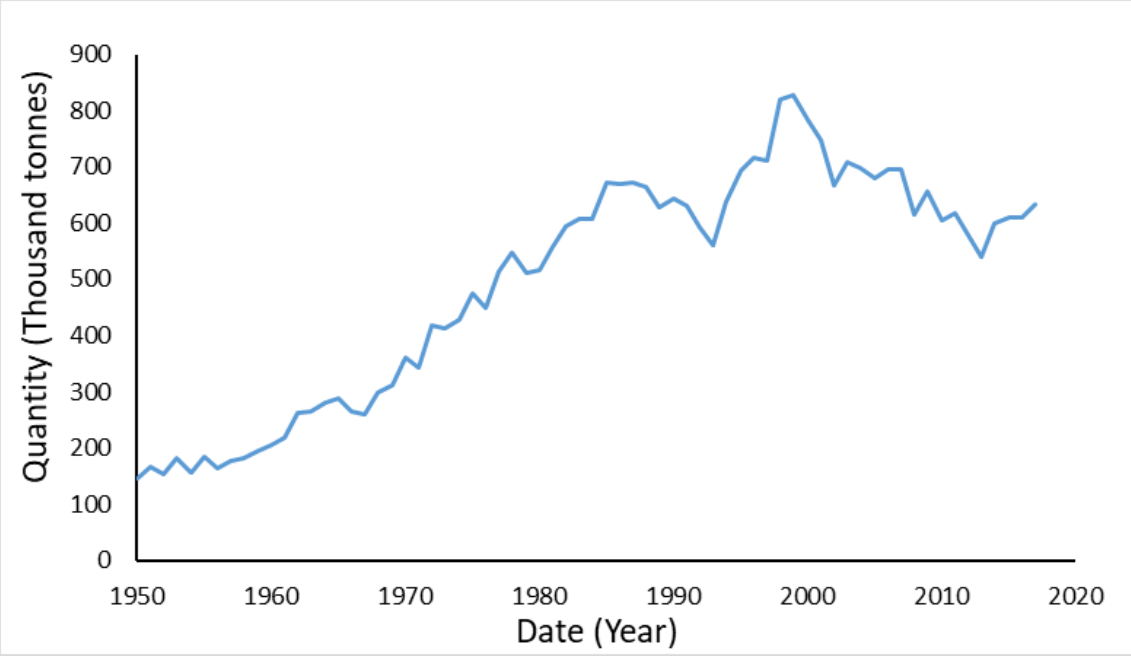


Fig. 1.1. Bivalve aquaculture production in Europe from 1950 to 2017 (data adapted from website FAO, 2019).

In Portugal, the production of bivalves has been increasing in the last decades (Fig. 1.2). In 2017, shellfish production corresponded to near 88 % of the aquaculture licensed establishments in Portugal. The statistical reference data point that, between 2016 and 2017, the production of bivalves increased 12 %, with clams culture reaching 3887 tones, mussels 1722 tones and oysters 1185 tones, an increase of 4.6 %, 16.8 % and 17.0 %, respectively (INE, 2018). Data from the official Statistics Portugal (INE, 2018) reveal that clams represent the primary bivalve group produced and harvested. Overall, these numbers reflect the growing importance of this industry for the European countries and Portugal in particular.

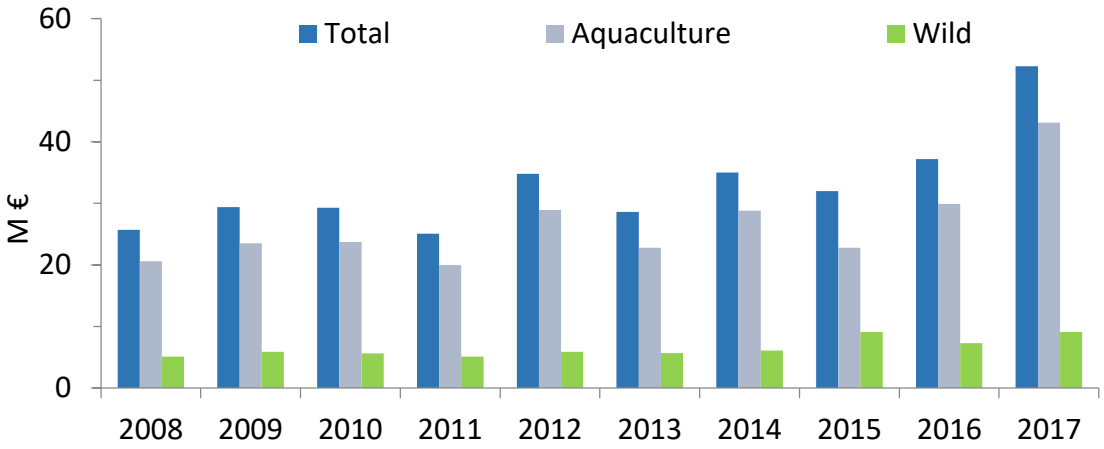


Fig. 1.2. Value of bivalve production, total, aquaculture and wild in Portugal between 2008 to 2017 (data adapted from website INE, 2018).

1.2. Present challenges

Bivalve production is considered a sustainable industry as is consisted by extensive aquaculture systems, where the animals produced are feeding on naturally available food sources and no compounds problematic to the environment are added, *e.g*. antibiotics (Wijsman et al., 2018). Nevertheless, several issues affect bivalve production and management. Shellfish industry also faces with the introduction of non-indigenous and invasive species. This issue presents a significant impact on the bivalve production, as these species can rapidly spread, dislocate or eliminate the native species yields (Chiesa et al., 2011; Karatayev et al., 2009; Mcneely and Schutyser, 2003; Sousa et al., 2009). Changes in the biotic and abiotic characteristics of the production sites, including dealing with anthropogenic contaminants (*e.g*. metals) and natural contaminants (*e.g*. marine biotoxins), are also regularly highlighted (Blanchet et al., 2019; Filgueira et al., 2016) as they may correspond to a decrease of revenue.

2. Introduction of non-indigenous and invasive species

Species introduction has been one of the critical ecological issues when it comes to biodiversity loss and ecosystems conservation (Karatayev et al., 2009; Mcneely and Schutyser, 2003; Sousa et al., 2009). The introduction of species into a new environment has and is still occurring. Most cases of species introduction are associated with human activities: shipping (fouling and ballast water), removal of biogeographical barriers (opening of new navigation channels), and import of new species for exploitation either accidental or intentional. While not unexpected, the aquatic ecosystems are particularly vulnerable to the introduction of non-indigenous species (Sousa et al., 2011; Wonham and Carlton, 2005). The new species can rapidly spread and become invasive severally affecting the local communities and even the local environment abiotic characteristics (Sousa et al., 2009).

Reports of invasive/introduced species exist in all groups of marine species, from micro and macro-algae to vertebrates; however, the invertebrates are the group presenting the highest number of non-indigenous species around the world. (Chainho et al., 2015; Chiesa et al., 2011; Karatayev et al., 2009; Mcneely and Schutyser, 2003; Sousa et al., 2011, 2009, 2008). Several non-indigenous shellfish species have been identified along the Portuguese coast, representing today 11 % of all the non-indigenous species registered for the Portuguese territory (Chainho et al., 2015).

Manila clam (*Ruditapes philippinarum*) is one of the most well-studied cases of shellfish introduction (Bidegain et al., 2015; Bidegain and Juanes, 2013; Blanchet et al., 2004, 2019; Chiesa et al., 2011; Dang et al., 2010; Delgado and Pérez-Camacho, 2007; Figueira and Freitas, 2013; Flassch and Leborgne, 1992; Freitas et al., 2012; Gosling, 2004; Goulletquer, 1997; Juanes et al., 2012; Laing and Child, 1996; Lopes et al., 2018; Moura et al., 2017; Velez et al., 2015a, 2015b, 2015c). This non-indigenous species was introduced to replenish the native Carpet Shell clam (*R. decussatus*) yields, whose natural populations collapsed due to intensive harvesting and mass mortalities events (Chiesa et al., 2011; Flassch and Leborgne, 1992; Gosling, 2004). The Indo-Pacific species was first introduced accidentally in North America around 1936. It was imported along with oyster seeds, and presented a high adaptation capacity. Its high reproductive and growth rates led to a deliberate introduction in Europe to support the local Carpet Shell

clam fisheries (Chiesa et al., 2011; Flassch and Leborgne, 1992; Gosling, 2004; Goulletquer, 1997).

The non-indigenous and native clams are congener species with very similar morphological traits (Fig. 1.3). This similarity allowed for indiscriminate harvesting and trade of the two clams. Possibly due to this reason, it was only in 2009 that the two clams appear as different species in Portuguese records (Figueira and Freitas, 2013). Also, their environmental requirements are within the same ranges, allowing for the two clam species to coexist in several locations along the European coast (Freitas et al., 2012; Karatayev et al., 2009; Lopes et al., 2018; Moreira et al., 2012; Ricciardi and Rasmussen, 1998; Velez et al., 2015c, 2015b).

Fig. 1.3. Picture of native (*Ruditapes decussatus*; on the left) and invasive clam (*R. philippinarum*; on the right), highlighting the key differences between species, such as siphon shape (separated in R. decussatus, merged in R. philippinarum) and external shell characteristics (deeper striations in the invasive clam).

The classification of *R. philippinarum* as non-indigenous or invasive is still not completely clear. While this clam is considered as non-indigenous species in Spain (Bidegain et al., 2015; Bidegain and Juanes, 2013), several field observations point out to an invasive behaviour of this species in Portugal, France and Italy (Blanchet et al., 2004; Chainho et al., 2015; Dang et al., 2010; Figueira and Freitas, 2013; Moura et al., 2017; Pranovi et al., 2006; Velez et al., 2015a, 2015c).

The invasive status attributed to *R. philippinarum* emerges not only due to the evident displacement occurring in the production and natural yields where it is conquering *R. decussatus* distribution areas, replacing almost completely the native

clam (Blanchet et al., 2004, 2019; Dang et al., 2010; Figueira and Freitas, 2013; Moura et al., 2017; Velez et al., 2015a). Also, *R. philippinarum* higher growth and reproduction rates (Chessa et al., 2005; Solidoro et al., 2000; Usero et al., 1997) and increased tolerance to environmental changes, xenobiotics and diseases point to the invasive classification.

3. Harmful algal blooms

One of the most important factors affecting bivalve production and harvesting all over the world is the occurrence of harmful algal blooms (HABs). From over 5.000 species of microalgae known, only 300 are able, under specific environmental conditions, to rapidly multiply, creating algae "clouds" with high cell densities, called algal blooms (Gerssen et al., 2010). Commonly known as "red tides" these algal bloom events naturally occur under propitious conditions, and while some algal blooms are benign, creating a beautiful spectacle of colour in the waters and boosting the ecosystem by increasing the resources available at the bottom of the trophic webs (Gerssen et al., 2010; Hallegraeff, 1993; M. Silva et al., 2015). Other may have severe negative ecological consequences, causing mass mortalities events through hypoxia/anoxia and altering food webs (Hallegraeff, 1993; Wells et al., 2015). These negative consequences may, however, be less visible as over 50, from these 300 algal species, are known to produce toxic compounds (Gerssen et al., 2010; Hallegraeff, 1993; Hinder et al., 2011; M. Silva et al., 2015). These blooms, which the proliferation may vary from the thousands to the millions of algal cells per litre of seawater, are frequently designated as Harmful Algal Blooms, HABs (Gerssen et al., 2010; M. Silva et al., 2015; T. Silva et al., 2015).

The toxic compounds produced by the phytoplankton are known as marine biotoxins. Despite their functional role not being completely established yet, it is known that these compounds are secondary metabolites of the algae, with a possible role on direct competition, for space and nutrients, or avoiding algae predation (Gerssen et al., 2010; M. Silva et al., 2015).

The occurrence of HABs represents a severe problem for the bivalve industry, as the biotoxins may be accumulated in bivalves and transferred to higher levels of the food

chain (Fig. 1.4) affecting from crabs and fish, to sea birds, marine mammals, and even humans (Costa, 2014; Gerssen et al., 2010; Hallegraeff et al., 1995; Kvitek et al., 2008; Landsberg, 2002; McCabe et al., 2016; Soliño et al., 2019).

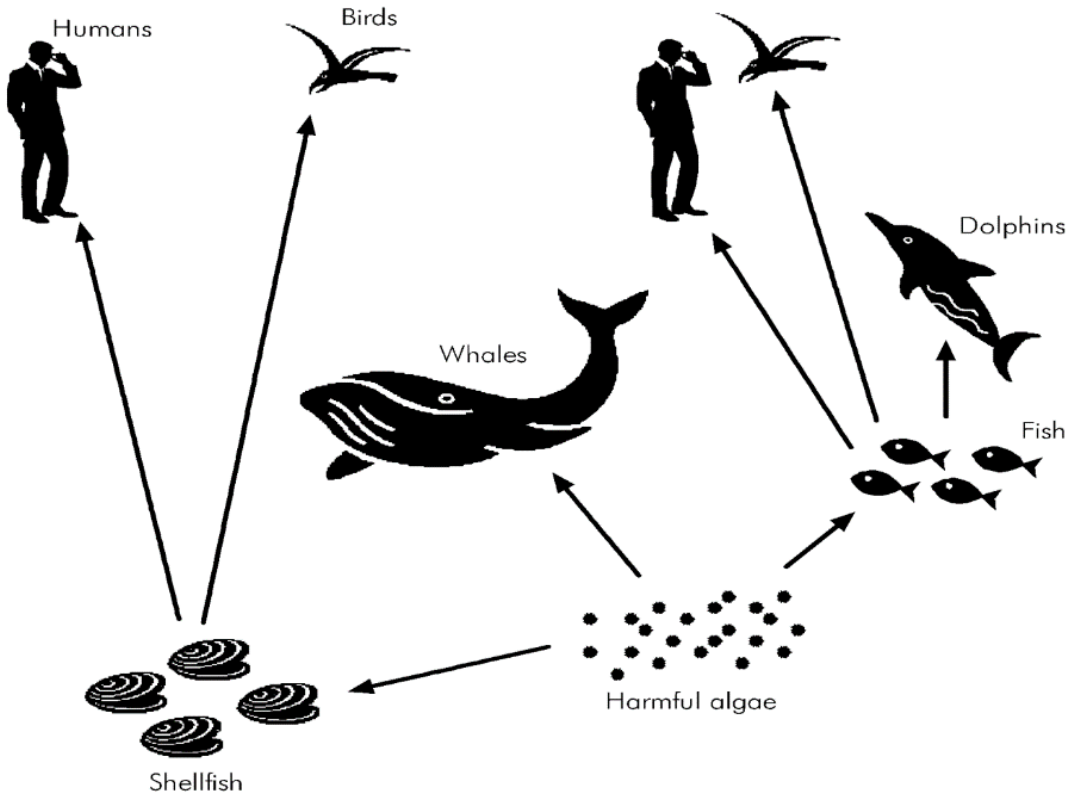


Fig. 1.4. Harmful algal blooms in the food chain and their routes of exposure, examples of toxins trophic transfer (adapted from Gerssen et al., 2010).

The human consumption of contaminated bivalve can originate several food poisoning syndromes. The most common are the diarrheic shellfish poisoning (DSP), the paralytic shellfish poisoning (PSP) and the amnesic shellfish poisoning (ASP) (Fig. 1.5). As indicated by the designation of the syndrome, biotoxins may cause different effects on humans, varying from gastrointestinal disorders to neurological problems. The toxins responsible for these syndromes can be divided according to their chemical properties in terms of water solubility. DSP toxins currently designated as lipophilic toxins in EU directives, a group that also includes yessotoxins (YTX) and azaspiracids (AZA). ASP and PSP toxins are hydrophilic toxins (EC, 2004a).

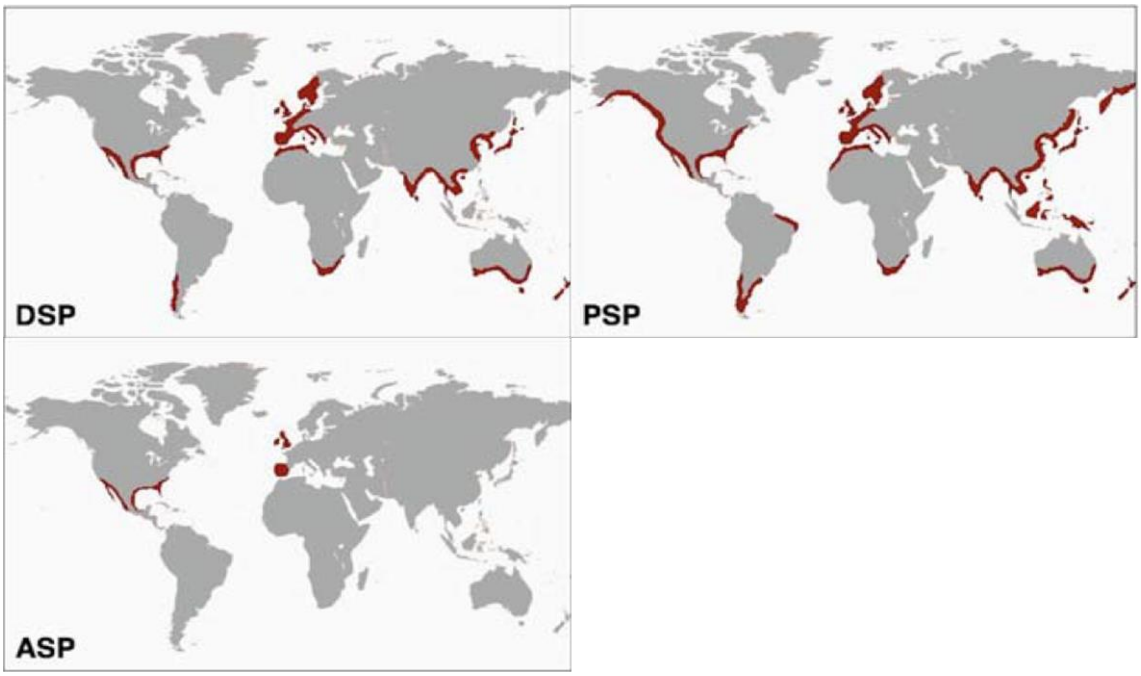


Fig. 1.5 Worldwide distribution of the most common shellfish poisoning syndromes, Diarrhetic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP) and Amnesic Shellfish Poisoning (ASP) (adapted from Manfrin et al., 2012).

3.1. Lipophilic toxins: producers, chemistry, effects on Humans

The lipophilic toxins were divided into several subgroups due to the similarities in the chemical structure: okadaic acid (OA) and dinophysistoxins (DTXs); the pectenotoxins (PTXs); the yessotoxins (YTXs) and the azaspiracids (AZAs) (Alarcan et al., 2018; EFSA, 2010).

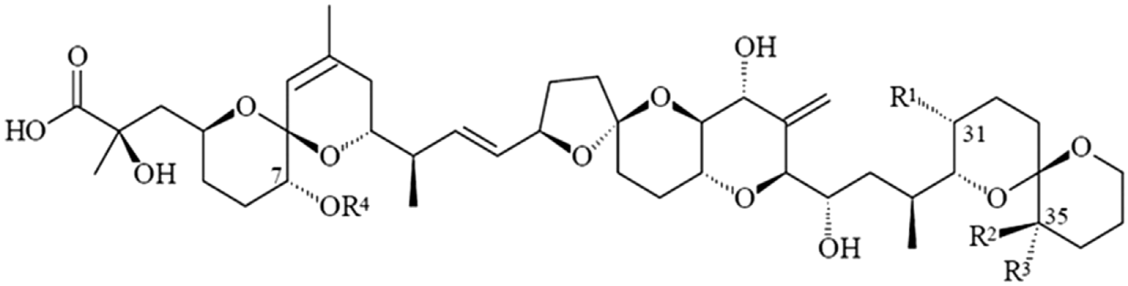
OA and DTXs are the most frequent toxins found in bivalves in Portugal (Vale et al., 2008). Initially known as diarrhetic shellfish poisoning toxins (DSP toxins), these compounds have been reported all over the world, with European and Japanese waters being particularly affected (Dominguez et al., 2010). OA is a polyether carboxylic acid and heat-stable compound that was first isolated from marine sponges of the genus *Halichondria* (Marr et al., 1992), but today is known to be produced by several microalgae species (Table 1.1).

Table 1.1. Marine lipophilic toxins, human syndromes and the phytoplankton species producer.

Toxin group	Toxins	Syndrome	Toxic Phytoplankton		
			Genus	**Species**	**References**
Okadaic Acid (OA) group	OA, DTX1, DTX2, and PTXs[1]	DSP	*Prorocentrum*	*P. lima*	Yasumoto et al. (1987)
				P. concavum	Dickey et al. (1990)
				P. hoffmannianum	An et al. (2010)
				P. rhathymum	An et al. (2010)
				P. belizeanum	Morton et al. (1998)
				P. faustiae	Morton et al. (1998)
				P. arenarium	Ten-Hage et al. (2000)
				P. maculosum	Zhou and Fritz (1993)
			Dinophysis	*D. miles*	Reguera et al. (2014)
				D. infundibula	Reguera et al. (2014)
				D. mitra	Lee et al. (1989)
				D. tripos	Lee et al. (1989)
				D. fortii	Murata et al. (1982)
				D. acuta	Lee et al. (1989)
				D. acuminata	Lee et al. (1989)
				D. norvegica	Lee et al. (1989)
				D. rotundata	Lee et al. (1989)
				D. ovum	Raho et al. (2008)
				D. caudate	Fernández et al. (2006)
				D. sacculus	Draisci et al. (1996)
Yessotoxins	*YTXs*	[2]	*Protoceratium*	*P. reticulatum*	Satake et al. (1999)
			Lingulodinium	*L. polyedrum*	Pistocchi et al. (2012)
			Gonyaulax	*G. spinifera*	Wang (2008)
Azaspiracids	*AZAs*	*AZP*[3]	*Azadinium*	*A. spinosum*	Tillmann et al. (2009)

Notes: [1] PTXs are not responsible for DSP but are co-produced by some of the phytoplankton responsible for OA-group toxins. [2] Yesssotoxins are listed as marine biotoxins due to their effects in mouse tests. Human poisonings were never reported. [3] AZP - Azaspiracid Poisoning.

The DSP toxin profile usually found in bivalves is composed by OA, DTX1 and DX2, which are present in the microalgae species, and by toxins fatty acid derivatives, collectively known as DTX3, that results from bivalve metabolism (Fig. 1.6). These derivatives result from the acylation of the hydroxyl group at C-7 with fatty acids. The carbon chain length can vary from 12 to 22 carbons with up to 6 unsaturated carbon-carbon bonds (Dominguez et al., 2010; Gerssen et al., 2011; Torgersen et al., 2008b, 2008a; Vale and Sampayo, 2002a).



	R1	R2	R3	R4
OA	CH_3	H	H	H
DTX1	CH_3	CH_3	H	H
DTX2	H	H	CH_3	H
DTX3	(H or CH_3)	(H or CH_3)	(H or CH_3)	Fatty acid

Fig. 1.6. Chemical structures of okadaic acid and dinophysistoxin 1, 2 and 3 (adapted from Larsen et al., 2007).

In humans and other mammals, the consumption of bivalves containing high levels of OA and DTXs can originate an array of gastrointestinal symptoms, including gastrointestinal disorders, diarrhoea, abdominal cramps, nausea and vomiting (EFSA, 2008). The mode of action of OA-group toxins consists in the inhibition of serine/threonine protein phosphatases, in particular for protein phosphatase 2A (PP2A). OA and DTX1 are potent inhibitors of these phosphatases, while DTX2 presented less inhibitory activity (Aune et al., 2007; Bialojan and Takai, 1988; Takai et al., 1992). OA and DTX1 are also considered potent tumour promoters (Fujiki et al., 1997; Suganuma et al., 1988; Yatsunami et al., 1993).

As for the 7-*O*-acyl fatty esters derivatives of OA-group toxin, toxicity and action mode are still unclear. Earlier studies indicated no significant toxicity, being considered weak inhibitors of serine/ threonine protein phosphatases, presenting reduced lethality in mice and moderate cytotoxicity (Takai et al., 1992; Yanagi et al., 1989; Yasumoto et al., 1985). However, after ingestion, they may be converted back into the toxic parental compounds by the human gastric reactions and cause human poisonings (Vale and Sampayo, 2002b).

3.2. Hydrophilic toxins: producers, chemistry and effects on Humans

Other dominant group of marine biotoxins in the Portuguese coast is that of hydrophilic toxins, which is divided into the toxins responsible for the paralytic and the amnesic shellfish poisoning.

3.2.1. Paralytic Shellfish Poisoning Toxins

The PSP toxins, also designated as saxitoxin group (STXs), are composed by a vast number of water-soluble neurotoxic alkaloids, derivate from the trialkyl tetrahydropurine (Llewellyn, 2006). These compounds are produced by several dinoflagellate species from the genera *Alexandrium*, *Pyrodinium* and *Gymnodinium* (Table 1.2). Saxitoxin (STX) and its derivatives are among the most frequent marine biotoxins in the North Atlantic (Bricelj and Shumway, 1998; Brown et al., 2010). In Portuguese waters, their presence is not as regular as the lipophilic toxins, occurring intermittently without a clear pattern (Vale et al., 2008). Nevertheless, their occurrence may drastically impact bivalve industry, and any inadvertent consumption of contaminated bivalves may cause severe human intoxications (Carvalho et al., 2019; Vale, 2019).

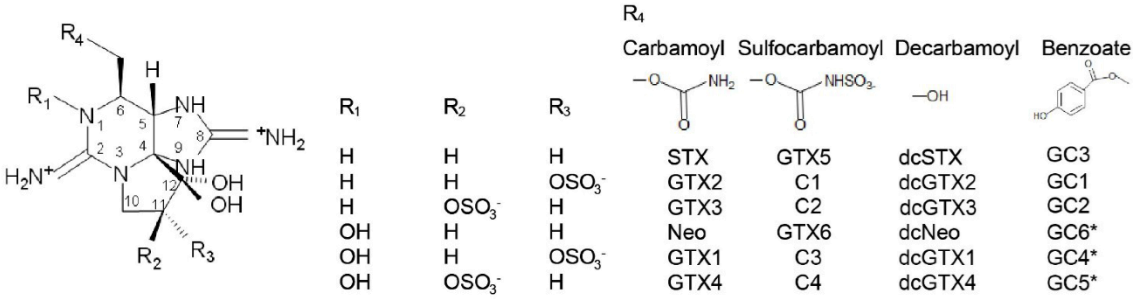
Table 1.2. PSP toxins and the responsible phytoplankton species.

PSP Toxins	Toxic Phytoplankton		References
	Genus	**Species**	
Carbamate: STX; NeoSTX; GTX1+4; GTX2+3 Decarbamoyl: dcSTX; dcNeo; dcGTX1+4; dcGTX2+3 N-sulfocarbamoyl: C1+2; C3+4; GTX5; GTX6	*Alexandrium*	*A. catenella* *A. fundyense* *A. lusitanicum* *A. minutum* *A. tamarense* *A. angustitabulatum* *A. tamiyavanichii*	Murray et al. (2015)
	Gymnodinium	*G. catenatum*	Hallegraeff et al. (2012)
	Pyrodinium	*P. bahamense*	Gedaria et al. (2007)

The paralytic shellfish poisoning (PSP) is characterised by symptoms that range from tingling, nausea, diarrhoea and vomiting to confused speech, muscular paralysis and, in extreme concentrations, respiratory paralysis and cardiovascular shock (Costa et al., 2018; Landsberg et al., 2006; Manfrin et al., 2012). These compounds are potent neurotoxins capable of disrupting the neurological impulse without destroying the cells. They bind to the voltage-gated sodium channels blocking the channel pore and preventing the formation of the Na^+ gradient. Also, the calcium and potassium channels are affected by the STXs with similar effects. The toxins bind themselves to the channels preventing the gradient required to the passage of the neurological impulse (Llewellyn, 2006; O'Neill et al., 2016).

The dinoflagellates listed in Table 1.2 produce a vast array of the STX analogues, and the toxin profile differs from species to species, and in some cases even within the algal strains (EFSA, 2009a; Hallegraeff et al., 2012). Differences on the toxins functional groups structure cause slight variations in the affinity to the binding sites of voltage-gated channels, what provoke different toxicities between compounds (EFSA, 2009a; Genenah and Shimizu, 1981; Llewellyn, 2006; O'Neill et al., 2016).

With more than 50 STX analogues and at least 18 with toxicological relevance (FAO/WHO, 2016), the STX-group toxins can be subdivided into three different subgroups: the carbamoyl toxins, the decarbamoyl toxins and sulfocarbamoyl toxins, taking into account their chemical characteristics (Fig. 1.7). The carbamoyl toxins subgroup is composed by saxitoxin (STX), neosaxitoxin (NeoSTX) and gonyautoxins (GTX1-4), which are considered the most harmful of the STX analogues presenting high toxicity after intra-peritoneal injection, and high affinity to the voltage-gated channels binding sites. The N-sulfocarbamoyl subgroup is considered to be the least toxic and includes the four C-toxins (C1–4), the gonyautoxin-5 (GTX5, aka. B1) and the gonyautoxin-6 (GTX-6, aka. B2). The third toxin subgroup, the decarbamoyl toxins subgroup, is constituted by the decarbamoyl-STX (dcSTX), the decarbamoyl-GTXs (dcGTX1–4) and the decarbamoyl-Neo (dcNeo) toxins. Recently, a new group of STX analogues was described, the hydroxybenzoate saxitoxin analogues (GCs toxins); however, these compounds seem to be produced only by *Gymnodinium catenatum* and little is known about their toxicity accumulation and metabolism in bivalves (Costa et al., 2018; Negri et al., 2003).



			R4			
			Carbamoyl	Sulfocarbamoyl	Decarbamoyl	Benzoate
R1	R2	R3				
H	H	H	STX	GTX5	dcSTX	GC3
H	H	OSO_3^-	GTX2	C1	dcGTX2	GC1
H	OSO_3^-	H	GTX3	C2	dcGTX3	GC2
OH	H	H	Neo	GTX6	dcNeo	GC6*
OH	H	OSO_3^-	GTX1	C3	dcGTX1	GC4*
OH	OSO_3^-	H	GTX4	C4	dcGTX4	GC5*

Fig. 1.7 Chemical structures of saxitoxin and their derivatives (adapted from Costa et al., 2015).

3.2.2. Amnesic shellfish poisoning toxins

Domoic acid (DA) is the main compound responsible for the human illness amnesic shellfish poisoning. Despite their worldwide distribution, and their significant impact in humans, marine mammal and bird's populations, DA is the least frequent toxin in Portuguese waters. Still, the occurrence of HABs containing DA-producing phytoplankton is felt in the Portuguese shellfisheries, provoking harvesting closures

every year. The main phytoplankton producers are diatoms of the genus *Pseudo-nitzschia* and *Nitzschia* (Table 1.3).

Table 1.3. Summary of domoic acid phytoplankton producers.

Toxin group	Syndrome	Genus	Species	References
Domoic Acid (DA)	ASP	*Amphora*	*A. coffeaeformis*	Bates and Trainer (2006) & Saeed et al. (2017)
		Nitzschia	*N. bizertensis*	
			N. navis-varingica	
		Pseudo-nitzschia	*P. australis*	
			P. brasiliana	
			P. caciantha	
			P. calliantha	
			P. cf lineola	
			P. cf. granii	
			P. cuspidate	
			P. delicatissima	
			P. fraudulenta	
			P. fukuyoi	
			P. galaxiae	
			P. heimii	
			P. inflatula	
			P. kodamae	
			P. multiseries	
			P. multistriata	
			P. plurisecta	
			P. pseudodelicatissima	
			P. pungens	
			P. roundii	
			P. seriata	
			P. subpacifica	
			P. turgidula	
			P. turgiduloides	

Domoic acid (DA) is a water-soluble cyclic amino with three carboxylic acid groups. From the three groups of toxins presented, DA possesses the lower toxic potency, and high DA concentrations are needed to cause illness in humans. Several analogues and geometrical isomers have been isolated from the producers (Fig. 1.8). However, the molecule is prone to spontaneous isomerisation, and the isomers contribution to toxicity is negligible (EFSA, 2009b; Saeed et al., 2017; Vale et al., 2008; Vale and Sampayo, 2001).

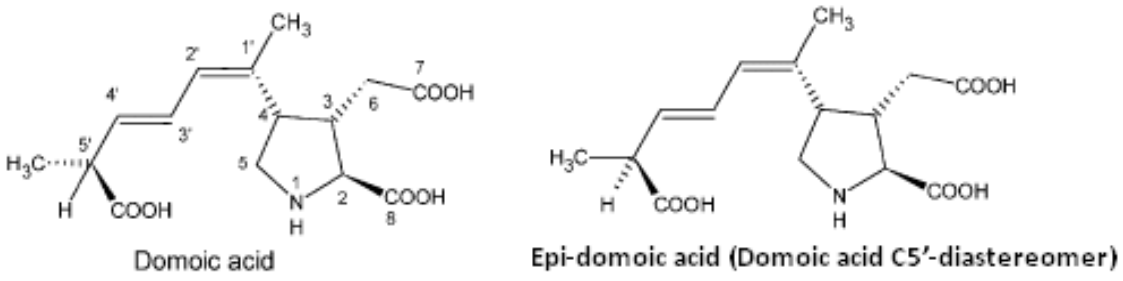


Fig. 1.8. Chemical structures of domoic acid and epi- domoic acid (adapted from EFSA, 2009b).

These compounds may have effects at gastrointestinal and cardiovascular levels and cause neurologic disorders characterised by behavioural changes and permanent short-term memory loss due to neuronal degeneration and hippocampal necrosis. DA effects may also include hallucinations, physical perplexity, coma and death in severe cases (Saeed et al., 2017).

DA action mode consists of membrane channels activity increase inducing excitotoxicity. Due to their similarity with glutamate (Glu), kainic acid (KAI) and aspartic acid (Asp), the more common excitatory aminoacids, DA presents high affinity to the ionotropic glutamate receptors. When DA interacts with the receptor, it prevents the desensitisation of the membrane channels, causing an excessive influx of Ca^{2+} into the neurons and an excessive release of Glu (Nijjar and Nijjar, 2000). The ion influx may then cause the disassociation of Ca^{2+}-dependent cascades resulting in membrane, cytoplasmic and nuclear events that induce neuron apoptosis (Nijjar and Nijjar, 2000). While neurological symptoms are the most characteristic symptoms of ASP illness, as disruptions affect mainly the hippocampus due to the high concentration of receptors, DA may also affect other organs as the kidneys, liver and lungs (Cendes et al., 1995; Nijjar

and Nijjar, 2000; Pulido, 2008; Ramsdell and Gulland, 2014; Saeed et al., 2017; Sheldon and Robinson, 2007).

3.3. Effects of biotoxins in bivalves

Although the mode of action of the three main groups of marine biotoxins in humans and other mammals is well-known and has been debated extensively in the last decades, the same cannot be said about the effects of the toxins in bivalves. Until recently, the general consensus was that bivalves were not affected at all by these compounds as exposure to toxic algae blooms very rarely causes high mortalities in bivalve populations. Only a hand full of studies had approached the effects of marine biotoxins in bivalves, and even them focusing mostly on behavioural and some very superficial physiological aspects (Landsberg, 2002; Svensson et al., 2003). Few studies have been performed assessing the impacts of marine biotoxins in the immune response in bivalves.

The immune system of bivalves, despite being characterised as an innate system, is very effective in the identification and elimination of potential pathogens. This complex defence system is comprised of a vast range of hemocytes and their sub-families as well as several humoral elements. These components interact with each other, with other systems in the bivalves and with exogenous information in order to prevent damage to the organism and maintain homeostasis. This complexity and interdependence, however, make the immune process in bivalves particularly vulnerable to environmental stressors, such as exposure to natural or anthropogenic xenobiotics (Harikrishnan et al., 2011; Hégaret and Wikfors, 2005a; Pipe and Coles, 1995).

While the predominant mechanism of defence against large exogenous bodies, as pathogens or parasites, involves phagocytosis, other responses to infection, including encapsulation and nacrezation, are also available to the bivalves. The immune response is typically initiated by activation of granulocytes and inflammatory factors, including the production of peroxides and other oxidative radicals (Harikrishnan et al., 2011; Hégaret and Wikfors, 2005a; Pipe and Coles, 1995). These reactive oxygen species (ROS), originated in several biological processes, are essential not only to the defence but also to the control of the normal functioning of cells (Choi et al., 2006; Fabioux et al., 2015; Guilherme et al., 2008; Livingstone et al., 1992; Qiu et al., 2013; Valavanidis et al., 2006).

Nonetheless, the presence of ROS in the cells and tissues may also correspond to a higher potential for damage. When the amount of ROS is not regulated and controlled, the cells are prone to suffer oxidative damage. This imbalance between the amounts of ROS created and neutralized is designated as oxidative stress. (Choi et al., 2006; Fabioux et al., 2015; Guilherme et al., 2008; Livingstone et al., 1992; Qiu et al., 2013; Valavanidis et al., 2006)

The peroxides, oxidative radicals and other free radicals composing ROS can react with a wide range of biomolecules in the cells causing oxidative damage. This damage may include protein degradation, enzymatic inhibition, lipid peroxidation (LPO), DNA damage and, in extreme conditions, cell death and tissue damage (Choi et al., 2006; Fabioux et al., 2015; Gerdol et al., 2014; Guilherme et al., 2008; Livingstone, 2001; Livingstone et al., 1992; Qiu et al., 2013).

To prevent oxidative stress in the cells and maintain the redox balance, an antioxidant system coevolved in the animals to neutralise and eliminate ROS. This system includes several antioxidant enzymes, as catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferases (GST), glutathione peroxidase (GPx) and glutathione reductase (GR), as well as non-enzymatic compounds as reduced glutathione (GSH) (Choi et al., 2006; Fabioux et al., 2015; Guilherme et al., 2008; Livingstone et al., 1992; Qiu et al., 2013; Valavanidis et al., 2006). The assessment of the antioxidant system activities and contents can be a very reliable way to understand the effects and impacts of exogenous stressors, particularly when coupled with the determination of LPO and DNA damage. Lipid peroxidation results from the degradation of the polyunsaturated fatty acids in cell membranes after reacting with ROS. From data collected in mammals this reaction causes membrane destabilisation and disintegration, producing toxic metabolites as malondialdehyde (MDA), that can induce protein synthesis inhibition, genotoxic effects, as DNA adducts, and also mutagenic and carcinogenic effects in the cells (Melegari et al., 2012).

As referred above, very little is known on how marine toxins affect bivalves, but even less is known when it comes to the impact of these compounds in the antioxidant system and their capacity to induce oxidative damage in bivalves. Data concerning the impact of OA-group toxins in bivalves are still scarce and very dispersed (Prego-Faraldo et al., 2013; Valdiglesias et al., 2013). Only recently, studies assessing the impact of lipophilic

biotoxins indicated that exposure to dinoflagellate producers of OA and DTXs causes specific effects in bivalves, such as induction of genetic damage and structural abnormalities in cells, impairing cell division and regeneration, compromising DNA integrity and reproduction, and possibly introducing disturbances at population level (Pinto-Silva et al., 2005, 2003) (Table 1.4).

A void of information remains, and further studies are needed in order to understand how HAB-toxins and species specificities affect not only the intensity but also the type of damage occurring in bivalves. Particularly, when physiological responses seem to be dependent of organism model and the approach selected, *i.e., in vitro* or *in vivo* (Flórez-Barrós et al., 2011; McCarthy et al., 2014; Prego-Faraldo et al., 2015, 2013; Talarmin et al., 2008).

Table 1.4. Effects of exposure to OA-group toxins in bivalves and their producers: summary of recent reports.

Toxin source	Species Targeted	OA-group Effects in Bivalves	Organ, Tissue or Cells	Reference
Prorocentrum lima	*Perna perna*	Increased frequency of micronucleus (MN) and Nucleotplasmatic bridges (NL) - genotoxic effect.	Haemocytes	Pinto-Silva et al. 2005
Toxin Standard	*Mytilus edulis*	Increase in DNA fragmentation in both cell types. No dose-response observed.	Haemocytes Hepatopancreas	McCarthy et al. 2014
Toxin Standard	*Crassostreas gigas*	Increase in DNA fragmentation in both cell types. No dose-response observed.	Haemocytes, Hepatopancreas	McCarthy et al. 2014
Toxin Standard	*Mytilus edulis*	Significant reduction of cell viability after 48 h – 50 % reduction after 3 days; Inhibit P-GP activity; Increase lysosomal compartments; Compromise lysosomal membrane integrity.	Haemocytes, Gills	Svensson et al. 2003
Prorocentrum lima	*Mytilus galloprovincialis*	Dow-regulation of SOD and CAT gene expression; Up-regulation of GST and selenium-dependent GPx gene expression. Increased antioxidant response and decreased LPO.	Hepatopancreas, Gills	Prego-Faraldo et al. 2017
Prorocentrum lima	*Mytilus galloprovincialis*	No genotoxicity lower concentrations (1,000 cells/L); Early increased oxidative DNA damage (100,000 cells/L); Concentration dependant genotoxicity; Apoptosis increased after 48h of exposure.	Haemocytes, Gills	Prego-Faraldo et al. 2016
Toxin Standard	*Perna perna*	Increased frequency of micronucleus – genotoxic effect.	Haemocytes	Pinto-Silva et al. 2003
Toxin Standard	*Ruditapes decussatus*	Increase in DNA fragmentation (2 h of exposure) and recovery (4 h of exposure).	Haemocytes, Gills	Flórez-Barrós et al. 2011

Toxin source	Species Targeted	OA-group Effects in Bivalves	Organ, Tissue or Cells	Reference
Prorocentrum lima	*Ruditapes decussatus*	Increase in DNA fragmentation in gills (1 and 4 days under 200 low concentration OA cells mL^{-1}); No significant response detected in hemocytes; Decrease in DNA fragmentation in gills (1 day under 200 and 20,000 high concentration OA cells mL^{-1}); No significant response detected in Hemocytes.	Haemocytes, Gills	Flórez-Barrós et al. 2011
Dinophysis acuminata	*Mytilus galloprovincialis*	Different responses between genders; Significant variation in CPR (increase in males), GST (increase in both genders), GR (increase in males and decrease females), GLO I (glyoxalases I) (increase in both genders), GPXtot (increase in both genders) and CAT (no significant response).	Hepatopancreas	Vidal et al. 2014
Prorocentrum lima	*Crassostreas gigas*	Concentration and time dependent modulation of genes expression involved in cell regulation (Cg-p21; Cg-CAFp55; and Cg-EF2) and immune system (Cg-LGBP).	Whole-body	Romero-Geraldo et al. 2014
Toxin Standard	*Mytilus galloprovincialis*	Hemocytes - Genotoxicity only occurred after 2 h when exposed to 10 n*M* OA; No changes in Apoptosis detected; Necrotic cells only increased after 2 h in 500 n*M* OA. Gills – Genotoxicity detected in all treatments > 10 n*M* OA after 1 h; (no data exist for apoptosis or necrotic cells).	Haemocytes, Gills	Prego-Faraldo et al. 2015
Natural Bloom (no specified species)	*Mytilus galloprovincialis*	Significant decrease in annexin V positive damaged and apoptotic hemocytes, at higher toxin concentrations.	Haemocytes	Prado-Alvarez et al. 2012

Toxin source	Species Targeted	OA-group Effects in Bivalves	Organ, Tissue or Cells	Reference
Toxin Standard	*Ruditapes decussatus*	Apoptosis increased after 4 h in the highest concentrations (100 and 500 nM); Cell death increased in the highest concentrations (100 and 500 nM) after 2 and 4 h; Haemocyte esterase activity decreased in all the concentrations studied after both 2 and 4 h; Phagocytosis decrease in all treatments after 4 h.	Haemocytes	Prado-Alvarez et al. 2013
Prorocentrum lima	*Ruditapes decussatus*	Apoptosis increased after 24 h and returned to near basal levels at 48 h; Cell death increased after 24 h and remained increased after48 h; Haemocyte esterase activity was increased after both 24 and 48 h.	Haemocytes	Prado-Alvarez et al. 2013
Toxin Standard	*Mytilus galloprovincialis*	Increased phagocytosis but only at the higher temperature (25˚C).	Haemocytes	Malagoli et al. 2008
Toxin Standard	*Crassostreas gigas*	No cytotoxicity or apoptotic cells were detected in cardiomyocytes after 24 h; p38 MAP kinases expression was not altered; p38 MAP kinases phosphorylation as induced after 1 h remaining increased up to 7 h; OA increased heart rate for 20 min; Ca^{2+} concentration-dependent increase.	Cardiomyocytes	Talarmin et al. 2008
Dinophysis acuminata	*Crassostrea gigas*	Total haemocyte count (THC) did not vary; Differential haemocyte count (DHC) unaltered; Apoptotic hemocytes unaltered; Hemagglutinating activity (HA) unaltered; Phenoloxidase (PO) activity unaltered; Total protein concentration unaltered.	Haemocytes	Mello et al. 2010
Dinophysis acuminata	*Perna perna*	THC increased 70 %; DHC presented a reduction in the granular haemocytes proportion; Apoptotic hemocytes unaltered; HA unaltered; PO activity decreased 30 %; Total protein concentration increased 22 %.	Haemocytes	Mello et al. 2010
Dinophysis acuminata	*Anomalocardia brasiliana*	THC increased 60 %; DHC unaltered; Apoptotic hemocytes unaltered; HA unaltered; PO activity unaltered; Total protein concentration increased 13 %	Haemocytes	Mello et al. 2010

Toxin source	Species Targeted	OA-group Effects in Bivalves	Organ, Tissue or Cells	Reference
Dinophysis acuminata	*Crassostrea gigas*	Under 4150 cells l^{-1} THC unaltered; DHC unaltered; Apoptotic hemocytes unaltered; Total protein concentration (PC) unaltered; PO decreased 60 %; HA unaltered; Histopathology: haemocytes migration, diapedesis and infiltration f into the stomach, intestine, and digestive tubules.	Haemocytes, Stomach, Intestine	Simões et al. 2014
Dinophysis acuminata	*Perna perna*	Under 4150 cells L^{-1} THC increased 44 %; DHC unaltered; Apoptotic hemocytes unaltered; PC increased 63 %; PO unaltered; HA unaltered; Under 2950 cells L^{-1} THC decrease 56 %; DHC unaltered; Apoptotic hemocytes unaltered; PC unaltered; PO increased 100 %; HA unaltered.	Haemocytes	Simões et al. 2014
Toxin Standard	*Mytilus edulis*	Protein Phosphatase (PP) activity was inhibited by 2nM OA in near 50 %; Glycogen synthase (GS) activity was not altered by 1 and 10 µM OA.	Hepatopancreas	Svensson and Förlin 1998
Prorocentrum lima	*Mytilus edulis*	GS activity was not altered.	Hepatopancreas	Svensson and Förlin 1998
Toxin Standard	*Mytilus galloprovincialis*	Gene Transcription Modulation of stress proteins; proteins involved in cellular synthesis.	Hepatopancreas	Manfrin et al. 2010

Among the limited data available regarding the effects of the three main groups of toxins in bivalves, there are some studies investigating the effect of STXs. These studies focused on behavioural and some physiological aspects (Table 1.5), with very little information on profound impacts in bivalves.

Table 1.5. Summary the initial reports on the effects of bivalve exposure to STXs producers (adapted from Landsberg, 2002).

Bivalves group	Bivalves Species	Main PSTs effect
Scallops	*Argopecten irradians*	↓ Clearance rate
	Chlamys farreri	↓ Egg hatching ↓ Larval survival
	Placopecten magellanicus	↑ Mucus ↑ Valve closure ↓ Oxygen consumption
Oyster	*Crassostrea gigas*	↓ Clearance rate ↑ Pseudo-faeces ↑ Valve closure
	Ostrea edulis	↓ Clearance rate ↓ Heart rate
Mussels	*Geukensia demissa*	↑ Valve closure ↓ Clearance rate ↑ Mucus ↓ Byssus production
	Mytilus edulis	↑ Valve closure ↓ Clearance rate ↑ Mucus ↓ Byssus production ↓ Heart rate
Clams	*Mercenaria mercenaria*	↑ Valve closure ↓ Clearance rate
	Mya arenaria	↑ Valve closure ↓ Clearance rate ↓ Burrowing ↓ Heart rate
	Spisula solidissima	↓ Clearance rate ↓ Oxygen consumption

↓ - Parameter decrease ↑ - Parameter increase

Recent studies demonstrated that STXs present acute toxicity for aquatic organisms, with extensive consequences, that range from reduction of responses to a stimulus to modulation of cellular and biochemical mechanisms, compromising detoxification processes, as well as immune and antioxidant responses (Table 1.6) (Borcier et al., 2017; Bricelj et al., 2005; Cao et al., 2018; Choi et al., 2006; Estrada et al., 2007; Fabioux et al., 2015; Galimany et al., 2008; González and Puntarulo, 2016; Hégaret et al., 2012, 2011, 2007a, 2007b; Hégaret and Wikfors, 2005b, 2005a; Lassudrie et al., 2014; Lesser and Shumway, 1993; Manfrin et al., 2012; Qiu et al., 2013). Responses observed in bivalves seem to be dependent not only on the species used as a model but also on the STX producer to which they were exposed. The majority of the reports were performed using dinoflagellate species from the genus *Alexandrium*, leaving a gap on how phytoplankton species from other genera may affect bivalves. It is also important be aware that recent studies with some strains of *Alexandrium* sp. revealed the existence of bioactive extracellular compounds (BECs) with allelopathic, hemolytic, cytotoxic or ichthyotoxic properties, that may be masking previous results (Castrec et al., 2018; Mello et al., 2013).

As for DA, almost no data are available on how the toxin affects bivalves, and the few reports approaching this subject are contradicting. For example, in mussels *Mytilus* sp. the toxin has no significant effect, either in larvae or adults, while, on the other hand, several behavioural and physiological responses were reported in oysters *C. gigas*, from shell closure to respiratory acidosis and increase in the phagocytic activity and the circulating haemocytes (De Rijcke, 2011; T.O. Jones et al., 1995; Trevor O. Jones et al., 1995)

Summarising the information collected~~,~~ regarding the OA-group toxins, the reduced number of studies available suggests that bivalves are affected at the DNA level with damage that may or not liable to be repaid by bivalve molluscs. Therefore, the need for a more profound analysis of these toxins' effects and their consequences for bivalves arises.

Table 1.6. Effects of exposure to STXs in bivalves and their producers: summary of the more recent reports.

Toxin source	Toxins	Species Targeted	STXs Effects in Bivalves	Organ, Tissue or Cells	Reference
Alexandrium catenella	GTX2, C2	*Crassostrea gigas*	Increased susceptibility to pathogens.	Whole body	Abi-Khalil et al. (2016)
Alexandrium catenella	na[a]	*Crassostrea gigas*	No time-dependent response observed in the hemocyte parameters. At increased temperature a positive correlation between death hemocyte and toxin concentration.	Hemocytes	Hégaret et al. (2007b)
Alexandrium catenella	na[a]	*Argopecten purpuratus*	Escape response: Higher clapping rates, but shorter duration of the response. Histopathology: increased melanisation; increased muscle fibers degeneration; increased rickettsiales-like organisms prevalence. Affect scallop interaction with pathogens and predators.	Hepatopancreas, Gills, Kidney, Gonad, Foot, Adductor Muscle	Hégaret et al. (2012)
Alexandrium catenella	na[a]	*Crassostrea gigas*	Increased hemocytes concentration, size and complexity; Increased ROS production. Inhibition of response to new microorganisms; Enhanced hemocyte mortality; Compromised hemocyte and physiological responses.	Hemocytes	Lassudrie et al. (2016)
Alexandrium catenella	na[a]	*Mytilus chilensis*	Increased SOD, CAT, Fer and heat-shock proteins gene expression; Modulation of the innate immune system.	Haemolymph	Núñez-Acuña et al. (2013)
Alexandrium fundyense	GTX1+4, GTX2+3, GTX5, NeoSTX, STX, C1	*Mytilus edulis*	Significantly increased Inflammatory response: hemocytes degranulation, diapedesis into the alimentary canal and, migration into the gonadal follicles connective tissue. Lipid peroxidation; Immunocompromised mussels, increased ceroidosis and trematodes prevalence. Spawned mussels showed more severe effects and remained paralyzed until post exposure.	Hemocytes, Digestive Diverticula, Gills, Mantle, Kidneys, Plicate Membranes, Byssus Gland	Galimany et al. (2008)

Toxin source	Toxins	Species Targeted	STXs Effects in Bivalves	Organ, Tissue or Cells	Reference
Alexandrium fundyense	na	*Argopecten irradians irradians*	Increased clearance rates; Open valve, filtering and producing biodeposites (feces only).	na[c]	Hégaret et al. (2007a)
Alexandrium fundyense	na	*Crassostrea virginica*	Similar clearance rates; Mostly closed shell valves, producing biodeposits (minimal fecal production).	na[c]	Hégaret et al. (2007a)
Alexandrium fundyense	na	*Mercenaria mercenaria*	Increased clearance rates; Partially closed shell valves, producing biodeposits (feces and pseudofeces).	na[c]	Hégaret et al. (2007a)
Alexandrium fundyense	na	*Mya arenaria*	Increased clearance rates; Open valve, siphons partially extended and producing biodeposites (feces only).	na[c]	Hégaret et al. (2007a)
Alexandrium fundyense	na	*Mytilus edulis*	Similar clearance rates; Open valves with extended foot, producing biodeposites (feces only).	na[c]	Hégaret et al. (2007a)
Alexandrium fundyense	na[a]	*Crassostrea virginica*	Adductor-muscle paralysis at higher algae concentrations.	Hemocytes	Hégaret et al. (2007b)
Alexandrium fundyense	na	*Mercenaria mercenaria*	Decreased Adhesion and the proportion of phagocytic haemocytes	Hemocytes	Hégaret et al. (2011)
Alexandrium fundyense	na	*Mya arenaria*	Decreased the percentage of phagocytic haemocytes.	Hemocytes	Hégaret et al. (2011)
Alexandrium fundyense	na	*Crassostrea virginica*	Increased dead haemocytes and inhibited phagocytosis	Hemocytes	Hégaret et al. (2011)
Alexandrium minutum	na[a]	*Pecten maximus*	Decreased clearance rates; Escape response compromised; Melanisation and hemocyte infiltration in mantel; Hyalinisation, hemocyte infiltration and atrophy in muscles; Morphological alterations in hepatopancreas	Hepatopancreas, Gills, Mantel, Muscles	Borcier et al. (2017)

Toxin source	Toxins	Species Targeted	STXs Effects in Bivalves	Organ, Tissue or Cells	Reference
Alexandrium minutum	C1+2, GTX2+3, dcGTX2+3, dcSTX	*Crassostrea gigas*	Disruption of the biological daily rhythm (compromised gene expression); Hepatopancreas inflammation; Hemocyte infiltration and diapedesis.	Gills, Hepatopancreas	Castrec et al. (2018)
Alexandrium minutum	na[a]	*Pecten maximus*	Increased valve movements (closures and expulsion)	na[c]	Coquereau et al. (2016)
Alexandrium minutum	GTX2+3, dcGTX2+3, C1+2, STX	*Crassostrea gigas*	Upper modulation of antioxidant system genes expression (GST, GR, and ferritin (Fer)).	Gills	Fabioux et al. (2015)
Alexandrium minutum	na[a]	*Mytilus galloprovincialis*	Scarcely modulated gene expression.	Hepatopancreas	Gerdol et al. (2014)
Alexandrium minutum	na[a]	*Crassostrea gigas*	Decreased neutral lipid content; Increased amylase specific activity (ASA); Increased total hemocyte count (THC); Increased size and complexity of granulocytes and hyalinocytes.	Hepatopancreas, Haemolymph	Haberkorn et al. (2010a)
Alexandrium minutum	na[a]	*Crassostrea gigas*	Decreased neutral lipid contents; Strong inflammatory response; Spermatozoa morphologically and functionally altered, with reduced motile and ATP available.	Hepatopancreas, Spermatozoa	Haberkorn et al. (2010b)
Alexandrium minutum	na[a]	*Crassostrea gigas*	Evidence of dose–response relationship behaviour and algae concentrations. Increased valve micro-closures. Feeding activity slightly decreased with increasing algae proportion. Valve opening duration was dose-related with PSP toxins concentration in hepatopancreas	Hepatopancreas	Haberkorn et al. (2011)
Alexandrium minutum	na[a]	*Crassostrea gigas*	Modulation of Neutral lipid contents, total hemocyte concentrations, phagocytosis and phenoloxidase (PO) activity in plasma.	Hepatopancreas, Haemolymph	Haberkorn et al. (2014)

Toxin source	Toxins	Species Targeted	STXs Effects in Bivalves	Organ, Tissue or Cells	Reference
Alexandrium minutum	na	*Crassostrea gigas*	Increased dead haemocytes; Decreased in the haemocyte's internal complexity and inhibited ROS production.	Hemocytes	Hégaret et al. (2011)
Alexandrium minutum	na[a]	*Crassostrea gigas*	Reduced valve-opening amplitude; Increased micro-closures; Gene expression repression; Genotoxic effects.	Gills, Hepatopancreas	Mat et al. (2013)
Alexandrium minutum	na[b]	*Crassostrea gigas*	Increased size in granular hemocytes; Reduced phagocytosis; Reduced ROS production; Gene expression modulation.	Haemolymph	Mello et al. (2013)
Alexandrium minutum	na	*Crassostrea gigas*	Increased daily valve-opening duration and micro-closure activity. Decreased valve-opening amplitude. Response to occurs, within 1 h after exposure, recovery to control patterns was observed within 4–5 days of post-exposure.	na[c]	Tran et al. (2010)
Alexandrium tamarense	C1+2, dcGTX2+3	*Ruditapes philippinarum*	Modulation of antioxidant system factors activity/content; Increased of LPO.	Gills, Hepatopancreas	Choi et al. (2006)
Alexandrium tamarense	na[b]	*Patinopecten yessoensis*	Temporary induction of ROS production; SOD activity inhibition; GPx activity induction	Muscle, Hepatopancreas	Qiu et al. (2013)
Alexandrium tamarense	na[b]	*Mytilus galloprovincialis*	Temporary induction of ROS production; CAT, SOD and GPx activity induction.	Muscle, Hepatopancreas	Qiu et al. (2013)
Gymnodinium catenatum	na[b]	*Nodipecten subnodosus*	Pseudo-faeces production; Partially closed shells; Increased melanisation and aggregate hemocytes; Modulation of antioxidant enzymes activity (increased GPx in gills; decreased SOD in gills and adductor muscle); Hydrolytic enzymes differential modulation.	Muscles, Gills, Mantel, Hepatopancreas	Estrada et al. (2007)

Toxin source	Toxins	Species Targeted	STXs Effects in Bivalves	Organ, Tissue or Cells	Reference
Gymnodinium catenatum	GTX2+3	*Nodipecten subnodosus*	Increased apoptosis; Morphological alterations of cytoplasmic membrane; Nuclear envelope damage; Chromatin condensation; DNA fragmentation; DNA fragments release to the cytoplasm; Translocation of phosphatidylserine to the plasma membrane; Activation of cysteine-aspartic proteases, caspase 3 and 8. Caspase-dependent apoptotic pathway.	Hemocytes	Estrada et al. (2014)
Gymnodinium catenatum	na[b]	*Crassostrea gigas*	Decreased clearance rate; Acute exposure: Differential modulation of antioxidant, detoxification and immune response gene expression.	Whole-body	García-Lagunas et al. (2013)
Gymnodinium catenatum	na[b]	*Crassostrea gigas*	Decreased clearance rate; Sub-chronic exposure: Upper modulation of antioxidant, detoxification and immune response gene expression.	Whole-body	García-Lagunas et al. (2013)
Gymnodinium catenatum (extract)	GTX2+3	*Nodipecten subnodosus* (Adults)	Adductor muscle paralysis without recovery after 40 days; Decreased total hemocyte count; Modulation of total hydrolytic enzymes activity (increased in hepatopancreas, gills and hemocytes, decrease in mantel); Modulation of lysozyme activity (Increased in gonad, mantel, muscle and hemocytes, decreased in gills); Modulation of antioxidant enzymes activity (Decreased SOD in gills, muscle and hemocytes; Increased CAT in hepatopancreas and gills; Decreased in gonad and hemocytes). Increased NO radicals in mantel and hemocytes; Increased LPO in gonad; mantle retraction; muscle contractions; Inability to close shells.	Hepatopancreas, Gills, Gonad, Mantel, Muscle, Hemocytes	Estrada et al. (2010)

Toxin source	Toxins	Species Targeted	STXs Effects in Bivalves	Organ, Tissue or Cells	Reference
Gymnodinium catenatum (extract)	GTX2+3	*Nodipecten subnodosus* (Juvenile)	Adductor muscle paralysis, with recovery; Decreased total hemocyte count; Decreased total hydrolytic enzymes activity; Increase in nitric oxide (NO) radicals;	Hemocytes	Estrada et al. (2010)
Toxin Standard	STX, GTX5, C1+2	*Crassostrea gigas*	Induced apoptosis through a caspase-dependent DNA fragmentation.	Hemocytes	Abi-Khalil et al. (2017)
Toxin Standard	STX	*Mytilus chilensis*	Modulation of phagocytosis and ROS production; Increased antioxidant, mitochondrial enzymes, and ion channel gene expression.	Hemocytes	Astuya et al. (2015)
Toxin Standard	STX	*Crassostrea gigas*	Antioxidant system modulation (increased CAT and GST, decreased SOD); Increased LPO; Decreased phagocytosis; Increased DNA damage; Upper modulation of gene expression (HSP 90 and CYP 450).	Hepatopancreas	Cao et al. (2018)
Toxin Standard	STX	*Chlamys farreri*	Antioxidant system modulation (increased CAT, SOD and GST); Increased LPO damage; Decreased phagocytosis; Increased ROS; Increased DNA damage; Upper modulation of gene expression (HSP 90 and CYP 450).	Hepatopancreas	Cao et al. (2018)
Toxin Standard	STX	*Crassostrea gigas*	Reduced phagocytosis; Reduced ROS production; Gene expression modulation.	Haemolymph	Mello et al. (2013)

na – Not Available.

[a)] Data presented in STX equivalents - no disclosure of individual toxins;

[b)] Data not provided;

[c)] Behavioural responses assessment

As for STX, the current information points to a significant impact of these toxins on bivalves, modulating a vast array of factors, from behaviour to the defence systems, gene expression and transcription. The information highlighted the species-specific nature of these impacts and revealed that only a hand full of studies approaching the effect of *G. catenatum* in bivalves has been performed, focusing mostly scallops.

4. The effects of environmental changes on Phytoplankton and Bivalves

4.1. The effects of environmental change drivers on phytoplankton and HABs

As referred before, an increase in the algal blooms' frequency, intensity, persistence and geographic distribution has been observed. The specific reason for this increment is still not completely clear, and several explanations have been proposed. Detection of HABs may have increased as a result of higher awareness and scrutiny for marine biotoxins in bivalves due to the elevated importance of this resource as a food source (Anderson et al., 2002; Botana, 2016). However, anthropogenic pressures and alterations of biotic and abiotic factors seem to be the major reasons behind the increase of algal blooms. Anthropogenic activities, such as shipping and agriculture, among others, seem to contribute to this increase by promoting algae dispersion, micronutrients increase or even eutrophication. Also, local, regional and global climate variations, regardless of whether natural or anthropogenic, seem to have an important role HABs increase (Anderson et al., 2002; Edwards et al., 2006; Hallegraeff, 2010, 1993; Moore et al., 2008; Sellner et al., 2003).

It is very difficult to attribute HABs increase to a single stressor/driver or even to differentiate the impact of different pressures in the algae communities, as this phenomenon is linked to the interaction of several climate change drives and other anthropogenic impacts (Anderson et al., 2002; Botana, 2016; Edwards et al., 2006; Fu et al., 2012; Hallegraeff, 2010, 1993; Moore et al., 2008; Sellner et al., 2003).

Coastal environments are particularly vulnerable to anthropogenic activities pressures and to climate change drivers. These areas are directly and indirectly affected by the impacts of greenhouse gases increase in the atmosphere, that range from sea level rise to water warming and acidification, changes on water column stratification, relaxation of upwelling events, changes on precipitation and evaporation patterns, as

well as fluctuations in river freshwater discharges and consequently salinity levels (Filgueira et al., 2016; Moore et al., 2008).

Different scenarios on how climate change drivers will affect phytoplankton have been proposed. Changes in the cyanobacteria and microalgae communities and biodiversity- mainly of species less tolerant to temperature variation - is a concern (Paerl and Huisman, 2009; Rasconi et al., 2017). Warm-water species, more tolerant to temperature variation, are expected to thrive and expand their distribution range. The increased water temperature and consequent enhanced stratification and relaxation of upwelling are expected to benefit bloom-forming species, among them toxic algae, whose growth is conditioned by temperature, light, and micronutrients availability (Hallegraeff, 2010; Moore et al., 2008). Additionally, to increase the frequency and intensity of HABs, climate change drivers also promote the enhancement of algae distribution ranges. Several species have already been reported where the expansion of its geographical area may be related with climate change drivers. For example, *Gambierdiscus toxicus* has been reported to expand its distribution due to warmer waters and extreme events (Chateau-Degat et al., 2005; Moore et al., 2008). Also, *Gymnodinium catenatum* has been showing a northward expansion from the NW Africa population into the Portuguese coast since the late XIX century. This natural expansion of their distribution seems to be linked with temperature increase in the NE Atlantic waters (Ribeiro et al., 2012).

4.2. The effects of environmental change drivers in bivalves

Phytoplankton is not the only group of species affected by changes in the environmental conditions. In fact, there are several studies approaching the impacts of warming or/and acidification in bivalves. Bivalves are sessile organisms or have low mobility, and cannot move or migrate to locations with better abiotic conditions. Also, bivalve larvae are known to be particularly susceptible to environmental changes, presenting thermal, and salinity tolerance ranges smaller than adults (Filgueira et al., 2016).

The effect of increasing temperature in bivalves may have positive and negative outcomes. If the rest of the environmental conditions are propitious, with the required

amount of oxygen and food available, the temperature increase leads to an increase of metabolic processes that may be translated into increased growth and reproduction (Filgueira et al., 2016). The coexistence of these conditions is however difficult to occur as the increase in water temperature causes a reduction in the concentration of dissolved O_2 in the water and the changes in the phytoplankton communities are somewhat unpredictable at the moment. Therefore, adverse outcomes of warming are expected to be more prominent. Between these outcomes, behavioural and metabolic changes are expected including the impairment of the immune response system, the reduction of energy available for the standard and somatic metabolism with the consequent reduction in growth rates and accumulation of xenobiotics, such as metals (including methylmercury), radionuclides and POPs - persistent organic pollutants (Alava et al., 2017; Anestis et al., 2007; Coppola et al., 2017; Dallas et al., 2016; Mohamed et al., 2014; Múgica et al., 2015b; Sokolova and Lannig, 2008).

Acidification can also impair these animals. The decrease of the water pH can directly compromise the structure of the shell by causing decalcification and preventing its formation due to ion sequestration. Indirectly, it may lead to physiological stress, as not only the external environment is affected. Environmental acidification may induce the decrease of pH in the extra- and inter-cellular fluids, impairing, even more, the shell structure as its dissolution may occur to compensate these acid-base disturbances. Also, several physiological and metabolic processes may be compromised, including lower tolerance to thermal stress, decrease of O_2 consumption, somatic growth and standard metabolic rates (Duarte et al., 2014; Gazeau et al., 2013; Griffith and Gobler, 2020; Michaelidis et al., 2005; Nikinmaa and Anttila, 2015; Parker et al., 2013; Pörtner, 2008).

The primary question is that, despite the existence of several studies approaching the impact of warming and acidification in bivalves, most are based only in one single driver and very little is known on the impacts of simultaneous exposure to several environmental changes (Anestis et al., 2010, 2007; Farrell et al., 2015; Fernández-Reiriz et al., 2012; Michaelidis et al., 2005; Múgica et al., 2015b; Nikinmaa and Anttila, 2015; Parker et al., 2013; Pörtner, 2008; Pörtner et al., 2007). Only in the last decade studies approaching exposure to multiple drives have been performed in bivalves, revealing that interactions of synergistic or antagonistic nature may occur (Benedetti et al., 2016; Coppola et al., 2017; Duarte et al., 2014; Freitas et al., 2017; Hu et al., 2015; Jarque et

al., 2014; Matoo et al., 2013; Matozzo et al., 2013, 2012; Nardi et al., 2017; Wu et al., 2016), reinforcing the need for this kind of studies when trying to predict the response to alterations in complex ecosystems, as coastal areas.

4.3. The impact of simultaneous exposure to environmental change drivers and HABs in bivalves

If only few studies are available on the impact of multiple environmental change drivers in bivalves, there are even less assessing the impact of multiple environmental change drivers combined with exposure to HABs. In fact, from all the studies mentioned above, only Benedetti et al. (2016) and Nardi et al. (2017) assessed the effects of exposure to warming and acidification, with a third stressor in both cases the metal cadmium.

Regarding the simultaneous exposure to environmental change drivers (warming and acidification) and HABs, very little is known and literature referring these two aspects is extremely scarce. In fact, to the best of our knowledge, there are no studies assessing the following three factors, warming, acidification and exposure to HABs and/or their biotoxins, simultaneously. The few reports addressing the exposure of bivalves to biotoxins/HABs under warming or acidification found in the bibliography were designed to understand the impact on toxins elimination (Bricelj et al., 2014; Svensson and Förlin, 2004). Under warming conditions, lower STXs levels were accumulated, elimination was slower, and no significant impacts were reported for routine metabolic rates (RMR), while the enzymatic activity seemed to be modulated by both temperature and exposure to STXs (Farrell et al., 2015).

Regarding the information reported, a major gap was identified: the lack of knowledge on how exposure to multiple drivers (warming and acidification) combined with exposure to HABs, may affect bivalves, regarding biotoxins kinetics (uptake, accumulation and elimination) and physiological aspects.

To approach and fill these gaps, it is essential to assess the impacts of each environmental driver individually and combined in wild bivalve populations and their ecosystems. Data will be of key importance to shellfisheries. Particularly in a scenario where the increase in HABs frequency, intensity and distribution is expected, a balance

between the resources demands *vs.* environment protection and human health safety is mandatory.

5. Human health safety and marine biotoxins monitoring

Outbreaks of diarrhetic, paralytic and amnesic shellfish poisoning have been reported over time all over the world. The earliest paralytic shellfish poisoning (PSP) recorded in Europe occurred in Norway in 1901 (FAO, 2004). For the XX century, at least seven PSP outbreaks were reported affecting 32 persons and causing two fatalities (FAO, 2004). In Portugal, PSP outbreaks have been reported since 1946. In that year, 100 victims and six fatalities were registered. Nine years later, a new episode of PSP occurred in Portugal, causing 21 victims and one fatality (Vale et al., 2008).

Similar worldwide outbreak reports occurred for diarrhetic shellfish poisoning (DSP), beginning in the 1960s in the Netherlands and the 1970s in Japan. Overall, in Europe, outbreaks have been consistently reported since 1981, with over 5,000 cases in Spain. Between 1984 and 1986, France reported over 2000 cases each year. Also, in 1984, in Norway, more than 300 cases were reported. DSP outbreaks are not limited to these countries, as Chile, Nova Scotia - Canada, Australia, Indonesia and New Zealand were also affected (Aune and Yndestad, 1993; FAO, 2004; Vale et al., 2008).

For the moment, very little information is known about ASP outbreaks. The first confirmed report of ASP outbreak dated from 1987 in Canada when at least 105 persons presented sins of acute intoxication, and four persons died. In 1992, 24 intoxication cases with symptoms similar to ASP occurred in the State of Washington (USA); however, the syndrome was not confirmed (FAO, 2004; Wekell et al., 2004).

In response to these events and in order to protect the populations from the threat of marine biotoxins, monitoring programmes have been implemented (EEC, 1991). These programmes are responsible for determining the presence and concentration of HABs and marine biotoxins in commercial bivalves, banning bivalve harvest and commercialisation until the toxin levels no longer represent a risk for the public health. In Portugal, the monitoring programme was implemented in 1986, screening for PSP toxins, while DSP toxins were added to the programme in 1987 and ASP toxins in 1996 (Quilliam and Wright, 1989; Vale et al., 2008).

Monitoring programmes initially relied on biological methods, such as the mouse bioassay (MBA), to detect marine biotoxins in bivalves. This bioassay, developed by Yasumoto et al. (1978), consisted of an intraperitoneal (i.p.) injection of bivalve extracts in a 20 g mice. The results were expressed in mouse units (MU), corresponding the MU to the toxicity of the compounds present in the extract injected. MU was defined as the amount toxin needed to kill a male mouse of 20 g body weight in 24 h period (Suzuki et al., 1996; Yasumoto et al., 1978).

While this method is able to detect a vast array of toxic compounds, providing a toxicity response that is almost proportional to human toxicity response, is a simple method not requiring extensive clean-up procedures or complex and expensive equipment. It has severe problems like the fact of being difficult to perform an accurate extrapolation of the results obtained when the exposure/administration route in the animal model and human differs, from i.p. injection to oral consumption (Fernandez et al., 2003). Also, this method requires a large number of animals for experimentation, raising ethical issues (EC, 2010). Therefore, the EU legislation regarding marine biotoxins determination was recently altered, implementing the use of chemical methods, based on liquid chromatography, to perform marine biotoxins detection and determination in bivalves (EC, 2017, 2011). The development and implementation of these methodologies created, however, a new set of problems as the lack of toxins standards and knowledge on toxicity equivalent factors, in addition to the need for trained expertise.

The European Commission (EC) established regulatory limits based on the initial toxicity reported from each of the toxin groups. The value of 160 μg of OA equivalents per kg of shellfish meat was established for the DSP toxins, the 800 μg of STX equivalents per kg of shellfish meat for the PSP toxins, and the 20 mg of DA per kg of shellfish meat for the ASP toxins (EC, 2004b). Bivalves containing toxin values exceeding the regulatory limits cannot be harvested or commercialised within the European borders, and a vast number of countries around the world.

So far, and much because of the effective work done by monitoring programs, quantitative data regarding marine biotoxins toxicokinetics in humans is scarce (EFSA, 2009a, 2009b, 2008). In addition, most of the studies approaching biotoxins toxicokinetics were performed in small mammals, such as rodents by abdominal or i.p.

injection and then extrapolated to humans (Aune et al., 2007; EFSA, 2009b, 2009a, 2008; Fernandez et al., 2003). There is a limited number of studies involving oral exposure to OA-group toxins. These studies were performed by administrating OA standards dissolved in water or methanol and not through bivalves or other naturally contaminated food matrices (Matias et al., 1999).

While the data obtained from this kind of studies may be indicative of toxicokinetic patterns in mice, the direct extrapolation of these patterns for humans may leave something to be desired. The studies performed using mice i.p. injection, for example, completely exclude the impacts of digestion and nutrient and other compounds retrieval from the food matrix (Fernandez et al., 2003).

As for the studies involving oral exposure, they include the impacts of food and toxin digestion. However, since they were performed using standards, the data collected exclude the effects of digestion in the OA-group toxins esterified with fatty acids (DTX3). These compounds only exist in naturally contaminated samples and despite being considered less toxic than the parental toxins, in some bivalve species correspond to the majority of the toxin profile. The 7-O-acyl derivatives present a high variability and complexity what hampers the production of standards for these compounds. They are also known to cause severe intoxications in humans, and to be prone to suffer hydrolysis when exposed to enzymes involved in human digestion (Doucet et al., 2007; Hallegraeff et al., 1995; Torgersen et al., 2005; Vale and Sampayo, 2002b).

Therefore, the need for more accurate information on how human digestion affects toxins profile and what percentage of toxins is released from the food matrix during digestion becoming accessible to be absorbed by the gut epithelial (bioaccessibility) arise, as this information provides more accurate data for the elaboration of human health risk assessments by the safety authorities (Cardoso et al., 2015; Metian et al., 2009; Versantvoort et al., 2005).

6. Main objectives and thesis structure

Taking into account the information presented above concerning marine biotoxins and their link to species introduction and environmental changes, the present work aimed to fulfil the knowledge gaps identified. Therefore, the primary goals pursued in this work were:

i) To determine whether native and invasive bivalve species cope with exposure to HABs differently, providing new insights on species-specificities and ecosystem functioning fragilities in the presence of invasive species and toxic algae.

ii) To evaluate how commercially valuable bivalve species cope with simultaneous exposure to several climate change drives and HABs, providing new insights on how environmental changes constrain toxicokinetics as well as physiological and genotoxic responses.

iii) To assess, marine biotoxins bioaccessibility, contributing for new information passively of being used in health risk assessments.

In order to achieve these general goals, the following specific objectives were considered:

- Assessment of toxicity and bioaccumulation patterns of marine toxins in naturally contaminated native (*R. decussatus*) and non-indigenous (*R. philippinarum*) bivalve species living in sympatry.
- Comparative evaluation of the native, *R. decussatus,* and the invasive, *R. philippinarum,* toxin accumulation and elimination patterns following exposure to the dinoflagellate *Prorocentrum lima* (producer of OA-group toxins).
- Comparative evaluation of the native, *R. decussatus,* and the invasive, *R. philippinarum,* to susceptibility to DNA damage in counterpoint with DNA damage repair capacity following exposure to the dinoflagellate *P. lima*.
- Assessment of the climate change drivers (warming and acidification) impacts, *per se* and combined, on the toxin's accumulation and elimination dynamics in *Mytilus galloprovincialis* following exposure to the dinoflagellate *Gymnodinium catenatum* (producer of STXs).
- Assessment of the organ-specific (gills and hepatopancreas) antioxidant response, oxidative and genotoxic damage modulation in *M. galloprovincialis*

exposed to *G. catenatum* under the climate change drivers~~,~~ warming and acidification.

- Determination of OA-group toxins bioaccessibility in two commercially important bivalve species naturally contaminated *M. galloprovincialis* and *Donax sp*. (donax clams).

The present thesis comprises 7 chapters, in which are included the General Introduction (Chapter 1) addressing background information on all the issues approached. The thesis also includes 5 chapters of original research work from chapters 2 to chapters 6.

The research work in the thesis starts with field observations aiming to assess the presence and accumulation patterns of regulated and non-regulated biotoxins in native and invasive species living in sympatry (Chapter 2). Laboratory experiments were then conducted under controlled conditions to better investigate the kinetics and genotoxic effects of OA-group toxins (Chapter 3), which were observed as the most common biotoxin in the Portuguese coast (Chapter 2).

The thesis continues by evaluating the effects of changes in the environmental conditions, such as warming and acidification (climate change drivers) on biotoxins kinetics (Chapter 4), and on the antioxidant and genotoxic responses of mussels simultaneously exposed to the climate change drivers and HABs (Chapter 5). The original research work in the thesis ends with an anthropocentric approach to biotoxins accumulation. Hence, in chapter 6, the potential of bivalves as vector of biotoxins into the human consumer is assessed and the biotoxins bioaccessibility evaluated.

The thesis finishes with a General Discussion and Conclusions (Chapter 7), where a comprehensive discussion of the data collected is performed, overall major conclusions and future studies perspectives are presented.

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Chapter 2

Native (*Ruditapes decussatus*) and non-indigenous (*R. philippinarum*) shellfish species living in sympatry: comparison of Regulated and Non-Regulated biotoxins accumulation

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Native (*Ruditapes decussatus*) and non-indigenous (*R. philippinarum*) shellfish species living in sympatry: comparison of Regulated and Non-Regulated biotoxins accumulation

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Abstract

The native *Ruditapes decussatus* and the non-indigenous *Ruditapes philippinarum* are an important target of shellfish industries. The aim of this study was to compare an invader with a native species living in sympatry in the view of marine biotoxins accumulation. Samples were analysed for regulated and non-regulated biotoxins. The consistently occurrence of okadaic acid-group toxins and BMAA, may cause human health problems and economical losses. A strong positive relationship was observed between species, with significantly higher DSP toxicity in *R. decussatus*. Similar toxin profiles dominated by DTX3 in both species suggests similar metabolic pathways. Lower DSP toxicity in *R. philippinarum* may favour their cultivation, but a tendency for higher levels of the non-regulated BMAA was observed, indicating risks for consumers that are not monitored. This study highlights the need to better understand the physiological responses and adaptations allowing similar species exposed to the same conditions to present different toxicity levels.

Keywords: Okadaic acid, BMAA, Dinophysistoxins, Tetrodotoxin, Diarrhetic Shellfish Poisoning

1. Introduction

Species introduction has been one of the major issues in the recent decades when it comes to biodiversity loss and ecosystems conservation, particularly in aquatic ecosystems, where non-indigenous species can rapidly spread and become invasive (Chiesa et al., 2011; Karatayev et al., 2009; Mcneely and Schutyser, 2003; Sousa et al., 2009). Shellfish is one of the groups with a higher number of non-indigenous species around the world. Shellfish species have been introduced either for their economic importance, or to offset the native population collapse (Chiesa et al., 2011; Flassch and Leborgne, 1992; Gosling, 2004).

Natural from the Indo-Pacific region, the Manila clam, *Ruditapes philippinarum* (Adams & Reeve, 1850) was first accidentally introduced in the North America Pacific coast (1936), imported along with *Crassostrea gigas* seeds. The species showed a high adaptation capacity, with high reproductive and growth rates, which led to a deliberate introduction in the coastal waters of France, England, Italy and Spain, in order to support the local clam fisheries where the native species, Carpet Shell clam, *Ruditapes decussatus* (Linnaeus, 1758), was declining (Chiesa et al., 2011; Flassch and Leborgne, 1992; Gosling, 2004; Goulletquer, 1997). In Ria de Aveiro, a major shellfish production site in Portugal, the two clam species, native *R. decussatus* and non-indigenous *R. philippinarum* live in sympatry, coexisting in the same harvesting areas. *R. philippinarum* is even considered invasive, presenting a larger distribution area and reaching mean densities of 16 ± 10 clams per m^2 (Velez et al., 2015a).

In fact, *Ruditapes philippinarum* has a high invasive potential, with a wide tolerance to environmental variability (Ricciardi and Rasmussen, 1998), organic pollution (Karatayev et al., 2009), and an increased resistance to bacterial infections (Moreira et al., 2012), which has contributed to its use by the shellfish industry, replacing irregular yields of the native clam.

The shellfish industry is also increasingly affected by the occurrence of marine biotoxins. Consumption of shellfish contaminated with biotoxins may cause intoxication and severe food-borne illnesses (Burri and Vale, 2006; Rodrigues et al., 2012). Therefore, to minimize the risk of acute intoxication, shellfish harvesting and sale is prohibited

whenever toxin levels reach a legal threshold, causing important economic losses to shellfish producers (Vale et al., 2008).

Lipophilic toxins, namely the okadaic acid group, are the most frequent and abundant marine biotoxins found in the shellfish from southern European coastal areas (Vale et al., 2008). The okadaic acid group of toxins, responsible for the human diarrhetic shellfish poisoning (DSP), consists mainly of okadaic acid (OA), its isomers dinophysistoxins 1 and 2 (DTX1, 2) and fatty acid ester derivatives of either OA or DTX1-2. These fatty acid ester derivatives are collectively designated as DTX3. Lipophilic toxins also include the azaspiracids (AZA), yessotoxins (YTX) and pectenotoxins (PTXs). A second group of marine biotoxins but with hydrophilic properties is constituted by domoic acid and saxitoxins. Domoic acid (DA) its epimer (5´-epi-DA) are responsible for the amnesic shellfish poisoning (ASP), and saxitoxin (STX) and its derivatives are responsible for the paralytic shellfish poisoning (PSP). These toxins also frequently occur in Ria de Aveiro, Portugal (Vale et al., 2008).

All the above mentioned marine toxins are today regulated by EU Directives (EC, 2004a). However, several non-regulated toxins, frequently designated as emergent toxins, are not included in the monitoring programs, even though they may represent a potential threat to the public health and have been reported in the North Atlantic waters (M. Silva et al., 2015; Vasconcelos, 2013). Among non-regulated toxins, BMAA, β-N-methylamino-l-alanine, has been found in Portuguese transitional water systems, including Ria de Aveiro (Baptista et al., 2011; Lage et al., 2014), and tetrodotoxin (TTX) has been described along the Portuguese coast associated to several gastropod species (Rodriguez et al., 2008; Silva et al., 2012).

The unusual non-protein amino acid BMAA was first associated with the high incidence of Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) in Guam, and has been implicated as a potential environmental factor in ALS, Parkinson's and Alzheimer's diseases, and other neurodegenerative diseases (Banack et al., 2007; Banack and Murch, 2009; Bradley and Mash, 2009; Paul Alan Cox et al., 2005; Murch et al., 2004; Pablo et al., 2009) . Cyanobacteria are not the only source of BMAA (Chiu et al., 2011; Cox et al., 2005), also a marine dinoflagellate (*Gymnodinium catenatum*) (Lage et al 2014) and several diatom species have been linked to BMAA production in aquatic ecosystems (Jiang et al., 2014; Lage et al., 2015; Réveillon et al., 2015). BMAA has been

found in seafood (i.e. crustaceans and mollusks) and fish (particularly those that feed on the benthic fauna) from several locations around the world, suggesting a widespread occurrence of BMAA in aquatic organisms. Tetrodotoxin is a potent neurotoxin, produced by a variety of bacteria and possibly by benthic dinoflagellates like *Prorocentrum minimum* (Rodríguez et al., 2017). TTX is well known in Japan as the pufferfish toxin causing nausea, ataxia, aphasia, cardiac arrhythmias, paralysis, coma and, ultimately, death by respiratory failure (Noguchi and Arakawa, 2008). Recently, TTX was associated with bivalve mollusks from European coastal waters and previously detected in gastropods from Portugal (Rodriguez et al., 2008; Silva et al., 2012; A. Turner et al., 2015; Vlamis et al., 2015).

The aim of this study is twofold: 1) to assess toxicity and bioaccumulation patterns of marine toxins in native (*Ruditapes decussatus*) and non-indigenous (*R. philippinarum*) shellfish species from the same production area and 2) to discuss recent trends of regulated and non-regulated marine toxins.

2. Methods

2.1. Study area

Ria de Aveiro, the major coastal lagoon system in Portugal, is composed of several channels, large areas of mud flats and salt marshes. The lagoon, approximately 45 km long, 10 km wide and 1 m deep, on average, is located in the northwest of the Portuguese coast (40º 38' 40.06" N; 8º 45' 9.82" W) and connected to the Atlantic Ocean by a channel with approximately 350 m wide, reaching depths of 20 meters and with an average tidal range of approximately 2.00 m (Dias et al., 2000). The water circulation in the lagoon is assured by the freshwater input from the rivers Antuã and Vouga and by the tidal inputs. This lagoon is one of the major shellfish harvesting sites in Portugal. It is divided in four classified shellfish production areas, designated as RIAV1, RIAV2, RIAV3 and RIAV4 (Fig. 2.1). This coastal lagoon system is recurrently exposed to several and prolonged blooms of toxic algae (Vale and Sampayo, 2003) that significantly impact the commercial harvesting of cockles (*Cerastoderma edule*), mussels (*Mytilus galloprovinciallis*), oysters (C*rassostrea gigas*), clams (*Venerupis pullastra*, *Ruditapes decussatus*, *Solen marginatus*) and the non-indigenous clam *Ruditapes philippinarum*,

recently introduced in the system. The two clams, *R. decussatus* (native) and *R. philippinarum* (non-indigenous), live in sympatry within the system and therefore are exposed to the same environmental pressures (Rocha et al., 2013; Velez et al., 2015b, 2015a).

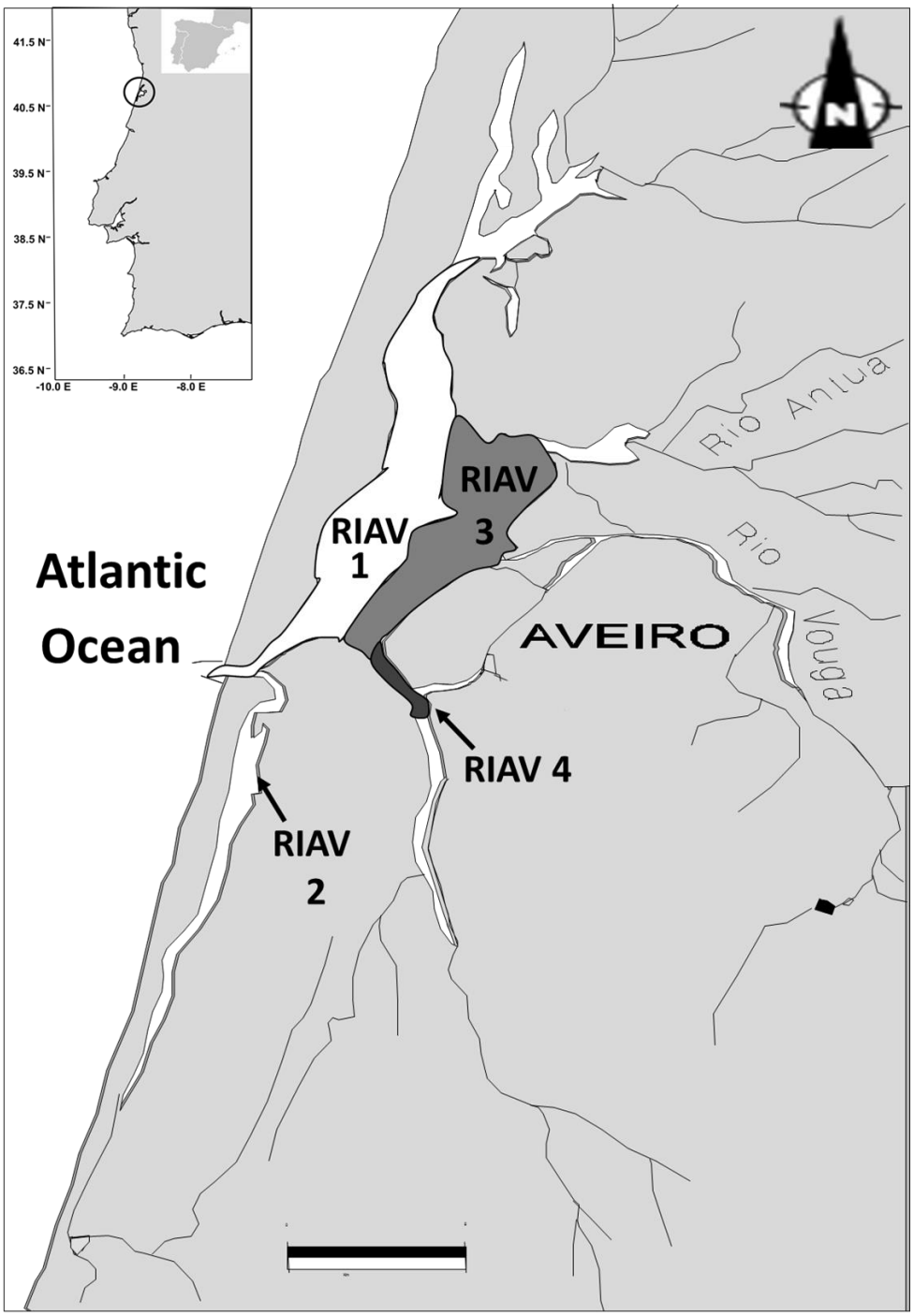

Fig. 2.1 Shellfish production areas in Ria de Aveiro, Portugal

2.2. Official control data

Data on DSP, ASP and PSP toxicity for both species, native (*R. decussatus*) and non-indigenous (*R. philippinarum*) clams, from classified shellfish producing areas of Ria de Aveiro are available at Instituto Português do Mar e da Atmosfera (IPMA) web site (www.ipma.pt) since 2014. Toxicity data for samples of native and non-indigenous species collected on the same day and in the same area from the period of January 2014 until December 2016 were used in this study. Toxicity data correspond to the sum of toxins concentration after applying a pre-established Toxicity Equivalent Factor (TEF) as is stated by European Food Safety Authority (EFSA 2009, 2008).

2.3. Sample collection and preparation

Since official data are reported only in terms of toxicity value and do not discriminate the variability of each compound or toxin derivative, 10 samples of Ruditapes decussatus and 10 samples of *R. philippinarum* collected from Ria de Aveiro, between April and September of 2015, were used to investigate the occurrence of the wide diversity of regulated and non-regulated toxins. To minimize differences on exposure to harmful algal blooms, samples of each species were collected in the same day and same location (Fig. 2.1). For each sample, at least 30 individuals were opened, removed from the shell, washed with running tap water to remove residues and drained. The samples were then homogenized in a blender and stored at - 20 ˚C until use for determination of regulated toxins – lipophilic toxins (OA-group toxins, AZA, YTX, PTX), amnesic shellfish poisoning toxins (DA and epi-DA) and paralytic shellfish poisoning toxins (STX and derivatives) – as well as for the determination of the non-regulated toxins BMAA and TTX.

Blue mussel, *Mytilus edulis*, collected near Askö Marine Research Station (Stockholm University, Sweden) was used as negative control and as matrix for BMAA standard curve preparation; after previous analysis with the BMAA extraction and analytical method describe in this study reveal no detectable BMAA. Mussel samples were grinded with mortar and pestle and subsequently lyophilized in a CoolSafe freeze-dryer (SCANVAC, Stockholm, Sweden).

2.4. Toxins extraction and analysis

2.4.1. Reagents

All reagents used for toxins extraction and analysis were of analytical grade or higher. Acetic acid glacial (100 %, p.a.), methanol (≥ 99.8 %, p.a.) and acetonitrile (Analytical grade) were obtained from Sigma-Aldrich; Methanol (LC-MS grade), acetonitrile (LC-MS grade), ammonium formate (≥ 99 % purity) from Fluka; Formic acid (98 %, p.a) from Roth; Hydrochloric acid (37 %) from Panreac; Hydrogen peroxide (30%) and sodium hydroxide (≥ 99 %, p.a.) from Merck. Water was purified using a Milli-Q 185 Plus system from Millipore. Toxins standard solutions for lipophilic toxins, domoic acid and PSP toxins were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council (NRC, Canada). Tetrodotoxin was purchased from Tocris Bioscience and BMAA from Sigma-Aldrich, Germany. The chemicals used for BMAA extraction were methanol (≥99,9 % purity, HPLC grade), water (HPLC grade), acetonitrile (≥ 99,9%, HPLC-Isocratic grade), ammonia (25 %, Analytical grade) and hydrochloric acid (37 %, analytical grade) all obtained from VWR Chemicals, Sweden. Formic acid (≥ 98 % purity) was from Sigma Aldrich.

2.4.2. Regulated toxins

Extraction and determination of the regulated toxins were carried out following the European official reference methods. Determination of lipophilic toxins was carried out according to the EU-Harmonised Standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS (EURLMB, 2015), which is further detailed in (Braga et al., 2016), the detection limit (LOD) for the lipophilic marine biotoxins was 0.5 ng mL^{-1} for OA, 0.33 ng mL^{-1} for AZA, 1.5 ng mL^{-1} for YTX and 0.6 ng mL^{-1} for PTX.

Determination of ASP toxins was carried out following the EU-Harmonised Standard Operating Procedure for determination of domoic acid in shellfish and finfish by RP-HPLC using UV detection (EURLMB, 2008), with modifications as described in Vale and Sampayo (2001), the LOD for the DA was 0.5 ng mL^{-1}. For PSP toxins, the AOAC Official Method 2005.06 (the so-called Lawrence method), a liquid chromatographic method

with fluorescence detection and pre-column oxidation of toxins was used, as described in (Costa et al., 2014), LOD for the PSP toxins was 1.5 ng STX mL $^{-1}$.

2.4.3. Non-Regulated toxins

2.4.3.1. β-N-methylamino-l-alanine (BMAA)

Samples were prepared according to Murch et al. (2004) with minor alterations (Lage et al., 2015; Masseret et al., 2013). Triplicate samples, 2 mg dry weigh (DW) each, were each dissolved in 1 mL of 0.1 mol L^{-1} TCA and then lysed by sonication for 3 min at 70 % efficiency. To prevent protein degradation, samples were kept in an ice-water bath throughout the sonication procedure. Samples were vortexed and incubated for 48 h at 4 °C. Subsequently, samples were vortexed and centrifuged for 10 min at 10,000 g and 4 °C. The supernatant was transferred to an Eppendorf tube and 500 µL of 0.1 mol L^{-1} TCA was added to the pellet, which was incubated for 1 h at 4 °C. The samples were vortexed and a 25 µL aliquot was taken from all samples to determine total protein concentrations using a BioRad RC/DC kit (Bio-Rad, Sundbyberg, Sweden). Afterwards, samples were centrifuged again for 10 min at 10,000 g and 4 °C. The supernatant was transferred to the Eppendorf tube, where both supernatant fractions were mixed, now constituting the total soluble BMAA. Both supernatant and pellet samples were lyophilized in a freeze-dryer and subsequently hydrolyzed in 600 µL of 6 mol L^{-1} HCl for 20 h, at 110 °C. After cooling, the acid solutions of both protein and total soluble fractions were separately filtered through a centrifugal filter unit (Ultrafree-MC centrifugal filter; Merck Millipore, Billerica, MA, USA) for 1 min at 10,000 g and then frozen at - 80 °C before lyophilization in the freeze-dryer. Samples were stored at - 80 °C until UPLC-MS/MS analysis.

Prior to UPLC-MS/MS analysis, the extracted lyophilized samples and standard curves spiked blank mussel matrix samples were reconstituted with 20 µL of 20 mmol L^{-1} HCl solution and dilutions were performed to obtain an optimum sample protein to derivatization ratio (Lage et al., 2015; Waters, 1993). Diluted samples of approximately 0.3 µg $µl^{-1}$ total protein content (i.e. quantified on protein bound fraction) were derivatized with AccQ-Tag using a WAT052880 AccQ-Tag kit (Waters, 1993), i.e. 70 µL of

borate buffer and 30 µL of AQC. UPLC-MS/MS analysis was performed within 2 – 10 h of samples derivatization.

Two standard curves were prepared, i.e. one with the protein fraction and one with the total soluble fraction. The calibration curves were prepared in triplicate from 2 mg DW of the blue mussel blank matrix, accordingly diluted, at five concentrations (i.e. 1.4; 7.1; 14.2; 71.4 and 142.3 ng mL^{-1}). Standards were added to a blank matrix immediately before derivatization.

Analysis by ultra-performance liquid chromatography–tandem mass spectrometry was performed using an Acquity UPLC system coupled to a Xevo-TQ-MS system (Waters). Separation was performed using an AccQ-Tag Ultra C18 column (100 × 2.1 mm, 1.7 µm particle size; Waters) with a binary pump. Eluent A was 0.01 % formic acid in 0.05 % ammonia in water and eluent B was 0.01 % formic acid in methanol. The gradient used was: 0.1 % B for 0.54 min, 45 % B for 4.00 min, 100 % B for 4.10 min, and 0.1 % B for 4.70-6.00 min. The flow was diverted to the mass spectrometer between 1.2 and 3.5 min to minimize interface contamination. Otherwise, it was diverted to waste. Injection volume was 10 µL. BMAA was identified using a highly selective UPLC-MS/MS method that distinguishes BMAA from its isomers N-(2-aminoethyl) glycine (AEG) and 4-diaminobutyric acid (DAB) in biological samples. Ionization was performed in positive ion mode and the mass analyser was run in selected reaction monitoring (SRM) scan mode using the following transitions to distinguish BMAA from its isomers AEG and DAB: general to all three analytes, *m/z* 459.1 > 119.08; DAB diagnostic fragment, *m/z* 459.1 > 188.1; AEG diagnostic fragment, *m/z* 459.1 > 214.1 and BMAA diagnostic fragment, *m/z* 459.1 > 258.09. To further ensure positive identification of BMAA, the retention time and SRM ratio of fragments 119.08 / 258.09 were monitored.

To prevent any error due to contamination and/or carryover response, approximately every tenth sample injection was followed by a 10 min column wash, a 3 min equilibration step and the injection of a blank sample (i.e. borate buffer).

All settings were optimized for BMAA detection as follows: cone voltage, 30 V; source temperature, 150 °C; desolvation temperature, 550 °C; cone gas flow, 20 L h^{-1}; desolvation gas flow, 1000 L h^{-1}; collision gas flow, 0.15 mL min^{-1}; collision energy, 26 V.; MassLynx V4.1 software (Waters) was used to analyse the acquired chromatographic data.

The standard curves (i.e. both protein and total soluble BMAA fractions) were generated with the chromatogram peak area of the general BMAA product ion (*m/z* 459.1 > 119.08) versus BMAA concentrations spiked in mussel blank matrix samples. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the signal-to-noise ratio (*S/N*) of the chromatographic peaks. The LOD was calculated for the diagnostic product ion of BMAA (i.e. *m/z* 459.1 > 258.09) and the LOQ for the general BMAA product ion (i.e. *m/z* 459.1 > 119.08). The LOD was established when the *S/N* was higher than 3.0 for the diagnostic product ion of BMAA. For the general BMAA product ion, the BMAA peak displayed an *S/N* of 10 or higher, thus the LOQ was described as equal to the LOD. For the total soluble and protein bound BMAA fractions of blank mussel matrix the LOD and LOQ was 1.4 ng·mL^{-1}and the calibration curves had an r^2 of 0.97 and 0.98, respectively. BMAA (i.e. 142.3 ng mL^{-1}) spiked into the total soluble and protein bound fractions of mussel blank matrix revealed a matrix effect, calculated in terms of matrix factor (Matuszewski et al., 2003) of 0.63 ± 0.18 and 0.44 ± 0.08, respectively.

2.4.3.2. Tetrodotoxin (TTX)

TTX extraction was performed according (McNabb et al., 2014). Briefly, 1.00 g aliquot of shellfish meat homogenate was extracted. 9 mL of acetic acid (0.1 %, v/v) were added to the sample and homogenized with Polytron mixer (Kinematica, Switzerland) for 0.5 min and centrifuged at 3000 g for 5 min. 1 mL of the resulting supernatant was then incubated with 9 mL of MeOH with 0.1 % (v/v) of acetic acid at 4 °C for 60 min. Following centrifugation (3000 *g* for 5 min) of the incubated supernatant an aliquot supernatant was removed and diluted (1:4) in MeOH with 0.1 % (v/v) acetic acid. The diluted aliquot was then filtered through 0.2 µm RC syringe filter (Phenomenex) and 2 µL injected to the LC-MS/MS system. The LC-MS/MS equipment consisted of an Agilent 1290 infinity LC system coupled to Triple Quadrupole 6470 Agilent. The chromatographic separation was performed using an Acquity UPLC BEH Amide column (3.0 × 50 mm, 1.7 µm), protected with a guard column Acquity UPLC BEH Amide (2.1 × 5 mm, 1.7 µm). The mobile phase A was water with 3 mM formic acid, and mobile phase B was 70 % acetonitrile with 2 mM formic acid and 2 mM ammonium hydroxide. An elution gradient

adapted from (Boundy et al., 2015) was used as follows: 0 - 2 min, 5 % eluent A at a flow rate of 0.250 mL min^{-1}; then a linear gradient from 5:95 to 50:50 A:B at 3.8 min. The composition was then held while the flow rate was linearly increased to 0.3 mL min^{-1} over 1.2 min and held by 0.50 min. The gradient was then decreased to 5:95 and flow rate increased to 0.450 mL min^{-1} during 0.50 min. From min 6 to 7 a further increase of flow rate to 0.6 mL min^{-1} was set and holding it by 1 min. Finally, flow rate was decreased to initial conditions and stabilized for 1 min before subsequent injection. The optimized source settings were as following: gas temperature 225 ºC, gas flow 12 L/min, nebulizer 45 psi, sheath gas temperature 375 ºC, sheath gas flow 11 L/min and capillary voltage 400 V. Two MRM transitions, in negative, mode were monitored: *m/z* 320 > 302 and *m/z* 320 > 162 for TTX and 4-epi TTX quantification and confirmation, respectively. The following transitions: *m/z* 302 > 162 and *m/z* 272 > 162 were set to check for the presence of 2,9-anhydroTTX and for 5,6,11-trideoxyTTX. Calibration standard solutions were prepared for a range from 0.5 to 50 ng mL^{-1}. The lowest calibration point was assumed as the limit of quantification. The limit of detection was 0.17 ng mL^{-1}.

2.5. Statistics

Data analysis was performed using the statistical program SigmaPlot Version 10.0. The Paired t-test was performed to assess significant differences between the OA equivalents toxins in the two clam species. The Wilcoxon Signed Rank Test was used to check for significant differences between free and esterified fractions of the OA group toxins in both clams.

The same test was used to check for significant differences between total soluble BMAA and protein-bound BMAA toxins. Data were previously tested for normality and homogeneity of variance by the Kolmogorov-Smirnov test and the Levene Median test. Differences were considered significant at $p < 0.05$. A last squares linear regression analysis was applied to data. The DSP toxicity determined in the non-native clam was considered as a function of increasing toxicity in the native species.

3. Results

3.1. DSP, ASP and PSP toxicity in native and non-indigenous clams from Ria de Aveiro

Data from the Portuguese Official Control reveal high levels of DSP toxicity in both species during 2014 and 2016, ranging from 15 to 482 µg OA eq. kg^{-1}. Fig. 2.2 illustrates the intensity of toxicity episodes registered for both species. This intensity is expressed by the ratio between the toxicity value and the regulatory limit, with values > 1 meaning the regulatory limit was exceeded. In this figure it is clearly shown that high DSP toxicity values were determined for native clam, which exceeded the Regulatory Limit (160 µg OA eq. kg^{-1}) in 13 samples. On the other hand, this limit was only reached by 10 non-indigenous clam samples. The significantly ($p < 0.05$) higher DSP toxicity levels observed in the native clam were strongly and positively correlated with levels found for the non-indigenous species ($y = 0.7373x + 19.147$, $R^2 = 0.6998$, $n = 88$).

Levels of ASP and PSP toxicity were markedly low (Fig. 2.2), in particular, if compared to levels of DSP toxicity. During the three-year period, 2014-2016, the ASP toxicity only exceeded the Regulatory Limit (20 mg DA kg^{-1}) once in early April 2014. However, according to the Official Control, only the non-indigenous species was analysed on this date (therefore not Included in this study, which only considers samples of both species collected in the same day). In the following week, when both species were analysed, similar levels of ASP toxicity were determined, presenting values well below the threshold level for human consumption. During 2015, a maximum toxicity level of 3.8 mg DA kg^{-1} was reported for native clam and always below quantification limit for non-indigenous species.

PSP toxicity was only reported in 2016 shellfish from Ria de Aveiro throughout the study period. During this period, the samples exceeded the Regulatory Limit (800 µg PSP eq. kg^{-1}) twice in December of 2016, reaching a maximum of 2330 µg STX eq. kg^{-1}. From the small data set, higher toxin concentrations were found in the native species.

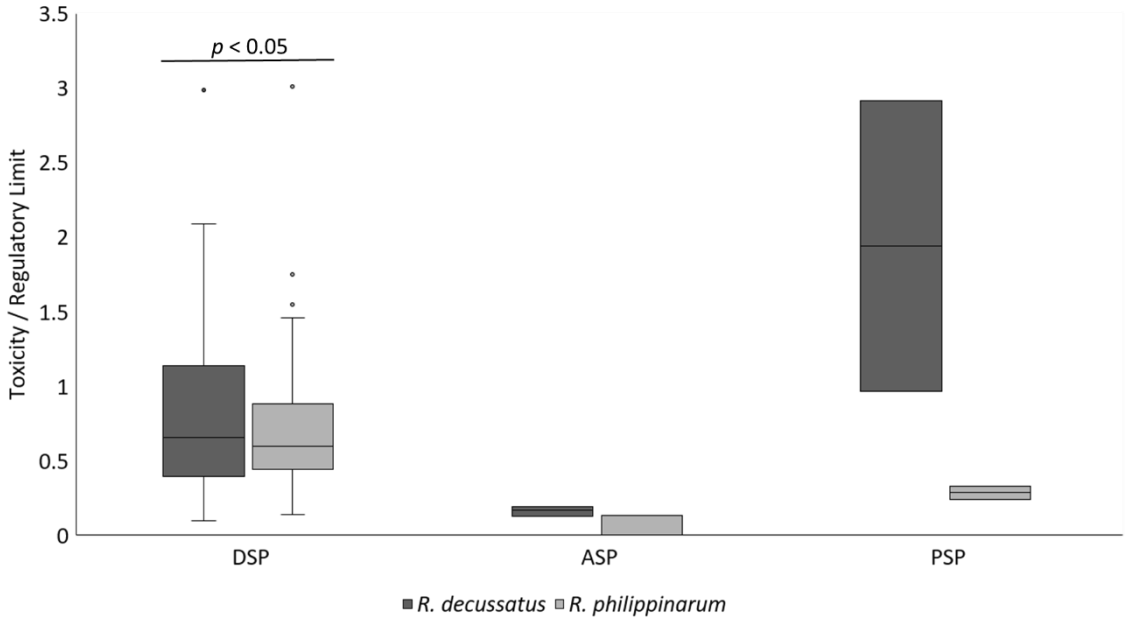

Fig. 2.2 Intensity of toxicity episodes expressed by the ratio between shellfish toxicity value and the regulatory limit for DSP, ASP and PSP, in native clam (*R. decussatus*) and non-indigenous clam (*R. philippinarum*), from Ria de Aveiro, Portugal, between January 2014 and December 2016, (data obtained form IPMA) (median, 25th and 75th quartiles, minimum, maximum and outliers, n = 88 for DSP, n = 3 for ASP and n = 2 for PSP). Significant differences between species display as $p < 0.05$.

3.2. Determination of regulated toxins in native and non-indigenous clams from Ria de Aveiro

DSP toxicity is caused by accumulation of OA-group toxins. Investigation of the toxin composition revealed okadaic acid (OA), 7-*O*-acyl fatty acid ester derivatives of OA, and, 7-*O*-acyl fatty acid ester derivatives of DTX2 in both species (Fig 2.3). The profiles of toxins were nearly identical showing similar esterification profiles. DTX3, that corresponds to a complex mixture of acyl ester derivatives of both OA and DTX2, was highly dominant (> 90 %) in the native and the non-indigenous clams. Small amounts of free OA were determined but free DTX2 was not observed (Fig 2.4).

Regarding the remaining lipophilic marine biotoxins, trace levels of YTX and PTX-1 were inconsistently found in both species, and AZAs were always below detection limit during the study period. As for the hydrophilic toxins, neither domoic acid nor saxitoxins were detected in the samples analysed during 2015.

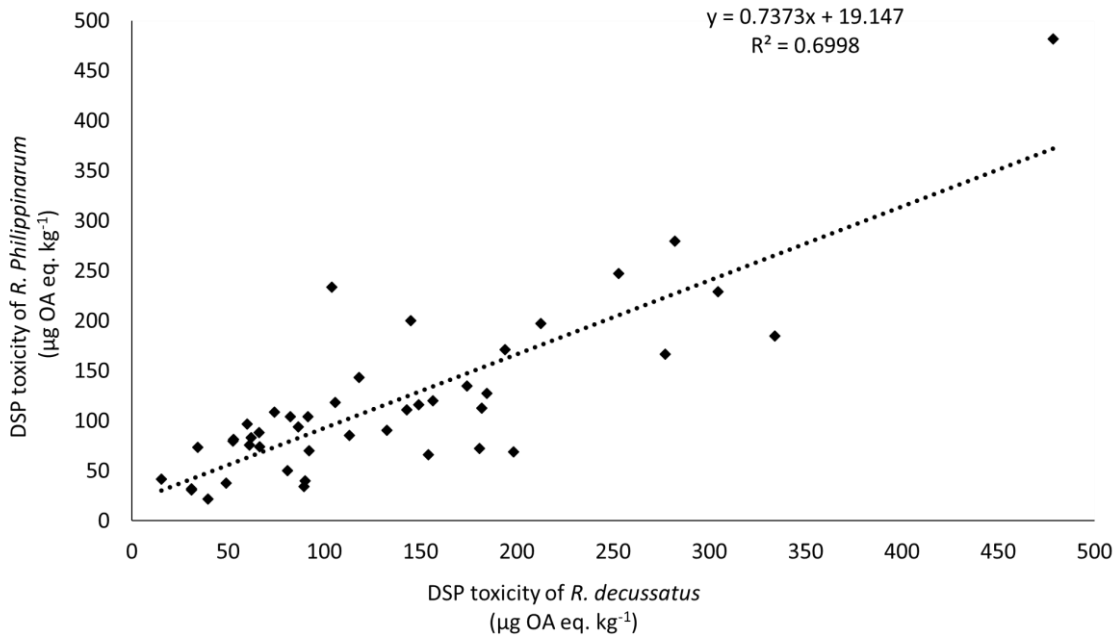

Fig. 2.3 Correlation between DSP toxicity (µg OA eq. kg^{-1}) in native clam (*R. decussatus*) and non-indigenous clam (*R. philippinarum*) from Ria de Aveiro, Portugal, between January 2014 and December 2016 (n = 88).

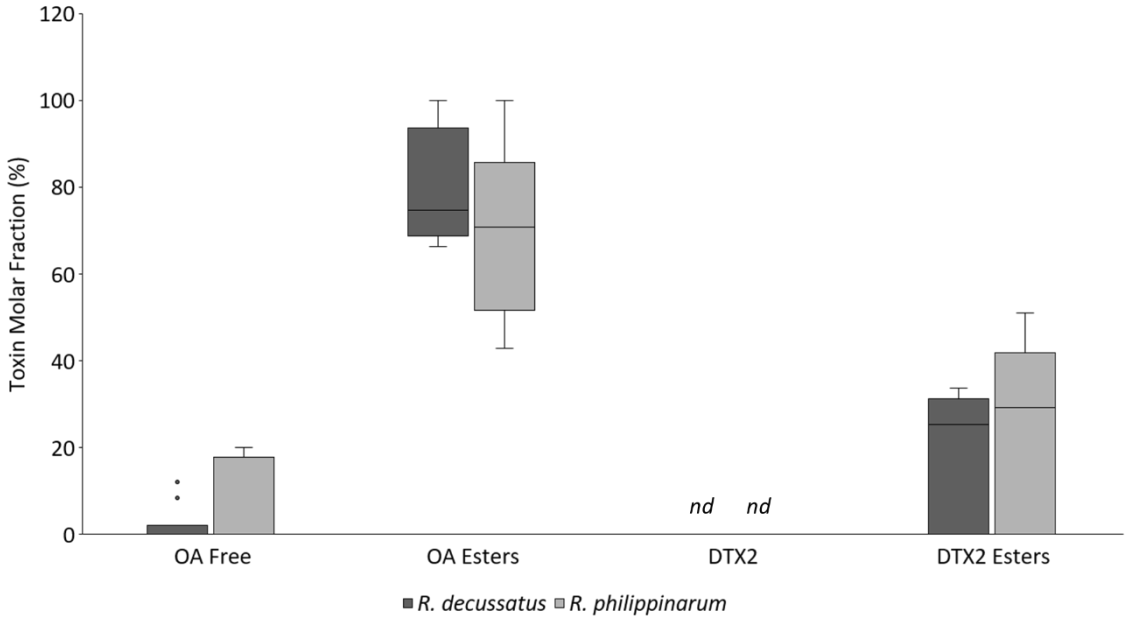

Fig. 2.4 Profile of OA-group toxins determined in *R. decussatus* and *R. philippinarum*, harvested during 2015 in Ria de Aveiro, Portugal (median, 25th and 75th quartiles, minimum, maximum and outliers, n = 20). nd - not detected.

3.3. Determination of non-regulated toxins in native and non-indigenous clams from Ria de Aveiro

BMAA was found in all samples of native and non-indigenous clam collected in 2015. The total amount of BMAA (i.e. the sum of the total soluble and protein-bound BMAA) ranged from 22 to 94 ng g^{-1} DW in *R. decussatus* and from 23 to 173 ng g^{-1} DW in *R. philippinarum*. Significant differences were found between the two species ($p < 0.05$), with higher BMAA concentrations in *R. philippinarum* than in *R. decussatus* was observed (Fig. 2.5). BMAA can be present in shellfish as free amino acid (quantified in this study as total soluble) or bound to proteins. In the collected samples, both free (i.e. total soluble) and protein-bound BMAA were detected in the native (≈ 51 % total soluble BMAA) and in the non-indigenous clams (≈ 54 % total soluble BMAA) without significant differences between the two species. BMAA isomers, namely AEG and DAB, were not detected in the analysed samples.

Tetrodotoxin and its derivatives were not detected in the native and non-indigenous clams analysed.

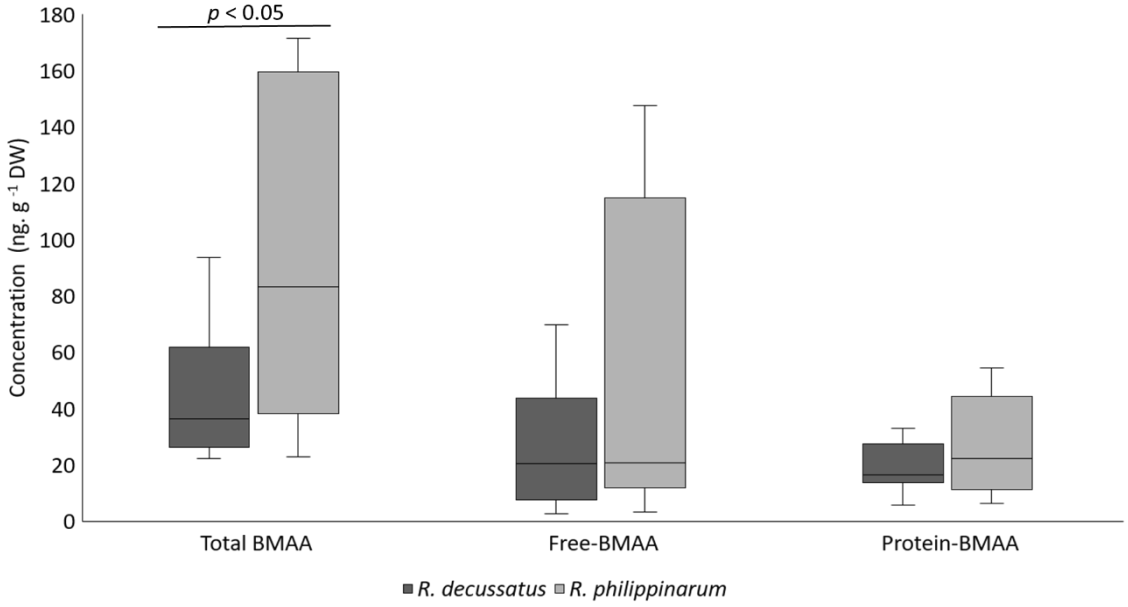

Fig. 2.5 Concentration (ng g-1 DW, Mean ± SD) of total BMAA, soluble BMAA and protein bound BMAA in the native clam (*R. decussatus*) and the non-indigenous clam (*R. philippinarum*, harvested in Ria de Aveiro, Portugal during 2015 (median, 25th and 75th quartiles, minimum and maximum, n = 20). Significant differences between species display as p < 0.05.

4. Discussion

This study focused on two clam species living in sympatry, one native (*Ruditapes decussatus*), with a declining stock, and the other non-indigenous (*R. philippinarum*) used by the shellfish industry to support irregular clam yields. Results of the present study indicate that consumption of the non-indigenous *R. philippinarum* is in general safer than consumption of the native clam *R. decussatus* in what regards to accumulation of regulated toxins, namely the lipophilic toxins of the OA group. However, since an effective monitoring program is currently running for these toxins, the contaminated samples are not allowed to reach the markets which ultimately reduce the risk for consumers to a minimum. On the other hand, the potential negative impact on the shellfish industry is higher for commercial farming of the native clam compared to the non-indigenous species, as prolonged closures of harvesting are foreseen in *R. decussatus* due to the higher DSP toxins accumulation (OA, DTX2 and their fatty acid ester derivatives).

Interestingly, the native and non-indigenous species showed significant differences in DSP toxicity levels, approximately 29 % higher in native species, despite having similar filtration rates, ranging from 1.2 to 6, and 1.5 to 5.1 dm^3 g^{-1} DW h^{-1}, respectively (Nakamura, 2001), and living in sympatry under the same conditions. While both species compete for the same resources, the non-indigenous species has a higher growth rate (Chessa et al., 2005; Solidoro et al., 2000; Usero et al., 1997), an increased tolerance to environmental changes, pollution and diseases (Karatayev et al., 2009; Moreira et al., 2012; Ricciardi and Rasmussen, 1998), and, as shown in the current study accumulates lower levels of marine toxins. This suggests that the non-indigenous clam is an important asset for aquaculture and intensive shellfish production.

Ria de Aveiro is a major shellfish production site in Portugal where the native clam is being replaced by the non-indigenous species in aquaculture farms due to reasons mentioned earlier. Nevertheless, studies elucidating the processes that lead to stress tolerance and ability to accumulate reduced levels of anthropogenic contaminants and natural toxins in *R. philippinarum* are scarce. Although higher levels of DSP toxicity are found in native clams, no differences in the profile of toxins, which was dominated (> 90 %) by esterified derivatives of OA and DTX2, were observed between the two species.

Since no differences were observed in the concentration of free OA, a similar toxin uptake is suggested. Free toxins are produced by toxic microalgae (e.g. *Dinophysis* spp.) and the ester derivatives are assumed to be a result of shellfish metabolism. Biotransformation of OA and DTX2 into 7-*O*-acyl fatty acid derivatives has been considered a pathway for toxins degradation and elimination (Rossignoli et al., 2011).

Since clams showed different toxicity levels, higher OA eq. concentrations in native species, it could be related with different rates of toxin biotransformation and their subsequent easier elimination in *R. philippinarum*. However, the clams that were under the same natural conditions at Ria de Aveiro did not show differences on the toxin profile. Therefore, further studies are needed to better understand the differences on bioaccumulation of OA-group toxins. It is also interesting to note that in freshwater environments, non-indigenous invasive species, such as *Dreissena polymorpha,* showed an increased ability to cope with microcystins, exhibiting a quicker and higher response in activation of biotransformation enzymes and a more effective cell protection against oxidative stress compared to the native species (Burmester et al., 2012).

Regarding the other regulated toxins, neither the lipophilic nor the hydrophilic were consistently detected in clams of Ria de Aveiro during the study period. However, investigation of non-regulated toxins revealed the presence of the neurotoxin BMAA in each sample analysed. Although at lower concentrations than previously detected in cockles (457 ± 186 ng g^{-1} DW) from Ria de Aveiro (Lage et al., 2014), BMAA was consistently detected in the native and non-indigenous clams collected for the present study. A tendency for higher BMAA accumulation was observed in the non-indigenous clam compared to native species. A possible reason for this tendency is the size of the BMAA producers especially if cyanobacteria are the source. While *R. decussatus* seems to be less efficient capturing smaller size particles, *R. philippinarum* can capture particles of the size range 2 - 200 µm with similar efficiency (Nakamura, 2001; Sobral and Widdows, 2000). Moreover, BMAA accumulation patterns might be dependent on other mechanisms, such as biotransformation, selective accumulation and/or depuration, which have already been reported for other well-known biotoxins (Kwong et al., 2006; Vale, 2006, 2004).

Although BMAA has been shown to be produced by cyanobacteria, diatoms and dinoflagellates (Paul Alan Cox et al., 2005; Jiang et al., 2014; Lage et al., 2014), the origin

of BMAA detected in marine molluscs is yet not clear (Lage et al., 2014; Réveillon et al., 2016, 2015). Ria de Aveiro is no exception; BMAA has been detected in cyanobacteria isolates from Ria de Aveiro (Baptista et al., 2011), however BMAA analyses were performed on HPLC with fluorescence detector, which has been reported to generate false positive response (Faassen, 2014). In addition, a previous study suggests the dinoflagellate *Gymnodinium catenatum* as the possible source of BMAA contamination in cockles growing in Ria de Aveiro (Lage et al., 2014). However, the presence of other BMAA producing algae species, such as diatoms, cannot be ruled out (Jiang et al., 2014). Therefore, contamination experiments in controlled conditions should be performed in order to support the transfer of BMAA from microalgae to shellfish. The BMAA neurotoxicity has been association to several neurodegenerative diseases, but a complete health risk assessment for this neurotoxin and baseline variability of BMAA in shellfish is still needed (Banack et al., 2007; Chiu et al., 2011; Paul Alan Cox et al., 2005).

Finally, it is important to note that tetrodotoxin, recently detected in bivalve molluscs from European coastal waters leading to urgent needs of toxin occurrence data to evaluate the risk for consumers, was not detected in clams from Ria de Aveiro.

In summary, in the interest of aquaculture farming, the replacement of the native clam *Ruditapes decussatus* by the non-indigenous species *R. philippinarum* is favoured based on lower DSP toxin accumulation by the non-indigenous clam, which, consequently leads to a lower number of precautionary days that shellfish harvesting may be banned. Nevertheless, higher levels of BMAA, which is not regulated in EU directives, therefore not leading to any temporary closures to harvesting, were observed in the non-indigenous species. A balance between the effective exploitation of shellfish resources and risk to consumers, as well as their confidence in the aquaculture products, should be obtained when decisions are to be made between cultivation of one or another species. In terms of scientific knowledge, this study highlights the need of future research to better understand the physiological responses of both species during blooms of toxic algae, the combined effect of multiple toxins accumulation and the processes that lead to differences on toxicity.

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Chapter 3

Invasive clams (*Ruditapes philippinarum*) are better equipped to deal with harmful algal blooms toxins than native species (*R. decussatus*): evidence of species-specific toxicokinetics and DNA vulnerability

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Invasive clams (*Ruditapes philippinarum*) are better equipped to deal with harmful algal blooms toxins than native species (*R. decussatus*): evidence of species-specific toxicokinetics and DNA vulnerability

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Abstract

This study aims to assess and compare the kinetics (accumulation/elimination) of the marine biotoxins okadaic acid (OA) and dinophysistoxin-1 (DTX1), between native (*Ruditapes decussatus*) and invasive (*Ruditapes philippinarum*) clam species, and their genotoxic effects and DNA recover capacity after, exposure to toxic dinoflagellates *Prorocentrum lima*. Clams were fed with *P. lima* for 5 days and then to non-toxic algae (post-exposure) during other 5 days. Toxin concentrations determined in clams by LC-MS/MS were related with DNA damage and repair assessment through the comet and base excision repair (BER) assays, respectively. Differential accumulation patterns were observed between the invasive and native species. The invasive species consistently and progressively accumulated the toxins during the first 24h of exposure, while the native clams showed drastic variations in the toxin accumulation. Nevertheless, at the end of a 5 days of exposure period, the native clams presented higher toxin concentrations, nearly reaching the legal regulatory limit for human consumption. In addition, native clams were vastly affected by OA and DTX1, presenting an increment in the DNA damage since the first day, with a correspondent increase in the repair activity. On the other hand, invasive clams were not affected by the dinoflagellate toxins, exhibiting only some

signs of the challenge, namely an increase in the DNA repair mechanisms in the post-exposure period. Invasive clams *R. philippinarum* are better adapted to cope with harmful algal blooms and OA-group toxins than native species. These results may increase farming interest and may lead to new introductions of the invasive clams. In sympatry sites, exposure to OA-group toxins may unbalance clams species biomass and distribution as exposure to toxic dinoflagellates affects the native clams from cellular to a population level, representing a significant threat to development and maintenance of *R. decussatus* populations.

Keywords: Marine toxins, Okadaic acid (OA), Dinophysistoxins; Genotoxicity; Comet assay; BER assay Ruditapes*;*

1. Introduction

The Manila clam*, Ruditapes philippinarum*, was deliberately introduced in Europe by shellfish producers due to its high growth performances and considerable economic value (Adams and Reeve, 1850). In the meanwhile, stocks of the native clam *Ruditapes decussatus* collapsed or are collapsing due to irregular yields and overexploitation (Chiesa et al., 2011; Flassch and Leborgne, 1992; Goulletquer, 1997).

The characteristics that made *R. philippinarum* so appealing for the shellfish industry in the early days, quickly became problematic when the high adaptation capacity and higher reproductive rate turned the species into an invasive organism. In many shellfish production farms over France and Italy, the native clam was almost completely replaced by *R. philippinarum* (Bidegain and Juanes, 2013; Blanchet et al., 2004; Dang et al., 2010). In other regions, such as certain production sites in Portugal and Spain, the invasive potential of *R. philippinarum* seems to be less pronounced as the two species appear to be able to coexist, even though *R. philippinarum* distribution continues to grow (Bidegain and Juanes, 2013; Velez et al., 2015c, 2015a).

Ruditapes philippinarum has been considered a generalist species when it comes to habitat requirements, presenting enhanced tolerance to environmental physical stress, organic and inorganic contamination, and bacterial infections (Bidegain et al., 2015; Figueira et al., 2012; Karatayev et al., 2009; Moreira et al., 2012). All these factors

contributed to the species high invasive potential. When the two clam species (*R. decussatus* and *R. philippinarum*) coexist, they seem to present different metabolic and biochemical responses to the same stressor (environmental or anthropogenic). For example, when exposed to metal contamination, different bioaccumulation patterns were observed. Higher levels of Cu, Zn, As, Cd and Hg in were observed in *R. decussatus*, whereas in *R. philippinarum* were higher for Ni and Pb (Figueira and Freitas, 2013). It has been reported that under exposure to low concentrations of Cd, *R. decussatus* accumulated higher levels, but they also had a higher metabolization capacity than *R. philippinarum* (Figueira et al., 2012). When exposed to Hg, the invasive species presented increased lipid peroxidation (LPO) levels in all metal concentrations tested. (Velez et al., 2015b). The metabolic responses appear to be both species- and metal-specific (Aru et al., 2016).

In addition to anthropogenic contaminants, clams are also recurrently exposed to natural toxins derived from harmful algal blooms (HAB). Field studies have shown that when living in sympatry, the two clam species presented correlated accumulation profiles with a tendency for significantly higher levels of okadaic acid (OA) toxins in the native species after exposure to blooms of toxic dinoflagellates *Dinophysis* spp. (Braga et al., 2017).

The OA group of toxins includes the OA, its isomers dinophysistoxins 1 and 2 (DTX1 and DTX2), plus a complex mixture of 7-*O*-acyl esters derivatives, which are the products of OA, DTX1 and DTX2 biotransformation through acylation with a fatty acid, collectively referred as DTX3 (Marr et al., 1992). These compounds are known to cause the diarrheic shellfish poisoning (DSP), characterised by gastrointestinal disorders with acute symptoms of diarrhoea, nausea, abdominal pain and vomiting (EFSA, 2008). To minimise the risk of acute human intoxications due to the consumption of contaminated shellfish, a regulatory limit of 160 µg of OA equivalents per kg of shellfish meat was established (EC, 2004b; EFSA, 2008). The toxins OA and DTXs are the most prevalent marine toxins in Southern Europe, being responsible for prolonged closures to shellfish harvesting (Braga et al., 2017).

Although the mode of action of OA and its effects on humans are well known, data concerning the impact of OA-group toxins in shellfish is still limited and mainly focussing on mussels and oysters (Prego-Faraldo et al., 2013; Valdiglesias et al., 2013). Bivalve

molluscs may have some resistance to these compounds (Svensson et al., 2003), but there are studies indicating that exposure to OA and DTX can cause genetic damage and promote severe structural abnormalities in the nucleus, impairing cell division, individual capacity for reproduction and, possibly, causing changes at the population level (Pinto-Silva et al., 2005, 2003). The intensity and type of harm appear to vary not only with the toxin concentration but also with the shellfish species, the analysed tissue and the experimental approach (*in vitro* or *in vivo*) (Flórez-Barrós et al., 2011; McCarthy et al., 2014; Prego-Faraldo et al., 2015, 2013).

Keeping in view the comparison between the native *Ruditapes decussatus* and invasive *R. philippinarum* clams, the present study aims to assess (i) the toxins accumulation and elimination patterns following exposure to the OA-group toxins-producing dinoflagellate *Prorocentrum lima* and (ii) the subsequent susceptibility to DNA damage, in counterpoint with (iii) the DNA damage repair capacity of each species, with the ultimate goal of providing new insights on the species-specificities towards the prediction of ecosystem functioning fragilities in the presence of invasive species and harmful algal blooms.

2. Methods

2.1. Clams collection and acclimation

Two hundred and six immature clams from each species, *viz*. the native Carpet Shell clam *Ruditapes decussatus* (28.9 ± 1.7 mm) from Ria Formosa and the invasive Manila clam *Ruditapes philippinarum* (30.4 ± 1.9 mm) from Sado Estuary, south and southwest of the Portuguese coast, respectively, were harvested in March 2018 during a period of no lipophilic toxicity. To ensure the absence of lipophilic toxins in the clam's samples from both species were analysed for toxin determination as described in point 2.4, and lipophilic toxins were not detected. Clams were shipped to IPMA facilities (Lisbon), checked for broken shells and cleaned from any sand or debris, and placed in four 150 L tanks. Each tank was equipped with a protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando

Ribeiro Lda, Portugal) to maintain seawater quality. The seawater used was filtered (0.35 µm) and UV sterilised.

The clams were maintained at a temperature of 18 °C and a pH of 8.0 and let to acclimatise for 19 days. The tanks were subdivided into replicates to allow exposure and control groups in triplicate. During acclimation, the clams were fed with approximately 200,000 cells/L^{-1} twice a day of the non-toxic freeze-dried *Tetraselmis* sp. (Necton, Olhão, Portugal). The following abiotic conditions were maintained in the tanks: *i*) dissolved oxygen (DO) > 5 mg L^{-1}; *ii*) salinity = 34 ± 0.7‰; *iii*) photoperiod = 12 L : 12 D. Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Ammonia, nitrite and nitrate levels were daily checked using colorimetric tests (Tropic Marin, USA), and kept below detectable levels (0.02 mg/L^{-1} for ammonia and nitrites, and 2.0 mg L^{-1} for nitrates) with daily water changes) with daily water changes, except nitrates, which were kept below 2.0 mg L^{-1}. A summary of seawater parameters during the experiment is reported in Fig. 1 of Supplementary Material (Annex B).

2.2. Prorocentrum lima cultivation

The benthic dinoflagellate *Prorocentrum lima* is a known producer of OA toxins, and it was chosen due to their feasibility to produce pure cultures and to their customary use in OA exposure studies (Flórez-Barrós et al., 2011; Pinto-Silva et al., 2005; Prado-Alvarez et al., 2013). The strain PL13v of *P. lima* isolated in 1996 in the Ria of Vigo, NW Iberian Peninsula, was kindly provided by Dr Paulo Vale (IPMA). Cells were cultured in 2 L flasks with seawater adjusted to 34 ‰, enriched with f/2 medium at 18 °C with a 12 L : 12 D cycle under fluorescent lights (Guillard, 1975; Guillard and Ryther, 1962). Cells were harvested during the exponential stage when cultures presented a density of approximately 3.4×10^7 cells per litre. The culture media was centrifuged for 10 minutes at 2000 *g* for cells collection and the pellets freeze-dried and stored at -20 °C.

The *P. lima* freeze-dried cells provided to clams contained OA (326.4 ± 23.2 µg g^{-1}) and DTX1 (165.1 ± 5.7 µg g^{-1}), which was determined by LC-MS/MS as described in section 2.4.

2.3. Exposure of clams to toxic dinoflagellates

Two experiments were performed:

Experiment 1: designed to assess the accumulation dynamics within a 24-hour timescale. A total of 90 clams of each species divided per 3 tank subdivisions of 5 L were used in this experiment. The total amount of toxic algae, 16 mg L^{-1}, was added at time 0 and samplings were performed after 0.5, 1, 2, 4, 6 and 24 hours of exposure. The *P. lima* freeze-dried cells were resuspended in seawater and vigorously mixed until no large fragments were present in the solution. This mix was then added to each tank. Toxins were determined as described in section 2.4.

Experiment 2: conceived to evaluate the toxin dynamics and the potential genotoxic effects over a prolonged period of exposure. A total of 116 clams of each species divided per 4 tank subdivisions of 5 L were used in this experiment. Clams were daily feed with 16 mg L^{-1} of freeze-dried toxic *P. lima* cells for 5 days and then with non-toxic freeze-dried algae *Tetraselmis* sp. (Necton, Olhão, Portugal) during another period of 5 days. Once a day the *P. lima* freeze-dried cells were resuspended in seawater and vigorously mixed until no large fragments were present in the solution. This mix was then added to each 5 L tank. The non-toxic diet was added twice a day, were 2 g of the commercial freeze-dried *Tetraselmis* sp. were resuspended in 1 L of seawater and vigorously mixed. From this stock solution, 42 mL were then added to each 5 L tank. The control group was fed throughout the experiment with the non-toxic diet. Clams of each species were sampled on days 1 and 5 corresponding to the initial (E1) and last (E5) day of exposure to toxic freeze-dried algae, as well as on days 6 and 10, that corresponds to days 1 (PE1) and 5 (PE5) of the post-exposure period. Toxins were determined as described in section 2.4, and genetic damage and the DNA repair were assayed as described in section 2.5.

2.4. Toxin analysis

2.4.1. Toxin extraction procedure

Extraction of toxins from *P. lima* freeze-dried cells was carried out by vortexing 0.05 g of algae in 5 mL of methanol. After centrifugation (10 minutes at 2000 *g*), the extract was filtered and stored at -20 °C until analysis.

For toxin extraction from the clams, in triplicated, five individuals were harvested at each sampling point were opened; the whole body removed from the shell, washed with running tap water to remove shell and sand residues, drained, immediately frozen in liquid nitrogen and stored until analysis. Later in the laboratory the samples were let to thawed and homogenised with a blender. A 2 g aliquot of clam tissue homogenate was extracted with 9.0 mL MeOH 100 % by vortexing for 2 min. After centrifugation at 2000 x *g* for 10 min, the resulting supernatant was transferred to a new 30 mL centrifuge tube. The remaining tissue was homogenised with a Polytron mixer (Kinematica, Switzerland) and re-extracted with 9.0 mL MeOH. After centrifugation, supernatants were combined, and the volume adjusted to 20 mL with MeOH. An aliquot of this extract was filtered through a 0.2 µm syringe filter, and 10 µL injected to the LC-MS/MS system for determination of free OA, DTX1 and DTX2 (EURLMB, 2015).

2.4.2. Hydrolysis of DTX3

Due to the lack of reference standards for the multiple 7-*O*-acyl ester derivatives (DTX3), an alkaline hydrolysis step is carried out to convert these compounds into their respective parental toxin. The hydrolysis is started by adding 125 µL of 2.5 M NaOH to a 1 mL aliquot of the sample extract in a test tube, which was homogenised for 30 s in the vortex and heated at 76 °C for 40 minutes in a heating block. The sample was let to cool down until reaching room temperature and neutralised with 125 µL of 2.5 M HCl. The sample was vortex for 30 s, and an aliquot was filtered through a 0.2 µm syringe filter, and 10 µL injected to the LC-MS/MS system(EURLMB, 2015).

2.4.3. Toxins determination by LC-MS/MS

Determination of OA-group toxins via liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection using Agilent 1260 Infinity LC system coupled to a Sciex Qtrap 4500 mass spectrometer was carried out following the Standardised Operating Procedure (SOP) of the European Reference Laboratory for Marine Biotoxins (EURLMB) for the determination of marine lipophilic biotoxins in bivalve molluscs (EURLMB, 2015) The LC-MS/MS equipment consisted of an Agilent 1260 Infinity LC system coupled to a Sciex Qtrap 4500 mass spectrometer. The chromatographic separation was performed using an XBridge BEH C18 2.5 µm 2.1 × 50 mm Column XP (Waters, Ireland).

The conditions of the LC-MS/MS analysis are summarised in the Supplementary Material (Table S1)

The detection of the toxins involved, was carried out in Multiple Reaction Monitoring (MRM) acquisition mode. Two MRM transitions were monitored in negative polarity: m/z 803.358 > 255.000 for OA and DTX2 quantification and m/z 803.358 > 112.700 for confirmation; *m/z* 817.380 > 255.000 for DTX1 quantification and m/z 817.380 > 112.700 for its confirmation. Calibration standard solutions included the following concentrations range levels: 0.5, 1.0, 5.0, 10.0, 20.0 and 40.0 ng mL^{-1}. The detection quantification limit (LQ) to 5 µg kg^{-1} for OA, DTX1 and DTX2. The toxicity equivalent factors (TEFs) stated by EFSA (2008) were used for calculation of OA-group toxins in terms of okadaic acid equivalents: OA = 1, DTX1 = 1, DTX2 = 0.6. For the 7-*O*-acylderivatives, TEFs corresponding parent toxins were used (EFSA, 2008).

The total toxin content was expressed as indicated in the EU Legislation (µg OA eq./kg) taking into account the TEF values for each particular toxin. 1) The total concentration of okadaic acid was determined in the hydrolyzated extracts, corresponding to the sum of free and esterified toxins determined after hydrolysis. 2) Free forms were determined in the raw extracts before hydrolysis. 3) The esterified toxin concentrations were calculated as the difference between total toxin and free toxin concentrations.

2.5. Evaluation of genetic damage and oxidative DNA damage repair

2.5.1. Sample collection and tissues preparation

The seven clams harvested on each sampling date were dissected and the hepatopancreas collected for genetic damage and oxidative DNA damage repair assessment. The hepatopancreas was divided into subsamples, and the section intended for genetic damage evaluation was slowly frozen in an antifreeze solution of PBS and DMSO (90:10, v:v) and kept in thermal insulation material, initially at -20 °C for 1 week and then at -80 °C until analysis. The hepatopancreas subsamples collected for oxidative DNA damage repair analyses (Base Excision Repair assay - BER assay) were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

2.5.2. Comet assay

Genotoxicity caused in the hepatopancreas by exposure to the OA-toxins producer *P. lima* was assessed by the comet assay. The alkaline version of this methodology was performed following Collins (2004) procedures with Guilherme et al. (2010) and Marques et al. (2016) modifications. Briefly, a portion of hepatopancreas was cut in a 2 mL PBS bath (pH 7.4; 0,01 M) to form a suitable cellular suspension. One mL of cell suspension in PBS was collected per sample and centrifuged at 200 × *g* , at 4 °C, for 5 minutes. The supernatant was discarded (990 µL) and the pellet resuspended in a new PBS (1 mL). Again, the cell suspension was centrifuged and 990 µL of the supernatant discarded. To the remaining 20 µL of cell suspension, 70 µL of 1 % agarose LMP (in PBS) was added. From this solution, two mini-gels, with 6 µL of cell suspension, were placed onto an agarose NMP pre-coated slide and refrigerated for 5 minutes at 4 °C. A system of eight gels per slide, with two replicates per sample, was adopted in order to improve the assay output.

The slides were then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1 % Triton X-100, and 10 % of DMSO, pH 10) at 4 °C, for at least 1 h, in the dark. To allow the discrimination between oxidative and non-specific DNA damage, a total of three sets of slides were prepared. From these slide sets, two were incubated with the DNA lesion-specific repair endonucleases for oxidative DNA damage detection. The

endonucleases EndoIII (endonuclease III) and FPG (formamidopyrimidine DNA glycosylase) were used for oxidised pyrimidine and purine detection, respectively. The third set was incubated with the enzyme buffer for non-specific DNA damage detection. To each slide set, 30 µL of enzyme or buffer were added per each mini-gel, coverslips were applied, to minimise evaporation, and the slides were incubated at 37 °C for 30 minutes in a humidified heater.

After incubation, the coverslips were removed, and slides were moved to the electrophoresis tank (Sub-Cell® GT, Bio-Rad), and immersed in the electrophoresis solution, for 20 min, for alkaline treatment. The DNA migration was performed per 15 minutes, at a fixed voltage of 25 V, a current of 300 mA (power supply PowerPac™, Bio-Rad), which results in 0.7 V cm^{-1} (achieved by adjusting the total volume of buffer). The slides were then neutralised in PBS for 10 minutes at 4 °C, followed by 10 minutes at 4 °C in distilled water and 10 minutes in ethanol (100 %) at 4 °C, and left to dry at room temperature overnight.

2.5.3. Base Excision Repair (BER) assay

The BER assay was performed as described in Marques et al. (2016) with slight modifications. Briefly, the comet assay was applied to substrate cells treated with an oxidative lesion-inducing agent and submitted to a cell-free extract obtained from the tissue sample to test. The cell-free extract initiates the DNA repair pathway, creating additional breaks in the DNA of the substrate cells. These additional breaks represent the cell-free extract capacity for DNA repair.

Cell-free extract preparation

The cell-free extracts were obtained by homogenising a portion of the hepatopancreas (approximately 50–100 mg) in 1 mL of extraction buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10 % glycerol, adjusted to pH 7.8 using 6 M KOH) diluted in distilled water (1:3) using a Potter-Elvehjem homogenizer. From the resulting cell suspension, 500 µL were collected, snap-frozen in liquid nitrogen, thawed, and mixed with 150 µL of a solution of Triton X-100 in extraction buffer (1:100). This

solution was then centrifuged at 11,200 *g* for 5 minutes at 4 °C, to remove nuclei and cell debris, and the supernatant collected. Total protein concentration was determined following the Biuret method (Gornall et al., 1949), and the final extract was obtained after dilution to a protein concentration of 0.3 mg mL^{-1}.

Substrate cell preparation

Hepatopancreas cells collected from *Ruditapes decussatus* and *R. philippinarum*, specimens harvested within 24 h from the Ria de Aveiro, when no harmful algal blooms were not occurring, presenting low DNA damage values (GDI baselines: *R. decussatus*, 92 ± 1 and *R. decussatus*, 96 ± 2), were used as substrate. The cellular suspension of 9 individuals was pooled and kept in ice until use. A system of 4 mini-gels with 6 µL of cellular suspension per slide was adopted in order to improve the assay output, preventing the cross-contamination of the cell-free extract in the slides.

After lysis, each mini-gel was treated with 30 µL of paraquat (PQ; 0.05 µM) and covered with a coverslip, for 15 minutes at 4 °C, to induce standard oxidative DNA damage. The PQ-treated slides were then washed twice with enzyme buffer.

Substrate cell exposure to cell-free extracts

To determine the repair enzymes activity, 20 µL of the cell-free extract was applied to each mini-gel in the substrate cells slides and covered with a coverslip. The slides were then incubated at room temperature, approximately 20 °C (adjusted to the shellfish body temperature) in a humidified chamber, for 30 min.

The enzymatic reaction was stopped by thermal shock, with the slides being placed on a cold plate over ice. The slides were then immediately placed in the electrophoresis solution for the alkaline treatment, and subsequent steps occurred as previously described (see Section 2.5.2).

Control slide sets for damage baselines determination

Two control slide sets, with a system of 8 mini-gels per slide, were prepared.

A first set of slides composed by undamaged substrate cells (no PQ treatment) were incubated with: (1) the enzyme buffer, to provide the DNA damage baseline of the substrate cells; (2) the enzyme FPG, to establish the initial DNA oxidation; (3) the extraction buffer plus Triton X-100, to assess that this control solution does not cause DNA damage.

A second set of slides treated with PQ were incubated with: (4) the enzyme buffer, to establish damage caused by PQ; (5) the enzyme FPG, to determine DNA oxidation caused by PQ; (6) the extraction buffer plus Triton X-100, to confirm that these solutions does not increase the DNA damage. Both slide sets were incubated at 37 °C.

2.5.4. Nucleoids counting and DNA damage quantification

Slides of both comet and BER assays were stained with ethidium bromide (20 μg mL^{-1}) and observed using a Leica DMLS fluorescence microscope (x 400 magnification). Visual classification of 50 nucleoids, per mini-gel, into five comet classes, according to the tail intensity and length (0 - no tail; 4 - almost all DNA in tail) was performed to quantified DNA damage (Collins, 2004).

In the comet assay, Genetic Damage Indicator (GDI) was calculated according to the formula:

$$\mathrm{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

where *i* is the number of each defined class (ranging within 0-4).

GDI values were expressed as “arbitrary units” in a range of 0–400 per 100 scored nucleoids (the average value for the two mini-gels observed per sample). In order to assess the DNA breaks corresponding to net enzyme-sensitive sites (NSS), the GDI in slides treated with FPG and EndoIII, corresponding to the parameters GDI_{FPG} and GDI_{EndoII}, as well the slides treated with the enzyme buffer were scored. The enzyme buffer slides scores were subtracted to GDI_{FPG} and $GDI_{EndoIII}$ values to obtain NSS_{FPG} and $NSS_{EndoIII}$, net enzyme-sensitive sites for FPG and EndoIII, respectively.

In the BER assay, the slides were scored for GDI as described above. The two previously described sets of control slides (treated and no-treated whit PQ) plus the two sets of slides exposed to the cell-free extract were scored. For BER sites determination, the PQ-induced damage was subtracted from the overall DNA breaks caused by PQ treatment plus the cell-free extract incubations. To the overall GDI values (the slides treated with PQ and the samples cell-free extracts) the control slide GDI (treated with PQ and the control solution - control 6) was subtracted, quantifying the cell-free extract-induced breaks.

2.6. Statistics

Statistica 7.0 software was used for statistical analysis. Data obtained in the different sets of the two assays were tested for outliers through the Grubbs Statistic Test and to normality through the Shapiro-Wilks test in order to meet the statistical assumptions. When the statistical assumptions were not satisfied, the data were transformed. The different sets of data were analysed within the exposure period or within the post-exposure phase. A two-way ANOVA followed by Tukey HSD test as post-hoc comparison tests were applied to assess the effects of each factor and their interaction. The factors considered were “Treatment” (clams exposed to toxic algae, and negative control group), and “Time” [Exposure for 1 (E1) or 5 days (E5), and Post-exposure for 1 (PE1) or 5 days (PE5)]. Statistical differences were considered significant at $p < 0.05$ (Zar, 1996).

Statistical results are presented in the respective figures with (*), (a) and (b), the complementary tables containing the Two-way ANOVA summaries were presented in the Supplementary Material (Annex A: Tables S2 to S7).

3. Results

3.1. Experiment 1: 24 hours of exposure to P. lima

3.1.1. Toxin accumulation in the native clam R. decussatus

Highly variable levels of toxins were determined in native clam *R. decussatus* during the 24 h period (Fig. 3.1a). At the first 30 min of exposure to toxic *P. lima*, clams presented a total toxin concentration (Total) of 37.8 µg OA eq. kg^{-1}. The profile observed at this initial sampling point was dominated by the esterified derivatives (DTX3) constituting 55.9 (± 18.2) %. However, following 1 hour of exposure, a marked decreased of toxins concentration to 11.7 µg OA eq. kg^{-1} was observed, being the profile of toxins completely dominated (100 %) by the esterified derivatives. At 4 hours, the toxin concentration increased to 70.4 µg OA eq. kg^{-1}, with DTX3 representing 97.5 (± 3.5) % of the toxin in the clams. Thereafter, toxin concentration deeply decreased to 3.0 µg OA eq. kg^{-1} at 6 hours of exposure, but increased again to 21.4 µg OA eq. kg^{-1} with the totality to the toxins in the esterified form at the end of the 24 h period of exposure to *P. lima*.

3.1.2. Toxin accumulation in the invasive clam R. philippinarum

Contrasting with the native clam, the invasive *R. philippinarum* showed a progressive accumulation of the toxins after the first hours of exposure, even though fluctuating levels were observed within the first hour. At the first 30 minutes of exposure, a concentration of 79.0 µg OA eq. kg^{-1} was determined and the profile of toxins was dominated (62.3 ± 4.2 %) by DTX3 (Fig. 3.1b). Then, toxin concentration decreased to 41.1 µg OA eq. kg^{-1} with the esterified forms increasing to 70.0 (± 4.4) %. During the subsequent sampling points there was a gradual increase of the toxin concentration, from 53.0 µg OA eq. kg^{-1} after 2 hours, to 62.4 µg OA eq. kg^{-1} after 4 hours, and 66.0 µg OA eq. kg^{-1} at 6 hours of exposure. DTX3 dominated the toxin profile varying from 82.9 % to 86.7 %. After 24 hours of exposure, the toxin concentration determined in *R. philippinarum* was 54.5 µg OA eq. kg^{-1} with the total amount of toxins in the esterified form.

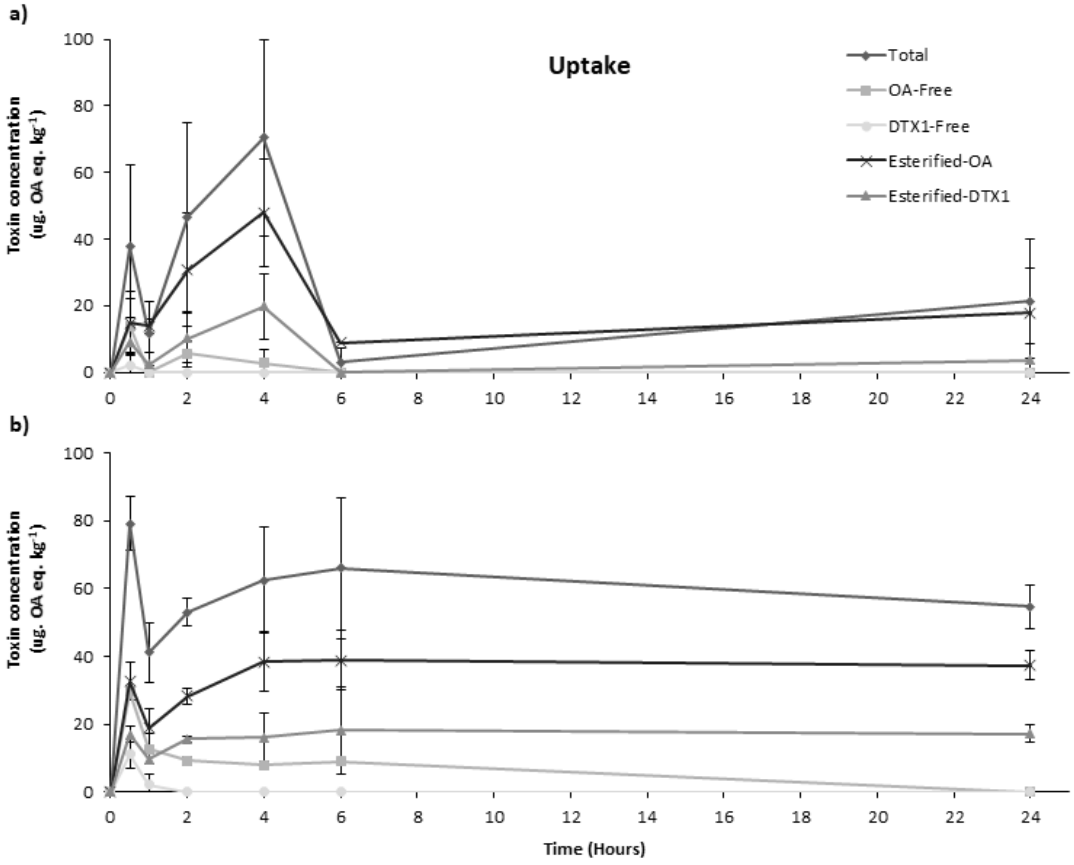

Fig. 3.1 Experiment 1 - Toxin concentration (µg OA eq. kg^{-1}, mean ± SD) determined in soft whole-body of a) native *Ruditapes decussatus* and b) invasive *Ruditapes philippinarum*, after 0.5, 1, 2, 4, 6 and 24 hours of exposure to the toxic dinoflagellate *Prorocentrum lima*. "Total" corresponds to the total toxin concentration, the sum of free and esterified toxins; "OA-Free" and "DTX1-Free" correspond to the concentration of OA and DTX1 in the parental conformation; while "OA-Esterified" and "DTX1-Esterified" correspond to the concentration of OA and DTX1 in the esterified conformation.

3.2. Experiment 2: 5 days of exposure to P. lima and post-exposure

3.2.1. Dynamics of toxin accumulation and elimination in the native clam R. decussatus

At the end of the first day of exposure to the toxic dinoflagellates (day 1, E1), the total amount of toxins accumulated by the native species were in the esterified form. 7-*O*-acyl derivatives of both OA and DTX1 were present, reaching a concentration of 56.5 µg OA equiv. kg^{-1} (Fig. 3.2a). After 5 days feeding on toxic freeze-dried *P. lima* (day 5, E5), the maximum concentration of this study was determined, reaching 158.9 µg OA

eq. kg^{-1}. Toxins in the parental conformation corresponded only to approximately 0.2 %, with OA being the only compound detected in this form. The concentration of DTX3 reached 156.4 µg OA eq. kg^{-1} and was mostly composed by fatty acid ester derivatives of OA (77.3 %), and 22.5 % derived from DTX1 esterification.

After changing the diet to non-toxic algae, *Tetraselmis* sp. (day 6, PE1), a slight decrease of toxin concentration was observed, with elimination of 1.6 % over the level displayed on the day before. However, on the last day of post-exposure (day 10, PE5), nearly 84.4 % of the toxins were eliminated. At this time point, the concentration of toxins reached only 24.8 µg OA eq. kg^{-1} and the toxin profile was exclusively constituted by the esterified forms (DTX3).

3.2.2. Dynamics of toxin accumulation and elimination in the invasive clam R. philippinarum

After 1 day of exposure to *P. lima* (E1), total toxin concentration reached 74.0 µg OA eq. kg^{-1}, with the esterified forms representing 95 % of the toxin profile (Fig. 3.2b). At day 5, which corresponds to the peak of toxins accumulation, a concentration of 105.5 µg OA eq. kg^{-1} was determined. This value is the sum of free OA, 4.5 µg OA kg^{-1}, and the esterified forms of OA and DTX1 (101.1 µg OA eq. kg^{-1}). The free forms represented 3.5 % of the toxin profile, while esterified OA represented 65.5 % and esterified DTX1 corresponded to 31.0 %.

Once the diet of clams was changed to non-toxic algae (day 6, PE1), only esterified compounds were detected, reaching a concentration of 64.4 µg OA eq. kg^{-1}, which corresponded to the elimination of 39.0 % of the toxins. At the end of 5 days feeding on a non-toxic diet (day 10, PE5), the overall concentration in *R. philippinarum* decreased nearly 76.5 % to 24.8 µg OA eq. kg^{-1}.

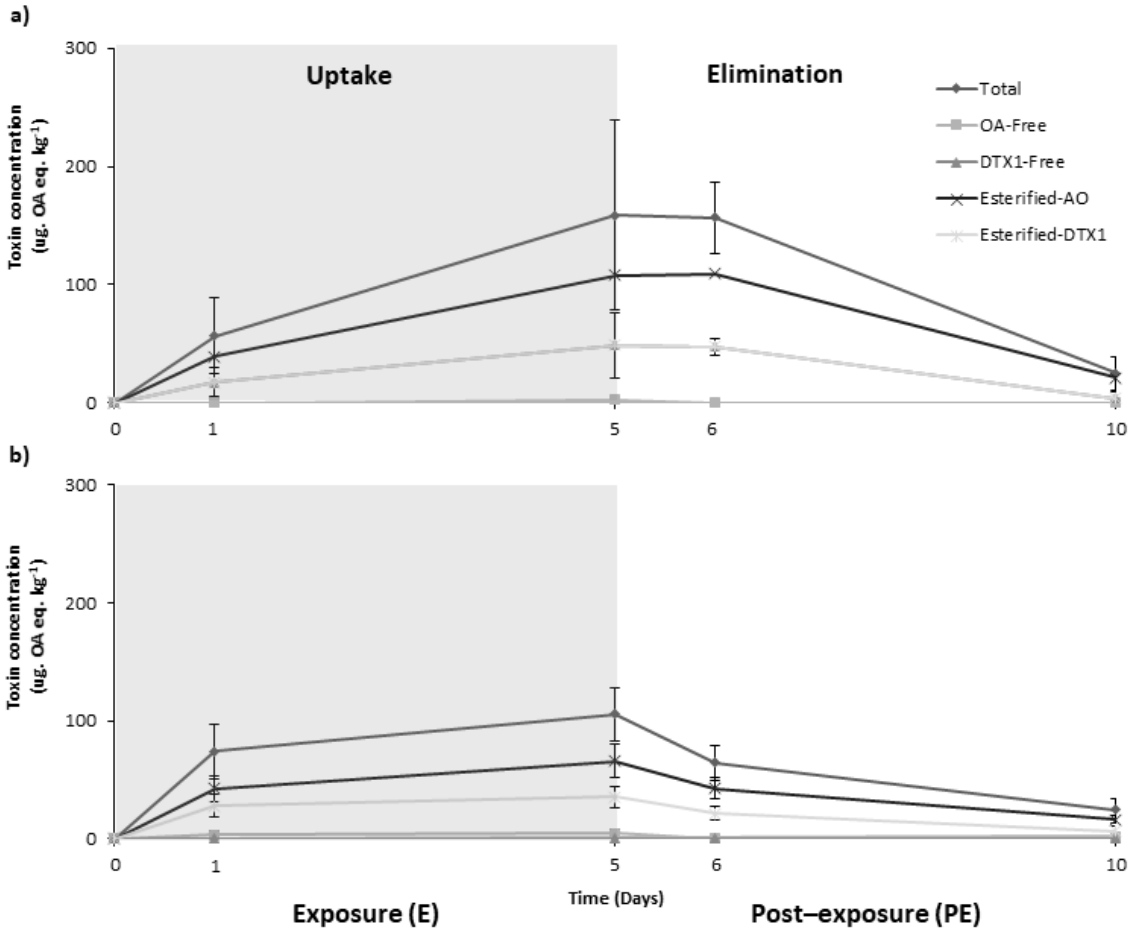

Fig. 3.2 Experiment 2 - Toxin concentration (µg OA eq. kg^{-1}, mean ± SD) determined in soft whole-body of a) native *Ruditapes decussatus* and b) invasive *Ruditapes philippinarum*, in days 1 and 5 of exposure to the toxic dinoflagellate *Prorocentrum lima* as well as the subsequent days 1 and 5 of post-exposure, with a non-toxic diet, for toxin elimination. "Total" corresponds to the total toxin concentration, the sum of free and esterified toxins; "AO-Free" and "DTX1-Free" correspond to the concentration of OA and DTX1 in the parental conformation; while "OA-Esterified" and "DTX1-Esterified" correspond to the concentration of OA and DTX1 in the esterified conformation.

3.2.3. Genotoxicity assessment in the clams' hepatopancreas

3.2.3.1. Effects in the native clam R. decussatus

Non-specific DNA damage

A clear impact derived from the exposure to the toxic dinoflagellate *P. lima* was observed in native clams *R. decussatus*, as expressed by the GDI values (Fig. 3.3a, Table S2). The GDI damage significantly increased during both exposure and post-exposure periods in comparison with clams exposed to non-toxic algae. However, no significant effects were observed over time during the exposure period, as GDI values were not different from E1 to E5. A significant decrease in GDI values was observed throughout the post-exposure period, with differences observed between PE1 and PE5 in both control and exposed clams.

Oxidative DNA damage

Detection of oxidised bases was accomplished through the slide sets incubated with DNA lesion-specific repair enzymes EndoIII and FPG. Regarding EndoIII, despite an overall increase in the DNA damage recorded in the exposed *R. decussatus*, no apparent effect of OA-group toxins was observed in the combined oxidative and non-specific damage ($GDI_{EndoIII}$) (Fig. 3.3b, Table S3). Nevertheless, a significant decrease of the EndoIII net enzyme-sensitive sites ($NSS_{EndoIII}$) was found at 5 days of exposure to the toxic algae (Fig. 3.3d, Table S4).

The GDI_{FPG} data presented a significant decrease in the damage observed in both exposed and control groups on E5 comparing to E1 (Fig. 3.3c, Table S5), but a significant increase in the last day of the experiment was reported for the control group (PE5) when compared to the first day of post-exposure (PE1). Assessing the toxin effect for this parameter, a significant increase in the damage was observed on the first day of post-exposure (PE1). The FPG net enzyme-sensitive sites (NSS_{FPG}) did not present any significant change during the entire experiment (Fig. 3.3e, Table S6).

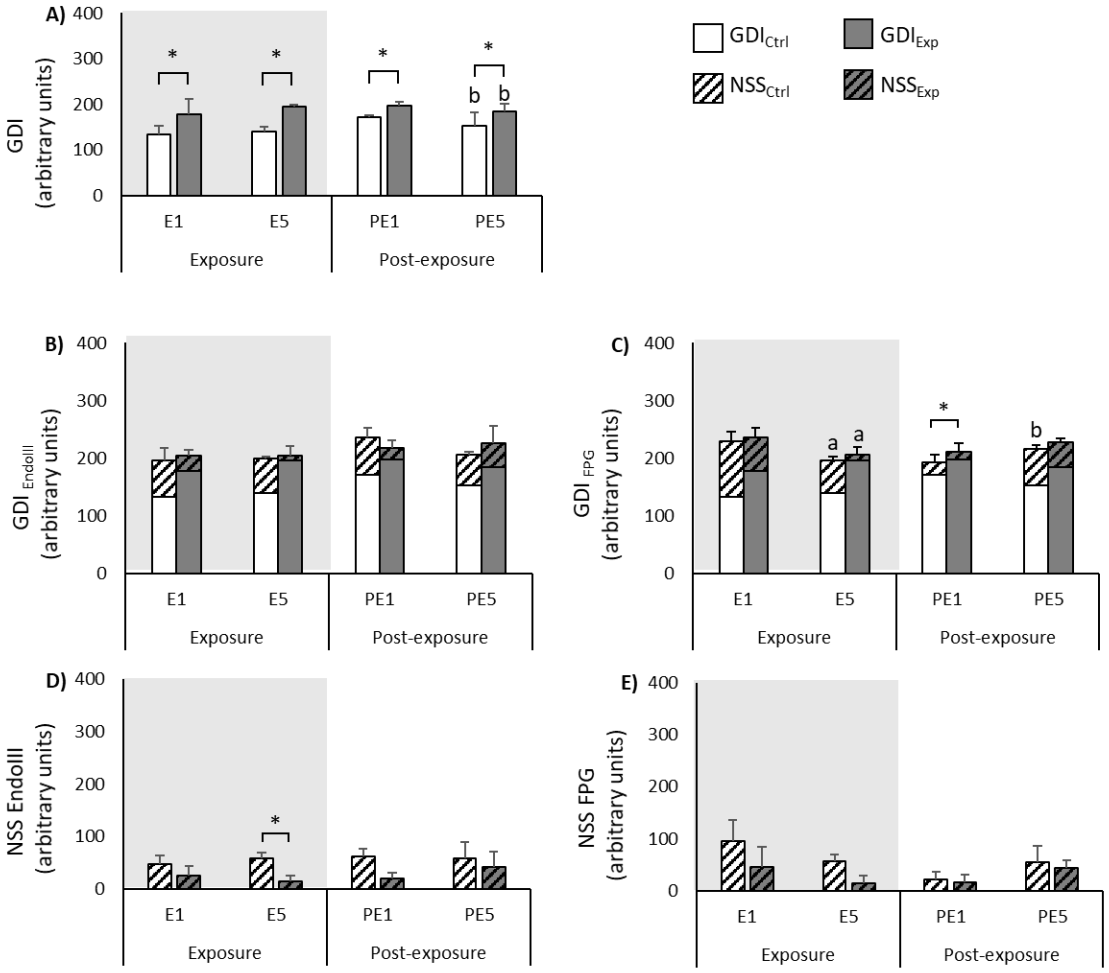

Fig. 3.3. Experiment 2 - DNA damage expressed through the genetic damage indicator (GDI) (mean ± SD, arbitrary units) in hepatopancreas of the native clam *Ruditapes decussatus* after days 1 and 5 (E1 and E5) of exposure to *Prorocentrum lima* as well as after days 1 and 5 (post-exposure; PE1 and PE5) feeding on non-toxic algae: A) non-specific DNA damage obtained by standard (alkaline) comet assay in control (in white) and exposed groups (in grey); oxidative DNA damage measured by the comet assay with the extra step of incubation with B) endonuclease III ($GDI_{EndoIII}$) and C) formamidopyrimidine DNA glycosylase (GDI_{FPG}), with partial scores shown, namely GDI (in full colour) and the DNA breaks resulting from enzyme net sensitive sites (NSS, in black dashed); D) magnifies the $NSS_{EndoIII}$ and E) NSS_{FPG} bars for clarity purposes. Significant differences ($p < 0.05$) are marked with (*) between control and exposed groups within the same point in time, while (a) and (b) point out differences between times within exposure and post-exposure, respectively.

3.2.3.2. Effects in the invasive clam R. philippinarum

Non-specific DNA damage.

No clear impact derived from the exposure to the toxic dinoflagellates *P. lima* was observed in the invasive clams *R. philippinarum*, as expressed by the GDI values (Fig. 3.4a, Table S2). The only significant difference observed was a decrease in the GDI throughout the exposure period (E1 *vs.* E5) affecting both control and exposed groups. In the post-exposure, no significant differences were observed either between control and exposed groups or over time (PE1 *vs.* PE5).

Oxidative DNA damage

Regarding the $GDI_{EndoIII}$ parameter, no significant changes were observed in *R. philippinarum* during the toxin uptake period (Fig. 3.4b, Table S3). However, a significant increase in the $GDI_{EndoIII}$ was found between the exposed groups throughout the post-exposure period (PE1 *vs*. PE5). A significant decrease in the EndoIII net enzyme-sensitive sites ($NSS_{EndoIII}$) was observed in clams exposed to the toxic algae during the uptake period (E1 and E5; Fig. 3.4d, Table S4) when compared with the respective control, but no changes were observed in the post-exposure period. Throughout the exposure period (E1 *vs*. E5), the $NSS_{EndoIII}$ sites significantly increased in both control and exposed groups.

Exposure to *P. lima* did not affect either GDI_{FPG} or NSS_{FPG} (Fig 3.4c, e; Table S5, 6) in the invasive clams.

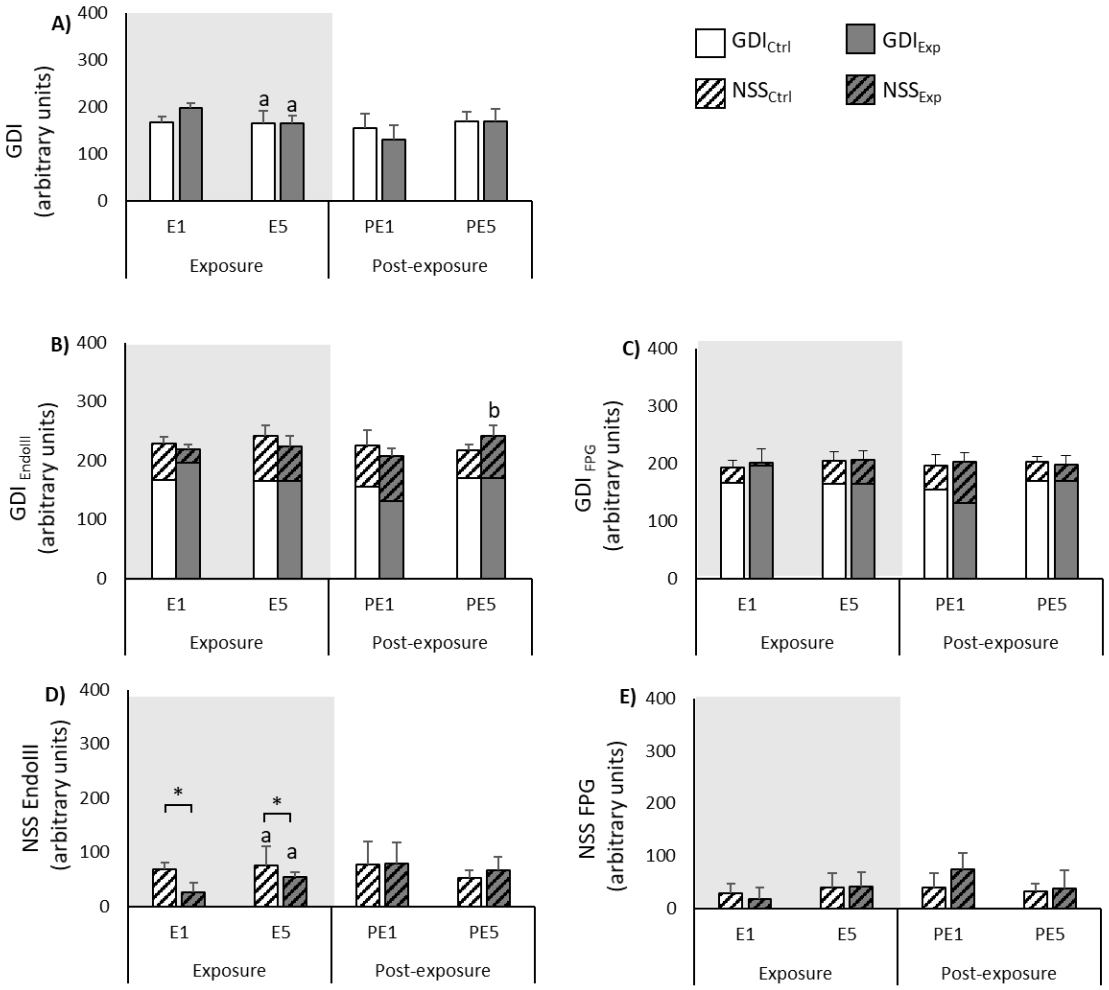

Fig. 3.4 Experiment 2 - DNA damage expressed through the genetic damage indicator (GDI) (mean ± SD, arbitrary units) in hepatopancreas of the native clam *Ruditapes philippinarum* after days 1 and 5 (E1 and E5) of exposure to *Prorocentrum lima* as well as after days 1 and 5 (post-exposure; PE1 and PE5) feeding on non-toxic algae: A) non-specific DNA damage obtained by standard (alkaline) comet assay in control (in white) and exposed groups (in grey); oxidative DNA damage measured by the comet assay with the extra step of incubation with B) endonuclease III ($GDI_{EndoIII}$) and C) formamidopyrimidine DNA glycosylase (GDI_{FPG}), with partial scores shown, namely GDI (in full colour) and the DNA breaks resulting from enzyme net sensitive sites (NSS, in black dashed); D) magnifies the $NSS_{EndoIII}$ and E) NSS_{FPG} bars for clarity purposes. Significant differences ($p < 0.05$) are marked with (*) between control and exposed groups within the same point in time, while (a) and (b) point out differences between times within exposure and post-exposure, respectively.

3.2.4. DNA repair assessment in clams' hepatopancreas

3.2.4.1. Effects in the native clam R. decussatus

The native clam, when exposed to the toxic algae (E1 and E5), presented a significant increase in DNA breaks caused by the cell-free extract, indicating a significant increase in DNA repair capacity (Fig. 3.5, Table S7). A similar increasing pattern was observed after the first day of toxin elimination (PE1). However, at the end of the post-exposure period (PE5), this effect was not noticed.

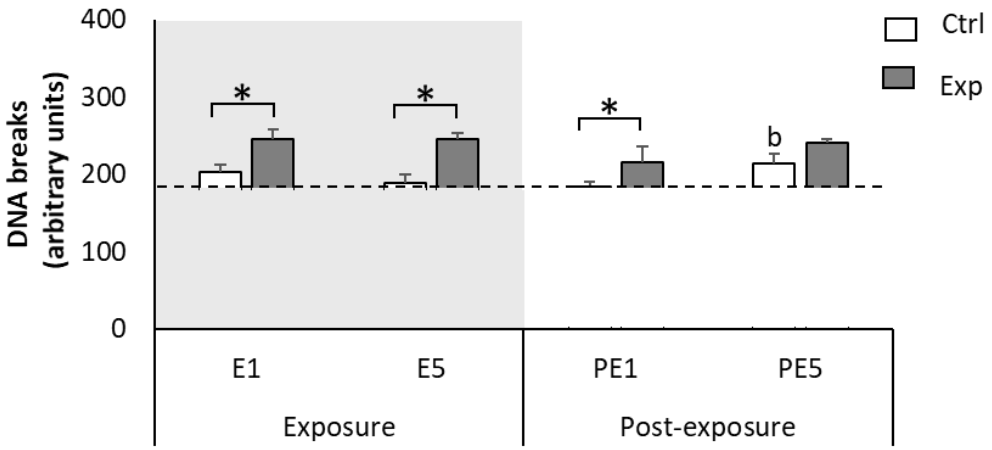

Fig. 3.5. Experiment 2 - DNA breaks after base excision repair (BER) assay of *Ruditapes decussatus*. Breaks (mean ± SD, arbitrary units) obtained in PQ-treated hepatopancreas cells (substrate cells) resulting from the activity of hepatopancreas extracts at day 1 (E1) and day 5 (E5) of exposure to *Prorocentrum lima* as well as after 1 (PE1) and 5 (PE5) days feeding on non-toxic algae (post-exposure). White (control) and grey (exposed) columns represent net extract-induced breaks (NEB) fitted on the level of PQ-induced breaks (dashed line). Significant differences ($p < 0.05$) for NEB are marked with (*) between control and exposed groups within the same point in time, while (b) points out difference between times within post-exposure.

3.2.4.2. Effects in the invasive clam R. philippinarum

Although the DNA repair capacity significantly increased throughout the exposure period (E1 *vs*. E5) in both control and exposed groups, the effects of the toxic algae were only observed on the post-exposure period, with a significant increase in the BER levels. It is noteworthy that DNA repair capacity was significantly reduced over time (PE1 *vs*.PE5) for both control and exposed clams (Fig. 3.6, Table S7).

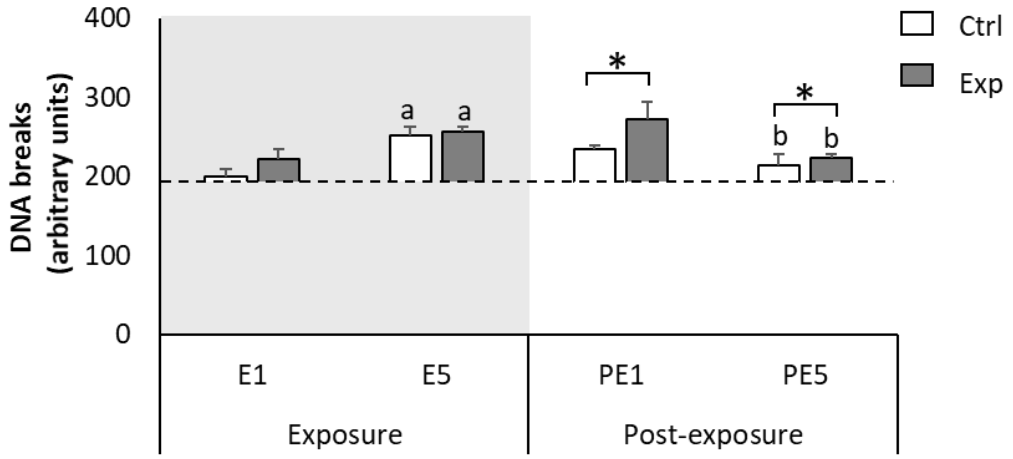

Fig. 3.6 Experiment 2 - DNA breaks after base excision repair (BER) assay of *Ruditapes philippinarum*. Breaks (mean ± SD, arbitrary units) obtained in PQ-treated hepatopancreas cells (substrate cells) resulting from the activity of hepatopancreas extracts at day 1 (E1) and day 5 (E5) of exposure to *Prorocentrum lima* as well as after 1 (PE1) and 5 (PE5) days feeding on non-toxic algae (post-exposure). White (control) and grey (exposed) columns represent net extract-induced breaks (NEB) fitted on the level of PQ-induced breaks (dashed line). Significant differences ($p < 0.05$) for NEB are marked with (*) between control and exposed groups within the same point in time, while (a) and (b) point out differences between times within exposure and post-exposure, respectively.

4. Discussion

In the last decades, biological invasions have been one of the major issues on conservation biology worldwide. The introduction of invasive species and the nearly complete replacement of the native species for the invasive ones has been a widespread phenomenon. The replacement of the native *R. decussatus* by the invasive *R. philippinarum* seems to be boosted by altered ecological, chemical or physical conditions caused by anthropogenic disturbances (Chiesa et al., 2011; Pranovi et al., 2006). In the present study we proposed to understand whether natural changes of ecological conditions, such as the occurrence of harmful algal blooms, affect the native / invasive species dichotomy and how species respond to this stressor.

To achieve this goal, the present study was designed and constituted by two interlinked experiments: one addressing toxin kinetics in the two clam's species *R. decussatus* and *R. philippinarum*, and the other combining a toxicokinetics approach with the evaluation of the effects of OA-group toxins regarding genotoxicity and DNA damage repair. Both experiments were performed by feeding the clams with the toxic algae *P. lima* in order to assess the accumulation of OA-group toxins by the two clam species and to understand if these congener species have different accumulation patterns as previously observed in the field (Braga et al., 2017) As an additional novelty element, a differential vulnerability of the two clam species to toxin genotoxicity was investigated, as well as critical underlying processes such as DNA damage repair, shedding light on the biochemical and physiological features associated to the ecological success of *R. philippinarum* as invasive species and in what extent HABs can invoke its opportunism, supported by the ability to withstand environmental disturbances.

4.1. Dynamics of toxin accumulation and elimination

Providing freeze-dried *P. lima* as feed to the clams showed to be a viable pathway of exposure, facilitating the experiments as timeliness of dinoflagellate cultures and possible cultures crash during prolonged experiments are avoided. The amount of *P. lima* provided to the clams allowed to simulate the beginning of the toxin accumulation

period and to reach a concentration near the threshold limit for shellfish harvesting (160 µg OA eq. kg^{-1}), as defined in the European Union by the Regulation (EC) No 853/2004.

To better understand the onset of the toxin accumulation process, short exposure periods were established during the initial 24 h of feeding on *P. lima* (Experiment 1). Although both species immediately accumulated OA-group toxins within the first 30 minutes of exposure, the accumulation patterns were highly irregular with increasing and decreasing toxin concentrations before reaching a stable and progressive accumulation pattern. A similar phenomenon was reported for oysters (*Crassostrea gigas*) that needed from 3 to 6 hours to adapt to the presence of the algae and reach the standard filtration behaviour (Romero-Geraldo et al., 2014).

The high variable levels observed during the initial period of exposure to toxic algae suggest the existence of a possible adaptation period to the presence of the *P. lima* cells in the water. Since shell closure behaviour, filtration rates and clearance rates, were not measured under our experimental conditions, it is not possible to determine whether the variability observed in the toxin accumulation profile was a response to the toxic algae or if it is an indirect effect of the shellfish adaptation to the increase in the organic matter in suspension in water. A negative correlation between clearance rates and increase of suspended particulate matter (SPM) in the water was demonstrated for *R. decussatus* and other shellfish species, including mussels and oysters (Sobral and Widdows, 2000).

The abrupt decrease of OA concentration observed after one hour of exposure to the toxic algae may be also associated with pseudofaeces production. The freeze-dried *P. lima* cells may be temporary stored in the clams, causing the toxin concentration increase, as observed after 30 minutes of exposure, but in response to the sudden increase of SPM in the water, caused by the adding of the freeze-dried algae, part of the algae filtered may be enveloped in a layer of mucus and expelled from the clams as pseudofaeces, without being digested or the toxin assimilated, causing the reduction observed after one hour of exposure.

The pseudofaeces production observed during this experiment may then help to understand the abrupt decrease of free OA and DTX1; nevertheless, the existence of active elimination mechanisms even at this early stage may not be discarded as the toxins in the esterified conformation also presented a decreasing tendency.

In the present study, *R. decussatus* reacted to the toxic algae with sudden changes in the accumulation of toxins throughout the first 6 hours of exposure, while *R. philippinarum* showed a faster adaptation.

Native clams were able to easily biotransform the toxins found in *P. lima*, *i.e.* OA and DTX1, into 7-*O*-acyl derivatives (DTX3) after the first hours of exposure, presenting 100 % of the toxin content in the esterified conformation, reinforcing the idea of an active elimination mechanism at this point. In contrast, invasive clams presented a steady increase in the proportion of toxin derivatives of shellfish metabolism, reaching 100 % of DTX3 only after 24 hours of exposure.

The second experiment, where the clams were exposed to toxic dinoflagellates for 5 days, allowed to better understand the dynamics of toxin accumulation and elimination. At day 1 of exposure to *P. lima, R. philippinarum* showed higher toxin concentration than *R. decussatus,* possibly due to the earlier stabilisation in algae filtration and consumption referred above. However, at the end of the 5 days of exposure, higher toxin concentration was found in *R. decussatus* than in the invasive clam species. This result is in agreement with previous studies based on field observations, showing that native clams collected from Aveiro Lagoon, NW Portugal, consistently reached higher levels of OA-group toxins than the invasive species (Braga et al., 2017). Toxicokinetic results of the present study suggest that native clam species has a quicker initial response to toxic algal blooms, with toxin biotransformation and elimination mechanisms being triggered at immediate and at lower concentrations

4.2. Differential vulnerability/resistance to OA-induced DNA damage of native and invasive clams

While the toxicokinetic study was performed in shellfish whole-body soft tissue in order to easily compare with most field studies and the regulatory limit for human consumption, the genotoxicity study focused only on the hepatopancreas. This strategy was selected because hepatopancreas is the main storage organ, presenting the highest levels of toxin accumulation in must shellfish species (García et al., 2015). This approach allowed to bridge a knowledge gap regarding the effect of OA in clams since, to the best of our knowledge, the approaches regarding OA genotoxicity in these species,

particularly *R. decussatus,* gave preference to other tissues, namely the gills and the haemolymph (Flórez-Barrós et al., 2011; Prado-Alvarez et al., 2013).

In addition to differences in accumulation also different genotoxicity patterns were observed between the native and invasive clam species. Assessment of non-specific DNA damage through comet assay clearly demonstrated that the two species react differently when exposed to the OA-group toxins. While *R. decussatus* showed a clear vulnerability in terms of GDI levels with a significant increase in the genetic damage in all sampling time periods, no effects were observed for *R. philippinarum* exposed to the toxic algae. To our knowledge, only two studies were performed assessing the genotoxic effects of OA in the native species, *Ruditapes decussatus* (Flórez-Barrós et al., 2011; Prado-Alvarez et al., 2013), and only one was performed in the invasive *R. philippinarum* (Huang et al., 2015). In both cases, adverse effects were reported. OA-group toxins caused an increase in DNA strand breaks but also induce DNA repair and other defence mechanisms in *R. decussatus* (Flórez-Barrós et al., 2011; Prado-Alvarez et al., 2013). In *R. philippinarum* toxins inhibit *P*-glycoprotein (*P*-gp), a mediator of the multixenobiotic resistance (MXR) mechanisms (Huang et al., 2015). However, none of these studies performed a comparative assessment of the two clam species. The genotoxicity of OA-group toxins has already been studied in *R. decussatus* gill cells and haemocytes (Flórez-Barrós et al., 2011). While haemocytes were not affected by exposure to *P. lima*, the gills presented contrasting responses under low and high toxin concentrations. Low concentrations increased the DNA damage, while high concentrations caused a reduction of DNA damage, suggesting a tissue- and dose-dependent response and stimulation of repair mechanisms or apoptosis in *R. decussatus* (Flórez-Barrós et al., 2011). Moreover, under 14.9 OA µg kg^{-1} the repair mechanisms or apoptosis should have been activated as the DNA damage in gills was significantly lower than in the control groups (Flórez-Barrós et al., 2011). In the present study, the results obtained in *R. decussatus* hepatopancreas after the accumulation of 158.9 (± 79.8) µg OA eq. kg^{-1} still showed increased in non-specific DNA damage. These data suggest that stimulation of repair mechanisms in the native clam hepatopancreas was not enough to cope with the damage induced by the toxins, and therefore the damage continued to increase. This indicates that *R. decussatus* hepatopancreas has a higher tolerance to the damage, needing an increased level of damage to trigger the repair mechanisms or that the toxin

presence compromises the repair mechanisms efficiency. The significant increase of DNA damage (as GDI) observed for *R. decussatus* is also in accordance with previous findings for other shellfish species. McCarthy et al. (2014) assessed the genotoxicity of OA in mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) and, although the exposure was performed through dilution of OA sodium salt in the algal solution used for feed the shellfish, a significant increase in the DNA damage was reported in both species, with oysters presenting a more considerable increase in DNA fragmentation than mussels.

Since no significant damage was observed as GDI, the invasive species seems to be better prepared to cope with the toxins and their genotoxic potential. Several processes may explain the lower vulnerability presented by *R. philippinarum* to the OA-group toxins. The lack of reaction to these compounds can be related to the toxicokinetic processes promoting toxin elimination prior to the occurrence of DNA damage or can be related to a higher efficiency of the antioxidant and other protection mechanisms that may prevent or inhibit the formation or reaction of genotoxic metabolites; Or even with a higher efficiency of the repair mechanisms involving non-oxidative damage.

From all hypotheses above, the data collected only supports differences in the toxicokinetic processes as a possible explanation. However, the other hypotheses presented should not be discarded as more information is needed to confirm that this is the only process involved in the vulnerability differences between the two clam species.

Investigation of oxidative DNA damage through incubation with the enzymes EndoIII and FPG revealed that, under the concentrations and conditions used in the present study, the damage caused by the OA-group toxins was not of oxidative origin, as no significant increase of $GDI_{EndoIII}$ and GDI_{FPG} was detected in both clam species. This is a noteworthy species similarity among the several dissimilarities detected. In fact, toxins seem to reduce the occurrence of oxidised pyrimidines, as both clams presented a significant decreased in the net sensitive sites (NSS) of EndoIII when toxin accumulation was at the highest levels (E1 and/or E5). In *R. decussatus*, this phenomenon can be explained by a downstream response. After DNA damage repair processes are induced by the toxins causing the decrease of NSS to levels below the control group. This response is in accordance with previous findings for gills in these species (Flórez-Barrós

et al., 2011). On the other hand, the decrease of NSS levels in *R. philippinarum* seems to be associated with upstream processes, occurring prior to the DNA damage, as neither the non-specific DNA damage nor the BER assay results were significantly affected by the exposure to toxic algae.

As referred, the lack of oxidative damage in *R. decussatus* seems to be related to the induction of DNA repair processes. While there are several studies indicating that *R. decussatus* clams do not produce ROS, particularly in haemocytes (Donaghy et al., 2009), several other studies report significant induction of antioxidant enzymes and the occurrence of oxidative DNA damage in hepatopancreas and gills (Jebali et al., 2007). Oxidative DNA damage caused by OA has been also reported in mammalian cell lines (González-Romero et al., 2012), in mussels (Prego-Faraldo et al., 2017, 2016) and scallops (Chi et al., 2016), confirming the capacity of OA to induce the production of these radicals in several organisms, including shellfish. Therefore, either the lack of ROS formation or the absence of oxidative DNA damage induction by the toxins may explain the lack of oxidative damage reported here. In fact, the results obtained in the present study regarding the base excision repair process support that the clams hepatopancreas were capable of response and therefore to produce ROS.

The activation of repair mechanisms was assessed through the BER assay and, in line with the results obtained for toxin dynamics and DNA damage, clear differences between species were observed. *R. decussatus* showed a significant increase in the activity of DNA repair enzymes targeting oxidative damaged bases since day 1, while in *R. philippinarum* such effect was only detected in the post-exposure period when the clams were eliminating the toxic compounds. This points out that DNA integrity was early challenged by toxic *P. lima* in the native clams. In *R. philippinarum*, the stimulation of DNA repair enzymes in the last day of the experiment, points to a time-dependent effect of OA-toxins, suggesting wear off in other protection mechanisms. The effects of both exposure time and toxin concentration were also reported in mussels feeding on *P. lima*; however, toxin concentration seems to have a greater impact in mussels response than the factor time (Prego-Faraldo et al., 2016).

The overall results point to the presence of an improved coping mechanism in *R. philippinarum* under low toxin concentrations, as the clams only react to the presence of the toxins in the post-exposure period. One may say that despite the increased DNA

damage observed in *R. decussatus*, this species has a favourable response to the toxin exposure since a significant increase of repair enzymes was detected. However, the activation of repair enzymes may not correspond to recovery. OA-group toxins are potent inhibitors of the phosphatases PP1 and PP2A (Swenson and Förlin, 1998), which are enzymes responsible for, among many other functions, signalling the end of the DNA repair process, leading to the offspring of the protein and enzymatic complexes involved in DNA repair and the chromatin condensation. In the presence of OA toxins, the action of these enzymes is impaired, and all these processes may be affected, compromising the genomic integrity, originating duplications, and in extreme cases mutations (González-Romero et al., 2012). Despite, PP1 more than PP2A is involved in the DNA repair process and OA is particularly known to affect PP2A (Herman et al., 2002), it is important to consider, that the expression of histones involved in the chromatin-remodelling process may also be affected by OA, contributing to the compromising of the genomic integrity (González-Romero et al., 2012).

5. Conclusions

In conclusion, after exposure of native and invasive clam species to *P. lima,* higher accumulation of toxins were observed for the native species, nearly reaching the EU regulatory limit for shellfish safety. These results confirm previous field observations, suggesting that invasive species *R. philippinarum* may be less affected by harmful algal blooms in terms of toxin accumulation and consequently on the number of days closed for harvesting, an important concern regarding the species farming management.

In addition, the invasive species seems not to be physiologically affected by the accumulation of OA toxins in the concentrations tested. In contrast, and despite the similarities between the two clams, exposure to *P. lima* induced a significant response in the native species not only causing increase of the DNA damage but also stimulating repair mechanisms since the very beginning of the exposure.

Although shellfish mortality events during blooms of *P. lima* or *Dinophysis* spp. is not commonly observed, it is important to recognize that, exposure to these toxins may cause significant disruptions in the ecosystems. The increased interest of *R. philippinarum* to the shellfish industry, coupled whit fewer harvest closures due to

marine biotoxins, may ultimately lead to the introduction of these invasive clams in new locations, promoting an increase in *R. philippinarum* distribution. In sympatry sites, exposure to OA-group toxins may unbalance the clams species biomass and distribution. As exposure to OA-group toxins compromise *R decussatus* genome integrity and may cause changes in the DNA information, possibly impairing reproduction and having a panoply of other effects in the future generations.

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Chapter 4

Combined effects of warming and acidification on accumulation and elimination dynamics of paralytic shellfish toxins in mussels *Mytilus galloprovincialis*

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Combined effects of warming and acidification on accumulation and elimination dynamics of paralytic shellfish toxins in mussels *Mytilus galloprovincialis*

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Abstract

Harmful algal blooms (HAB) have been increasing in frequency and intensity most likely due to changes on global conditions, which constitute a significant threat to wild shellfish and its commercial farming. This study evaluated the impact of increasing seawater temperature and acidification on the accumulation/elimination dynamics of HAB-toxins in shellfish. *Mytilus galloprovincialis* were acclimated to four environmental conditions simulating different climate change scenarios: i) current conditions, ii) warming, iii) acidification and iv) interaction of warming with acidification. Once acclimated, mussels were exposed to the paralytic shellfish toxins (PSTs) producing dinoflagellate *Gymnodinium catenatum* for 5 days and to non-toxic diet during the subsequent 10 days. High toxicity levels (1493 µg STX eq kg^{-1}) exceeding the safety limits were determined under current conditions at the end of the uptake period. Significantly lower PSP toxicity levels were registered for warming- and acidification-acclimated mussels (661 and 761 µg STX eq kg^{-1}). The combined effect of both warming and acidification resulted in PSP toxicity values slightly higher (856 µg STX eq kg^{-1}). A rapid decrease of toxicity was observed in mussels at the current conditions after shifting to a non-toxic diet, which was not noticed under the predicted climate change scenarios.

Variability of each PST analogue, measured throughout the experiment, highlighted different mechanisms are associated with changes of each environmental factor, although both resulting in lower toxicity. Warming-acclimated mussels showed lower accumulation/elimination rates, while acidification-acclimated mussels showed higher capability to accumulate toxins, but also a higher elimination rate preventing high toxicity levels. As different mechanisms are triggered by warming and acidification, their combined effect not leads to a synergism of their individual effects. The present work is the first assessing the combined effect of climate change drivers on accumulation/elimination of PSTs, in mussels, indicating that warming and acidification may lead to lower toxicity values but longer toxic episodes. PSTs are responsible for the food poisoning syndrome, paralytic shellfish poisoning (PSP) in humans. This study can be considered as the first step to build models for predicting shellfish toxicity under climate change scenarios.

Keywords: Harmful algal blooms; Climate change; Seafood safety; Saxitoxins; *Gymnodinium catenatum.*

1. Introduction

Most coastal areas are highly productive and simultaneously vulnerable ecosystems that have been continuously affected by multiple man-made pressures. The impacts of climate change are likely to deteriorate environmental conditions and worsen ecological problems in these sensitive coastal ecosystems, where bivalves play a significant role. Bivalves are of high ecological importance, increasing local biomass, promoting biodiversity, and being responsible for some ecosystem engineering processes (Sousa et al., 2009, Fernández-Reiriz *et al.*, 2012). In addition, these molluscs are an import source of protein for human consumption, representing valuable resources for coastal populations.

Warming, sea level rise, changes on circulation and salinity patterns, nutrient and sediment regimes, and acidification have been pointed out as the major climate change drivers affecting coastal areas (Filgueira et al., 2016). The Intergovernmental Panel on Climate Change (IPCC) indicates that, not only, the water temperature increased in the

last century, but also acidification occurred, with a decrease of the water pH by almost 0.6 pH units (IPCC, 2013). These trends are projected to worsen, with temperatures increasing over 4.5 degrees Celsius and pH decreasing from 0.3 to 0.5 pH units by 2100 in the worst case scenarios (IPCC, 2013). While IPCC values are projected for the end of the century, global ocean conditions, coastal areas and intertidal organisms, such as shellfish, are nowadays being frequently challenged with abrupt changes of environmental conditions (Filgueira et al., 2016).

Thermal tolerance with changing temperatures has been the focus of several studies (Pörtner et al., 2007). The increase of temperature usually leads to an increase in feeding and metabolic rates, promoting growth and reproduction when values are within the species thermal optimum range and in the presence of sufficient food and oxygen (Filgueira et al., 2016). However, when these conditions are not met, the increase in temperature may negatively affect shellfish. Changes in mussels behaviour, immune response, standard metabolic rate, reduction of energy available for the somatic metabolism, and negative impacts on growth rates have been associated with increasing temperature (Anestis et al., 2007; Coppola et al., 2017; Múgica et al., 2015a). Increase of temperature may also promote the uptake, accumulation and metabolization of some pollutants, like metals (e.g. Ni and Cd) and radionuclides, as well as persistent organic pollutants (POPs) and methylmercury (Alava et al., 2017; Banni et al., 2014; Coppola et al., 2017; Dallas et al., 2016; Múgica et al., 2015a; Sokolova and Lannig, 2008). Regarding marine biotoxins, namely paralytic shellfish poisoning toxins, temperature increase seems to affect their accumulation and elimination reducing both in oysters (Farrell *et al.*, 2015) and increasing elimination surf clams (Bricelj et al., 2014).

In coastal upwelling zones, such as the Iberian Peninsula, the west coast of Americas, and the northwest and southwest coast of Africa, ocean acidification is anticipated to rise with the intensification of upwelling regimes (Bakun et al., 2015). Prolonged exposure to hypercapnia (acidified waters) may decrease the inter- and extra-cellular pH values and the consequent shell dissolution to compensate these acid-base disturbances (Parker et al., 2013). It also leads to changes in several physiological and metabolic processes like the reduction of thermal tolerance, decreased O_2 consumption, increased standard metabolic rates (SMR) and reduction of somatic growth rates (Michaelidis et al., 2005; Nikinmaa and Anttila, 2015; Parker et al., 2013).

The eastern boundary upwelling systems are among the most productive marine ecosystems. The nutrient pulses from deeper waters into the coastal photic zone, often resulting in phytoplankton blooms that are the basis of the marine food web. Among phytoplankton species blooming, some produce toxins as secondary metabolites, which are then accumulated in the biota, particularly by filter-feeding bivalves, and can become a public health concern. Recently, the largest and longest harmful *Pseudo-nitzschia* diatom bloom was observed along the west coast of the US associated with anomalously warming ocean conditions, being the bloom sustained by upwelled nutrients from the seasonal spring transition (Bond et al., 2015; McCabe et al., 2016). On the other hand, based on data from the last three decades, Pérez *et al.* (2010) pointed out a decline of coastal upwelling in the inner shelf of the Iberian Peninsula induced by sea surface warming. The upwelling decline and consequent less turbulent environment and persistence of stratified conditions may result in an increase in the frequency of toxic dinoflagellates blooms, such as *Dinophysis* spp. and *Gymnodinium catenatum,* profoundly impacting shellfisheries (Vidal et al 2017). The bloom-forming dinoflagellates *Dinophysis* spp. and *G. catenatum* are linked to diarrhetic shellfish poisoning (DSP) and paralytic shellfish poisoning (PSP) outbreaks in humans, respectively. *G. catenatum* produces highly potent neurotoxins designated as paralytic shellfish toxins (PSTs). Its life-cycle includes a microreticulate resting cysts stage that facilitates their widespread distribution, either promoted by changes in natural conditions or by anthropogenic activities (Hallegraeff et al., 2012, Gobler et al., 2017). Harmful algal blooms (HAB), including *G. catenatum*-blooms, seem to be emerging and expanding in recent decades, which negatively affects the marine ecosystems and local economies due to increasing closures to shellfish harvesting (Gobler et al., 2017).

Research on the combined effects of warming seawater and acidification and exposure to harmful algal blooms on a bivalve production context is still scarce. Therefore, this study aims to assess the impact of both relevant climate change drivers *per se* and their combined effect on the dynamics of PSTs accumulation and elimination in *Mytilus galloprovincialis*.

2. Methods

2.1. Mussel collection and acclimation

One hundred and twenty immature Mediterranean mussels, *Mytilus galloprovincialis* (53.78 ± 6.22 mm), were harvested in Aveiro Lagoon in July 2016, when blooms of *G. catenatum* were not occurring. Upon collection, mussels were immediately shipped to IPMA facilities (Lisbon) in a thermally isolated container. Mussels were cleaned from macro-algae, barnacles or any other epibiont, and placed in four 150 L tank systems, subdivided into 6 replicates. Each tank systems was equipped with a protein skimmer (Reef SkimPro, TMC Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), biological filtration (model FSBF 1500, TMC Iberia, Portugal) and chemical filtration (activated carbon, Fernando Ribeiro Lda, Portugal) to maintain seawater quality. Each tank was used to simulate a specific treatment: 1 - current conditions (CC: 19 ºC; pH 8.0), 2 - warming (W: 24 ºC; pH 8.0), 3 - acidification (A: 19 ºC; pH 7.6) and 4 - warming and acidification (WA: 24 ºC; pH 7.6) (Fig. 4.1). The seawater used was filtered (0.35 µm) and UV sterilised. Mussels were gradually adapted to the conditions of each treatment, by increasing 1 ºC and decreasing 0.1 pH units per day. The animals were then acclimatised for 21 days, being fed with non-toxic and freeze-dried *Tetraselmis sp*. diet (Necton, Olhão, Portugal). Temperature and pH were automatically adjusted whenever needed. Water temperature was cooled through an automatic seawater refrigeration system (± 0.1 °C; Frimar, Fernando Ribeiro Lda, Portugal) or heated by submerged digital heaters (200W, V2Therm, TMC Iberia, Portugal). Water pH was measured through individual pH probes (GHL, Germany) connected to a computerized pH control system (± 0.1 pH units; Profilux 3.1N, GHL, Germany), which monitored each tank every 2 s, and adjusted them whenever need, via submerged air stones, by injecting CO_2 (Air Liquide, Portugal; to decrease pH) or filtered air (to increase pH) using air pumps (Stella 200, Aqua One Pro, Aqua Pacific UK Ltd, United Kingdom).

Tanks were kept under the following abiotic conditions: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) salinity = 35.7 ± 0.4 ‰; iii) photoperiod = 12L:12D (12 hours light:12 hours dark). Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Ammonia, nitrite and nitrate levels were daily checked by means of colorimetric tests (Tropic Marin, USA), and kept

below detectable levels with daily water changes, except nitrates, which were kept below 2.0 mg L^{-1}. Seawater total alkalinity was also measured in every tank on a weekly basis, spectrophotometrically at 595 nm, following a protocol previously described elsewhere (Sarazin et al., 1999) and the combination of total alkalinity (AT) and pH was used to calculate carbonate system parameters. A summary of seawater parameters is reported in the Supplementary Material (Appendix C: Table S.1 and Fig. S.1).

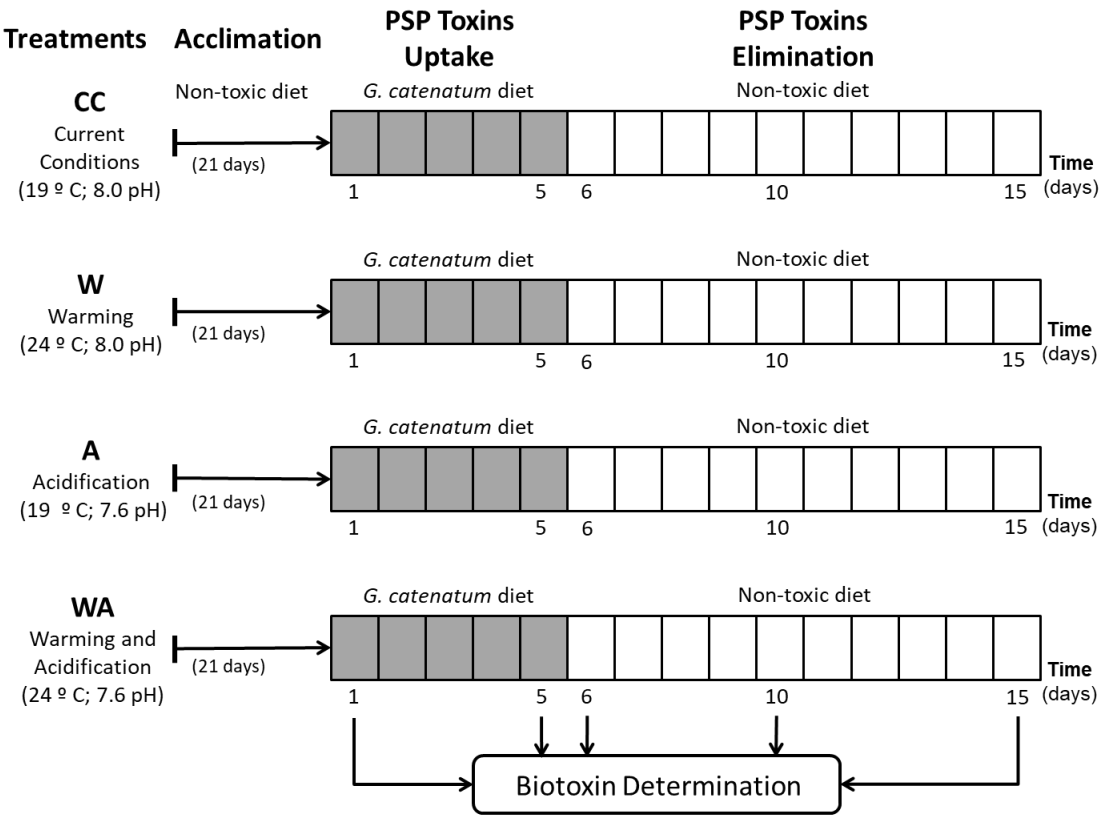

Fig. 4.1 Design of mussels feeding experiments with toxic dinoflagellates *Gymnodinium catenatum* under four environmental conditions (CC: 19 ºC and 8.0 pH; W: 24 ºC and 8.0 pH; A: 19 ºC and 7.6 pH; WA: 24 ºC and 7.6 pH), showing sampling dates during uptake (grey) and elimination (white) phases.

2.2. Gymnodiniun catenatum cultivation

G. catenatum strain IO-13-04, obtained from the algae culture collection at Lisbon University (ALISU), was isolated from a bloom in Espinho, NW Portuguese coast, in September 2005. Cells were mass cultured in 2 L flasks with seawater adjusted to 30 ‰ and enriched with GSe medium as in Doblin et al. (1999) without the soil extract. Seawater and nutrients solutions were filtered and autoclaved to minimise contamination. The cultures were grown at 18 °C with a 12:12 L:D cycle under fluorescent lights. Cells were harvested when cultures presented a density of approximately 2.5 × 106 cells per litre, concentrated using 10 µm mesh sieve. The toxin profile of the algae was composed by sulfocarbamoyl toxins and decarbamoyl toxins (Table 1). Toxins were determined in the algae cell culture as described below (section 2.4).

Table 4.1 PST content and toxin profile of the *Gymnodinium catenatum* culture used to feed mussels.

Toxin	Concentration (fmol.cell^{-1})	Molar Fraction (%)
C1+2	40.80	95.7
GTX5	1.01	2.4
dcNeo	0.42	1.0
dcGTX2+3	0.31	0.7
dcSTX	0.1	0.2
C3+4	*	*
GTX6	*	*

* - Not Quantified

2.3. Mussels exposure to toxic dinoflagellates

During acclimation, mussels were fed with 100,000 cells per day per animal of the non-toxic and freeze-dried, *Tetraselmis* sp. diet (Necton, Olhão, Portugal). The mussels were then fed for 5 days with toxic dinoflagellates from a *G. catenatum* culture under the conditions indicated in the previous section and following the experimental design shown in Fig 4.1. Mussels were exposed to approximately 91,000 *G. catenatum* cells per

day per animal. After the 5 days of exposure to the toxic diet, mussels were fed again with 100,000 cells per day per animal of *Tetraselmis* sp. during 10 days in order to assess the elimination of toxins accumulated during the 5-days exposure period. Two mussels exposed to *G. catenatum* were collected in triplicate for toxin analysis on days 1 and 5, corresponding to the uptake period, and on days 6, 10 and 15, corresponding to the elimination period.

2.4. Determination of PSTs

2.4.1. Reagents

All reagents used for toxins extraction and analysis were of analytical grade or higher. Acetic acid glacial (100 %, p.a.), methanol (> 99.8 %, p.a.) and acetonitrile (Analytical grade) were obtained from Sigma-Aldrich; ammonium formate (> 99 % purity) from Fluka; Hydrochloric acid (37 %) from Panreac; Hydrogen peroxide (30 %) and sodium hydroxide (> 99 %, p.a.) from Merck. Water was purified using a Milli-Q 185 Plus system from Millipore. Toxins standard solutions for dcGTX2 + 3, C1 + 2, dcSTX, GTX2 + 3, GTX5 and STX, were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council (NRC, Canada).

2.4.2. Extraction and determination of PSTs by liquid chromatography with Fluorescence detection

Extraction of toxins from *G. catenatum* cell cultures followed the methodology described by Silva *et al.* (2015). Briefly, an aliquot of *G. catenatum* cell culture (500 mL) was filtered onto 47 mm Whatman GF/C with a nominal pore size of 1.2 mm, under low vacuum. Toxins were extracted in 4 mL of 0.05 M acetic acid and sonicated for 4 min at 25 Watts, 50 % pulse duty cycle (Vibracell, Sonic & Materials, Newtown, CT, USA) in an ice bath. Cell lysis was confirmed with light microscopy. The extract was then centrifuged (3000 *g*) for 10 min, and 1 mL of the supernatant was used for the determination of PSTs. The supernatant was cleaned by solid-phase extraction (SPE) with an octadecyl bonded phase silica (Supelclean LC-18 SPE cartridge, 3 mL, Supelco, USA). Briefly, the

SPE cartridge was conditioned with 6 mL of methanol, followed by 6 mL of water and 1 mL of extract was loaded to the cartridge and the PSTs eluted with 2 mL of water.

Both extraction of toxins from mussels and the determination of the PSTs followed the EU reference method (AOAC Official Method 2005.06) which consists on a Liquid chromatographic separation coupled to fluorescence detection (LC-FLD) with precolumn derivatization, to convert the toxins into the correspondent fluorescent derivatives. Briefly, 5 g of shellfish whole soft tissue homogenate was double extracted with 1 % acetic acid solution (first extraction with heating), and the extracts were cleaned using solid-phase SPE C18 cartridge (Supelclean, Supelco, USA). Before LC/FLD analysis, 100 µL of C18 extract from both algae and mussels was derivatized with peroxide and periodate oxidants.

LC-FLD analysis were performed on a Hewlett-Packard/Agilent LC system (Germany) constituted by a Model 1100 quaternary pump, Model 1100 in-line degasser, autosampler, column oven, and Model 1200 fluorescence detector. The Hewlett-Packard Chemstation software performed data acquisition and peak integration. Toxins separation was performed using a reversed-phase column Supelcosil C18 column 150 × 4.6 mm id, 5 µm (Supelco, USA) equipped with a column Supelguard Supelcosil C18, 20 mm id × 4.0 mm id, 5 µm (Supelco, USA) and kept in a column oven at 30 ºC. The flow rate was 1 mL min^{-1} and the mobile phase gradient used to elute the PSTs oxidation products consisted of 2 mobile phases under the following conditions: 0–5 % B (0.1 M ammonium formate in 5 % acetonitrile, pH = 6) in the first 5 min, 5–70 % B for the following 4 min and 100 % mobile phase A (0.1 M ammonium formate, pH = 6) used during 5 min before the next injection. The injection volume was 50 µL, detection wavelengths were set at 340 nm for excitation and 395 nm for emission. PSTs were identified by comparison with standards retention times and quantified by interpolation in the calibration curves obtained with the factor response peak areas *vs.* toxin concentration (see an illustrative LC-FLD chromatogram of PSTs in Appendix C. Fig. S.2). The limits of detection (signal-to-noise ratio of 3) ranged from 0.004 µM for C1 + 2, GTX5 and STX to 0.020 µM for dcNEO. The toxicity equivalent factors stated by EFSA (2009) were used for calculation of PSTs in terms of saxitoxin equivalents. The total PSP toxicity in the sample corresponds to the sum of all toxin analogs quantified. For toxins co-eluting and determined together (C1 + 2, dcGTX2 + 3, and GTX2 + 3) the higher toxicity

equivalent factor of the co-eluted compound was used. Low potency PSTs analogues for which analytical standards were not available, namely C3+4 and GTX6, were not considered for this study.

2.5. Statistics

Two-Way Analysis of Variance followed by a Multiple Comparisons *versus* Control Group (Bonferroni t-test) was used to assess significant differences on toxin content and toxicity between the current conditions, warming, acidification and the combined effect of warming and acidification. Also, a Two-Way Analysis of Variance followed by All Pairwise Multiple Comparison Procedures (Bonferroni t-test) was used to assess significant differences between the sampling dates.

For the empirical kinetics of PSTs elimination, a one-compartment model was used to describe elimination kinetics using a single component first-order kinetic model:

$$\frac{dC_m}{dt} = -k_{el}C_m \quad (1)$$

where C_m is the toxin concentration in mussels and k_{el} denotes the elimination rate. Solving this differential equation gives:

$$C_m = C_{m0}\, e^{-k_{el}t} \quad (2)$$

The toxin concentration decreases according to an exponential decay, with the steepness of the decay being determined by the elimination rate (k) and the size of the curve depending of the initial concentration of the toxin (C_{m0}) at the beginning of the elimination period, when mussels diet was changed from *G. catenatum* to non-toxic algae.

Data were tested for normality and homogeneity of variance by the Kolmogorov-Smirnov test and the Levene Median test. Differences were considered significant at $p < 0.05$. Data analysis was performed using the statistical program SigmaPlot Version 10.0.

3. Results

3.1. Impact of warming and acidification on mussels toxicity

A marked increase of PSTs accumulation was observed in mussels during the uptake period in all treatments (Fig.4.2). The highest toxin levels were determined in mussels at the current conditions of temperature and pH (CC: 19 ºC and pH 8), which in terms of shellfish toxicity expressed as saxitoxin equivalents after multiplying the toxins concentration with the respective toxicity factor, reached levels of 1,493.8 ± 202.4 µg STX eq. kg^{-1} at day 5. PSP toxicity was significantly ($p < 0.001$) lower in acidification- and warming-acclimated mussels, reaching values of 761.2 ± 62.3 and 661.9 ± 22.8 µg STX eq. kg^{-1}, respectively. Although similar to the toxicity observed for treatments 2 and 3, where the effect of each climate change driver was evaluated individually, the combined effect of both components, tested in treatment 4, resulted in PSP toxicity values slightly higher (855.8 ± 61.4 µg STX eq. kg^{-1}).

When algae provided to mussels was changed to non-toxic species, a decrease of toxicity was immediately observed in all treatments. The decrease of PSP toxicity was particularly evident in mussels at current conditions. For these mussels, a significant decrease ($p< 0.001$) was observed after 24 hours feeding on non-toxic algae, corresponding to a toxicity reduction of 57.4 ± 3.2 %. The highest toxicity decrease in the first 24 hours was found in acidification treatment (66.9 ± 5.0 %), and the lowest decrease of only 27.3 ± 12.9 % was registered for mussels maintained in the warming treatment. The interaction between the two variables conducted to a decrease of 47.6 ± 25.7 %. Toxicity reached similar levels for all treatments in the subsequent days of the elimination period (Fig. 4.2).

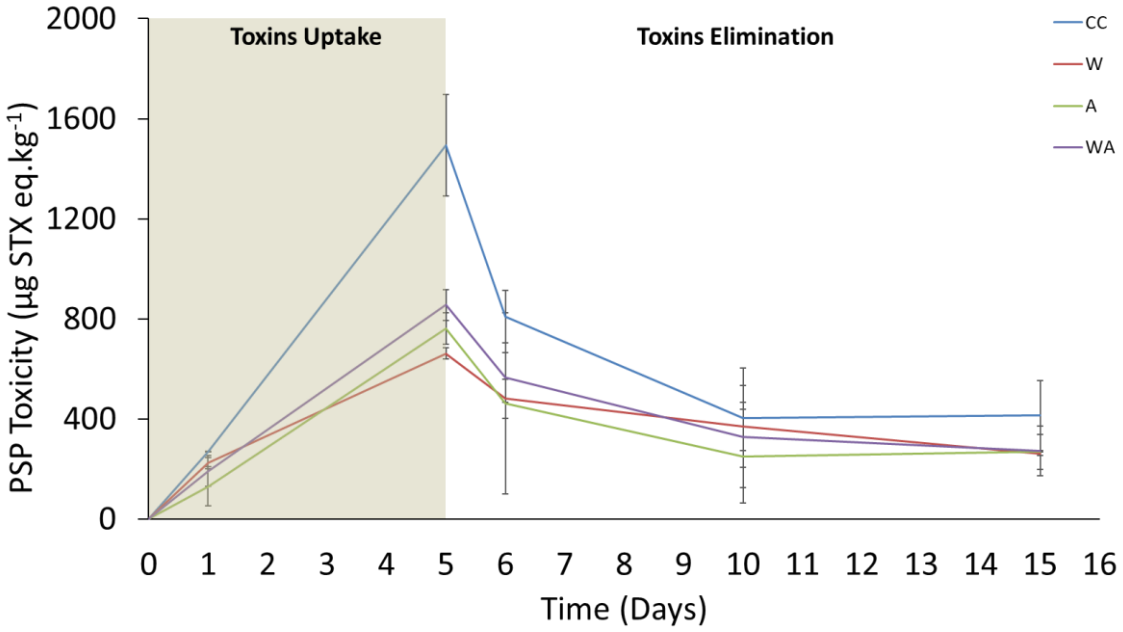

Fig. 4.2 PSP toxicity (µg STX eq. kg^{-1}, mean ± SD) determined in mussels exposed to toxic Gymnodinium catenatum during 5 days and to non-toxic diet during the subsequent 10 days, under four environmental conditions (CC: 19 ºC and 8.0 pH; W: 24 ºC and 8.0 pH; A: 19 ºC and 7.6 pH; WA: 24 ºC and 7.6 pH)

3.2. Accumulation and elimination kinetics of PSTs under conditions of warming and acidification

The profile of PSTs detected in mussels, as illustrated in Fig. 4.3, was constituted by the same toxins found in the toxic algae given as food, namely C1+2, GTX5, dcGTX2+3, dcSTX and dcNeo.

At the end of the 5-day uptake period, significantly higher concentrations of each PST congener were observed in mussels acclimated at current conditions than in the other treatments. Exception occurred for the concentration of C1+2, which was determined at levels similarly high in mussels at current (CC) and acidified (A) conditions.

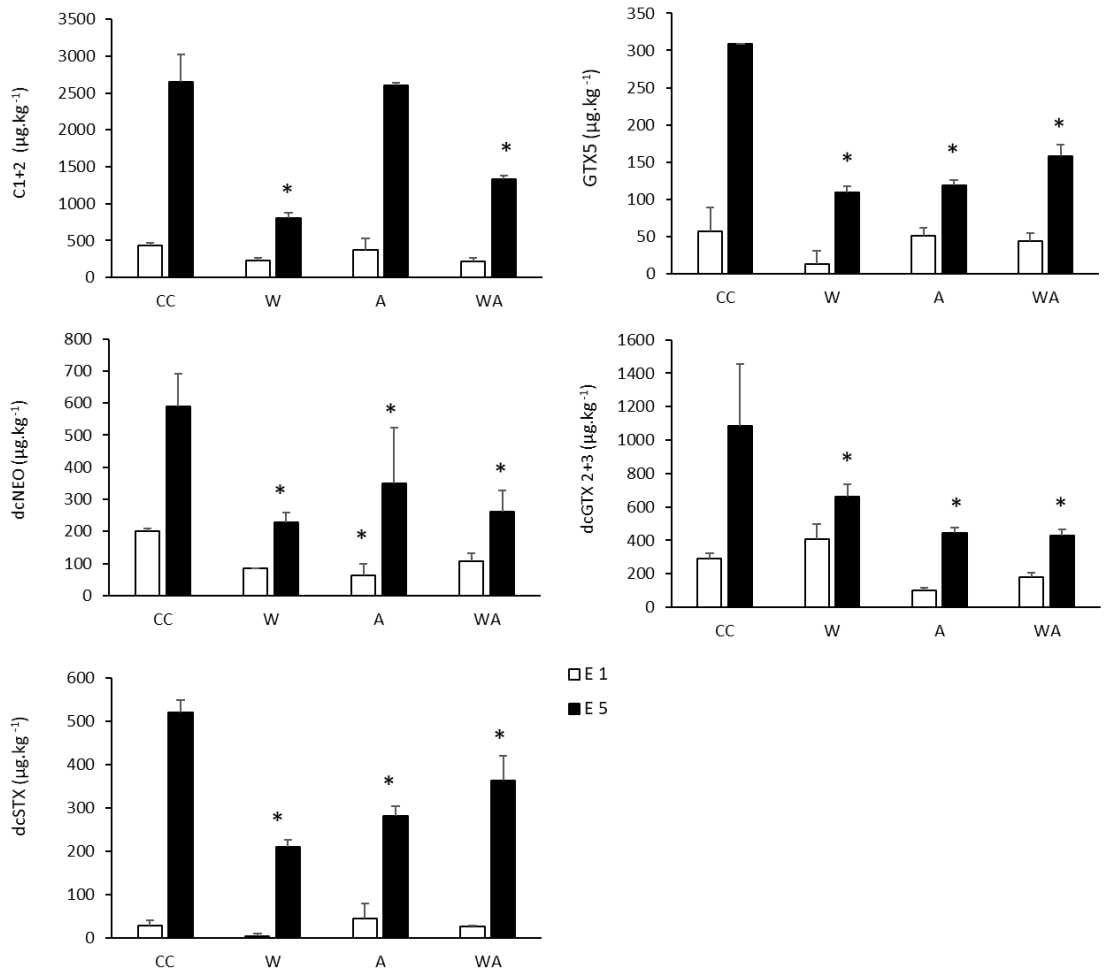

Fig. 4.3 Concentration (µg kg^{-1}, mean ± SD) of PSTs (C1+2, GTX5, dcNeo, dcGTX2+3 and dcSTX) determined in mussels exposed to *Gymnodinium catenatum* at day 1 (white bars) and 5 (black bars), under four environmental conditions (CC: 19 ºC and 8.0 pH; W: 24 ºC and 8.0 pH; A: 19 ºC and 7.6 pH; WA: 24 ºC and 7.6 pH). Values marked with an asterisk represent significant differences ($p < 0.05$) from current conditions (vs. CC) within the same exposure time.

Significant differences in concentration of PSTs were also found between the combined effect of warming and acidification (WA) with each component tested individually (W or A). Indeed, higher C1+2 concentrations were determined in WA than in W, but reaching lower levels than in A. Higher GTX5 and dcSTX concentrations were determined in WA than in W and A. Finally, concentrations of dcGTX2+3 reached lower values than W, but not statistically different to A.

After reaching the peak of toxins accumulation in day 5, the shift to non-toxic diet resulted in a decrease of toxins concentrations. However, toxins were not completely eliminated during the studied period (10 days). The timeline of toxins elimination in each

treatment is illustrated in Fig 4.4. All toxins fitted well to an exponential decay model, except dcGTX2+3. Elimination rates calculated from the best fit are indicated in table 4.2. The highest elimination rates were generally obtained for mussels under acidifying conditions, and lower elimination rates were registered in mussels under warming conditions. The combination of both climate change drivers resulted in an attenuation of effects, leading to elimination rates between mussels at current and acidified conditions.

Table 4.2 Elimination rate (kel, d-1) (± standard error) and coefficient of determination R2 of each PST determined in mussels under four environmental conditions (CC: 19 ºC and 8.0 pH; W: 24 ºC and 8.0 pH; A: 19 ºC and 7.6 pH; WA: 24 ºC and 7.6 pH).

Treatment	Toxin	Elimination Rate		R^2
CC Current Conditions	C1+2	0.173	(± 0.052)*	0.81
	GTX5	0.131	(± 0.035)*	0.82
	dcNeo	0.578	(± 0.076)*	0.98
	dcSTX	0.234	(± 0.071)*	0.82
W Warming	C1+2	0.087	(± 0.016)*	0.89
	GTX5	0.074	(± 0.024)*	0.73
	dcNeo	0.300	(± 0.037)*	0.98
	dcSTX	0.105	(± 0.018)*	0.90
A Acidification	C1+2	0.898	(± 0.219)*	0.93
	GTX5	0.063	(± 0.027)*	0.63
	dcNeo	0.658	(± 0.243)*	0.90
	dcSTX	0.231	(± 0.064)*	0.85
WA Warming and Acidification	C1+2	0.355	(± 0.114)*	0.86
	GTX5	0.064	(± 0.021)*	0.71
	dcNeo	0.904	(± 0.194)*	0.95
	dcSTX	0.174	(± 0.062)*	0.73

* Represents values within the confidence limit ($P < 0.05$).

C1+2, that were initially the dominant PSTs found in algae and mussels from the four treatments, was easily eliminated in mussels maintained in acidification conditions. On the other hand, these toxins were slowly eliminated from mussels under warming conditions. The lowest elimination rates were calculated for GTX5 in all treatments. The most potent compound from this set of toxins, *i.e.* dcSTX, was rapidly eliminated in mussels under current conditions compared to any other treatment.

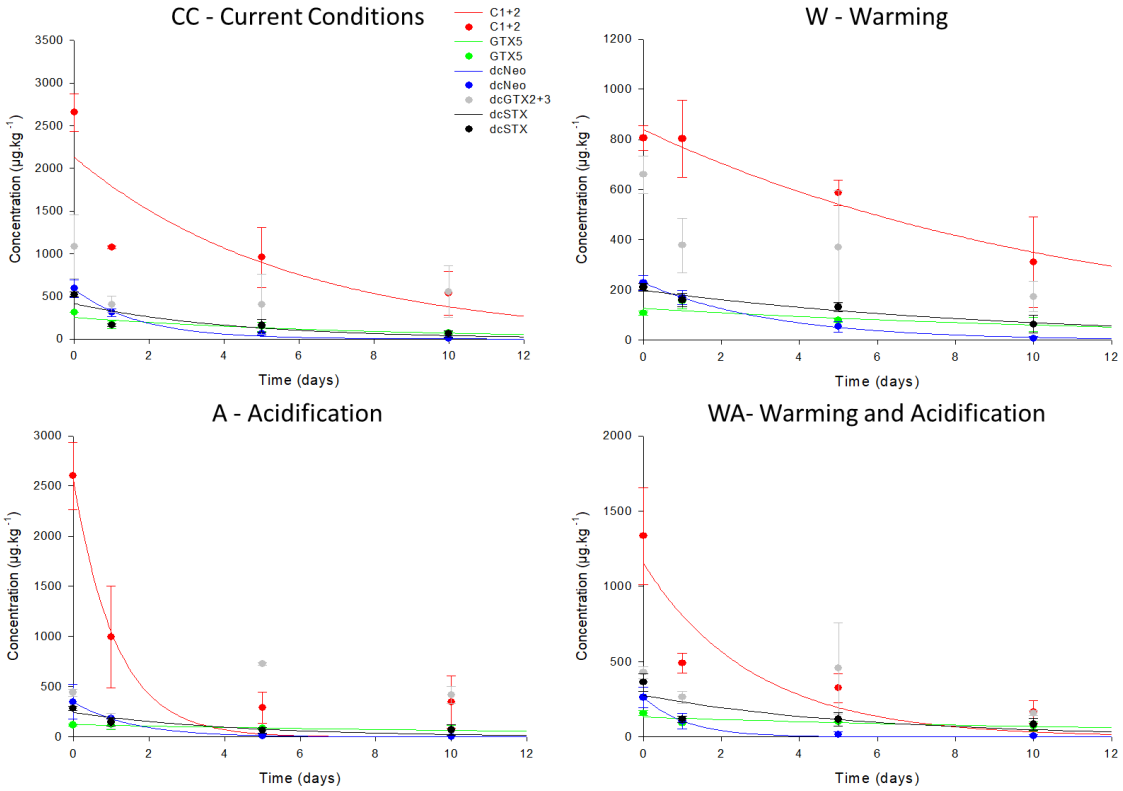

Fig. 4.4 Elimination of the PSTs (C1+2, dcSTX, GTX5, dcNeo and dcGTX2+3) in mussels under four environmental conditions (CC: 19 ºC and 8.0 pH; W: 24 ºC and 8.0 pH; A: 19 ºC and 7.6 pH; WA: 24 ºC and 7.6 pH). Dots and error bars represent experimental data; the coloured lines represent the output of the simulation model ($Cm = Cm0\ e^{-kel\ t}$).

4. Discussion

Climate change is a complex phenomenon, resulting from alterations on several environmental drivers. Temperature might be the easiest parameter to follow, but climate changes include several other parameters with direct and indirect impact on pH, salinity, oxygen levels, current regimes and even frequency and intensity of harmful algal blooms (HAB). Therefore, it is essential to understand the interaction of these variables, which may lead to either antagonistic or synergistic responses of marine organisms (Duarte et al., 2014; Lischka et al., 2011).

HAB-toxins represent a significant threat to commercial shellfish farming, frequently leading to closures to harvesting due to toxins accumulation at levels above the international regulatory limits. In this study, mussels feeding during 5 days on PSTs-producing dinoflagellate *Gymnodinium catenatum* exceeded the EU regulatory limit of 800 µg STX eq. kg^{-1} when maintained under the current conditions of temperature and pH and in the combined acidification and warming conditions. In contrast, mussels acclimated solely to warming and acidification displayed significantly lower PSP toxicity levels, i.e. below but close to the safety limits. Seafood demand has grown in the last decades and is expected to increase at a higher level with the global population growth. For this reason, sustainable increased production, *i.e.* aquaculture, is seen as the solution to meet the demand for shellfish that cannot be supported by the declining wild stocks. According to PSP toxicity results in this study, one could suggest that climate change, regarding warming and acidification, may benefit shellfisheries as lower toxicity levels are reached. Nevertheless, the decrease of toxicity during the elimination period was significantly slower in acclimated mussels than in mussels maintained at current environmental conditions. Assuming that HAB are increasing in frequency and intensity (Fu et al., 2012) and that toxin-producing dinoflagellates under acidified conditions are prone to increase their toxicity (Tatters et al., 2013), shellfish are expected to become more frequently contaminated. Moreover, shellfish may remain contaminated for longer periods, although not reaching the actual exacerbated levels.

Most studies on climate change effects in shellfish have focused on a single driver (*e.g.* temperature, acidification, salinity) (Anestis et al., 2010, 2007; Farrell et al., 2015; Fernández-Reiriz et al., 2012). A reduced number of studies addressed the combination

of two variables. In this study, the individual impact of each parameter was tested to better understand the effects of their interaction. Analyzing warming as a single climate change driver in mussels exposed to toxic algae lead to a reduction of toxicity levels. The same trend was previously described by Farrell *et al.* (2015) in one of the few studies combining climate change drivers, namely increased temperature and biotoxin accumulation. A reduction in both accumulation and elimination of the PSTs GTX1+4 and GTX2+3 were observed in warming-acclimated oysters fed with toxic *Alexandrium minutum.* Reduction of oysters PSP toxicity under warming conditions was not concurrent with decrease of clearance rates and routine metabolism (Farrel *et al*. 2015). In contrast, a reduction of nearly 50 % of clearance rates were reported in mussels *M. galloprovincialis* under warming conditions (Anestis et al., 2010). This reduction was suggested to be related with a metabolic depression mechanism in order to balance the increase in energy demand due to warmer water temperatures (Anestis et al., 2010). Although clearance and metabolic rates were not assessed in the present study, lower PSP toxicity values in warming-acclimated mussels may result from changes on these physiological components.

In addition to increasing temperature, the effect of acidification was also investigated. Ocean acidification is not plausible without temperature rising. However, to better understand the role of this climate change driver, mussels were acclimated to an acidification scenario with and without warming. Testing this parameter individually also allowed to investigate the hypothesis that lower pH conditions may promote the elimination of PSTs. This hypothesis was raised in 1965, but not positively tested (Hayes, 1966; Shumway *et al.*, 1995). Suspecting that pH could affect shellfish physiology and favor toxins elimination, Hayes (1966) submitted PST contaminated butter clams (*Saxidomus giganteus*) to extreme acidified pH values (5 and 6) for 15 hours at 7º C without promising results. In the present study, significant decrease in mussel toxicity was observed, reclaiming this hypothesis as a way to promote PSTs elimination.

In the present study, the lower toxicity levels found for *M. galloprovicialis* under acidified conditions appear to be related with different mechanisms than those associated with toxicity reduction at increasing temperatures. *M. galloprovicialis* acclimated to seawater acidification conditions (*i.e.* to pH decrease of 0.3 or 0.6 units) have shown to maintain their clearance, ingestion and metabolic rates (Fernández-Reiriz

et al., 2012). Alternatively, some digestive enzymes may present higher activities (Areekijseree *et al.,* 2004; Fernández-Reiriz *et al.,* 2012). Indeed, induction of enzymatic activities and antioxidants was found at a pH around 7.3 and 7.7 (Hu et al., 2015). In *M. coruscus*, an increase in the reduced glutathione (GSH) consumption was assessed under acidification conditions (Hu et al., 2015). The consumption of this antioxidant is particularly relevant since GSH has been found to have an important role on biotransformation and elimination of PSTs analogues (Costa et al., 2012; Sakamoto et al., 2000; Sato et al., 2000). Assessing the variability of each PST analogue throughout the experiment was essential to better understand the individual effects of warming and acidification. Although both climate change drivers resulted in lower PSP toxicity levels, the individual effects of warming and acidification differed on the accumulation and elimination of several PST analogues determined.

The *N*-sulfocarbamoyl analogues C1+2 were the most abundant toxin congeners found in mussels during the uptake period in all treatments, as they were the most abundant PST identified in the micro-algae. However, C1+2 concentration was significantly higher in mussels maintained under acidification conditions than in warming-acclimated mussels. In fact, the concentration of C1+2 was similar to mussels under current conditions, suggesting high uptake rates and no effects on clearance rates, thus supporting previous findings mentioned above (*i.e.* Fernández-Reiriz *et al.*, 2012).

The C1+2 are the least potent of the PST derivatives, making variations in their concentrations less relevant for total PSP toxicity. On the other hand, variability of decarbamoyl toxin analogues, in particular, dcSTX that is the most potent compound of this set of toxins, notably impacts mussels PSP toxicity. The accumulation of these analogues was identically reduced in mussels maintained at either warming or acidification, which was the main factor for decreasing toxicity levels in mussels subjected to climate change drivers compared to mussels at current conditions.

The effects of warming and acidification also differed during the elimination period. The experimental data concerning the concentration of toxins determined in mussels during this period fitted well to the dynamic model, providing a good description of the kinetics of C1+2, GTX5, dcNeo and dcSTX in mussels. C1+2 that dominated mussels profile in all treatments are the less stable (Kodama, 2010) and easier eliminated PST

derivatives (Botelho et al., 2010; Li et al., 2005; Lopes et al., 2014; Yu et al., 2007). The highest C1+2 elimination rates were calculated in mussels under acidification conditions (A), which highly accumulated these toxins during the uptake period. Mussels of this treatment (A) revealed similar or slightly higher elimination rates of dcSTX and dcNeo than mussels maintained at current environmental conditions. The lowest elimination rate in mussels at A was calculated for GTX5. Reduced elimination rates of GTX5 were also obtained for warming-acclimated mussels and under the combined effect of warming and acidification. These elimination rates support the theory discussed before referring acidification as a possible way to promote toxins elimination. Despite no clear biotransformation pattern was detected in our study, an enhancement of biotransformation enzymes described under acidified conditions (Hu et al., 2015) could be behind these rates. Warming-acclimated mussels presented the lowest or nearly the lowest elimination rates in all toxin congeners, pointing out a decrease in mussels metabolic activity as suggested by Anestis *et al.*(2010).

The effects of both climate change drivers, *i.e.* warming and acidification, lead to a reduction of PSP toxicity, but mussels subjected to the combined effect of both drivers showed only slightly higher toxicity levels than mussels subjected solely to warming or acidification. This fact highlights the need for more studies approaching the exposure to multiple climate change drivers.

5. Conclusion

The present work is the first study assessing PSTs accumulation and elimination in mussels under the combined effects of warming and acidifying environments, indicating that such climate change scenarios may lead to lower but prolonged PST contamination in *Mytilus galloprovincialis*. Although the combined effects of warming and acidification promoted lower PSP toxicity levels than the current conditions, the underlying kinetics differed among them. While warming-acclimated mussels accumulated low toxin levels and may retain them due to low elimination rates, mussels at acidification conditions showed higher ability to accumulate PSTs, but their high elimination rates prevented mussels to reach high toxicity levels as mussels maintained under current conditions.

This study also highlights an approach with potential interest for shellfisheries industry affected by *Gymnodinium catenatum* blooms, as lower seawater pH may promote elimination of PSTs. Although further studies are needed to confirm this hypothesis and to set the optimal parameters to promote such elimination without conditioning shellfish shell growth and well-being in future depuration facilities.

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Chapter 5

DNA damage and oxidative stress responses of mussels *Mytilus galloprovincialis* to paralytic shellfish toxins under warming and acidification conditions – Elucidation on the organ-specificity

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DNA damage and oxidative stress responses of mussels *Mytilus galloprovincialis* to paralytic shellfish toxins under warming and acidification conditions – Elucidation on the organ-specificity

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Abstract

Commonly affected by changes in climate and environmental conditions, coastal areas are very dynamic environments where shellfish play an important ecological role. In this study, the oxidative stress and genotoxic responses of mussels (*Mytilus galloprovincialis*) exposed to paralytic shellfish toxin (PST) - producing dinoflagellates *Gymnodinium catenatum* were evaluated under *i*) current conditions (CC: 19 °C; pH 8.0), *ii*) warming (W: 24 °C; pH 8.0), *iii*) acidification (A:19 °C; pH 7.6) and *iv*) combined effect of warming and acidification (WA: 24 °C; pH 7.6). Mussels were fed with *G. catenatum* for 5 days, and to a non-toxic diet during the following 10 days. A battery of oxidative stress biomarkers and comet assay was performed at the peak of toxin accumulation and at the end of the post-exposure phase. Under CC, gills and hepatopancreas displayed different responses/vulnerabilities and mechanisms to cope with PST. While gills presented a tendency for lipid peroxidation (LPO) and genetic damage (expressed by the Genetic Damage Indicator - GDI), hepatopancreas seems to better cope with the toxins, as no LPO was observed. However, the mechanisms involved in hepatopancreas protection were not enough to maintain DNA integrity. The absence of LPO, and the antioxidant system low responsiveness, suggests DNA damage was not oxidative. When exposed to toxic algae under W, toxin-modulated antioxidant responses were observed

in both gills and hepatopancreas. Simultaneous exposure to the stressors highlighted gills susceptibility with a synergistic interaction increasing DNA damage. Exposure to toxic algae under A led to genotoxicity potentiation in both organs. The combined effect of WA did not cause relevant interactions in gills antioxidant responses, but stressors interactions impacted LPO and GDI. Antioxidant responses and LPO pointed out to be modulated by the environmental conditions in hepatopancreas, while GDI results support the dominance of toxin-triggered process. Overall, these results reveal that simultaneous exposure to warming, acidification and PSTs impairs mussel DNA integrity, compromising the genetic information due to the synergetic effects. Finally, this study highlights the increasing ecological risk of harmful algal blooms to *Mytilus galloprovincialis* populations.

Keywords: Harmful algal blooms; Climate change; Genotoxicity; Oxidative stress responses; Saxitoxins; *Gymnodinium catenatum.*

1. Introduction

Coastal areas and estuaries are highly productive ecosystems where shellfish species play a fundamental role linking planktonic and nektonic communities and acting as ecosystem engineers capable of changing the abiotic environment around them (Sousa et al., 2009). However, coastal areas are recurrently under intense pressures that may affect shellfish well-being and survival. Therefore, shellfish are frequently exposed to pressures that range from anthropogenic contamination to pathogens and parasites, to variations on the abiotic environmental conditions, such as seawater temperature increase, pH decrease (a.k.a. acidification), salinity and nutrient fluctuations, changes in freshwater input, and exposure to toxic phytoplankton blooms (González and Puntarulo, 2016; Hégaret et al., 2007a).

Harmful algal blooms (HABs) can have profound impacts on shellfish settlements and populations, leading in extreme cases to mass mortalities (Hégaret et al., 2011). HABs constitute a significant problem for shellfish farming, due to their adverse effects in metabolic fitness and organisms' health, and due to its impacts on human health as shellfish may act as vectors of HAB-toxins.

There are a vast number of phytoplankton species capable of producing marine biotoxins, among these are the dinoflagellates from the genera *Alexandrium, Pyrodinium* and *Gymnodinium* producers of saxitoxin (STX) and their derivatives (Gedaria et al., 2007; Hallegraeff et al., 2012; T. Silva et al., 2015). STX and related compounds are among the most frequent biotoxins accumulating in filter-feeding organisms, such as shellfish, in the North Atlantic (Bricelj and Shumway, 1998; Brown et al., 2010). These toxins are responsible for the human illness paralytic shellfish poisoning (PSP) which is characterised by symptoms that range from nausea and diarrhoea to confused speech, paralysis and, in extreme concentrations, death (Landsberg et al., 2006; Manfrin et al., 2012).

While the impacts of these toxins in humans are well known, and their action mode almost completely described, the same cannot be said for biotoxins impacts in shellfish. Only in the last decades the effects of paralytic shellfish toxins (PST), and other biotoxins in shellfish gained relevance. Initially, this research started by focusing mostly on behavioural aspects and only some physiological parameters were investigated. A wide range of responses, ranging from reduction in the clearance rates, shell valves activity, byssus production, O_2 consumption and heart rate, as well as hatching and larval survival rates, have been reported for several shellfish species exposed to PST (Landsberg, 2002; Shumway and Gainey, 1992).

Shellfish defence mechanisms are composed by an innate immune system, that includes hemocytes and humoral elements, and is the first response to the presence of exogenous compounds based on neutrophil activation and inflammatory factors initiation, including peroxides and oxidative radicals production (Harikrishnan et al., 2011; Hégaret and Wikfors, 2005a). Peroxides, oxidative radicals and other reactive oxygen species (ROS) have their origin in several biological processes, being essential to the physiological control of critical cellular functions (Fabioux et al., 2015; Guilherme et al., 2008a; Qiu et al., 2013). ROS also present a high potential to cause oxidative damage, since they can react with a wide range of biomolecules and induce protein degradation, enzymatic inhibition, lipid peroxidation (LPO), DNA damage and, in extreme conditions, cell death and tissue damage (Choi et al., 2006; Fabioux et al., 2015; Gerdol et al., 2014; Guilherme et al., 2008a). In order to maintain the redox balance and minimise oxidative damage, an antioxidant system evolved in the animals to prevent the overgeneration

and neutralise ROS. This system includes antioxidant enzymes, as catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR), and non-enzymatic compounds as the reduced glutathione (GSH). Although the high efficiency of this system, not all ROS produced in the cells are deactivated, the imbalance between the formed and neutralised ROS is designated as oxidative stress (Fabioux et al., 2015; Guilherme et al., 2008b; Valavanidis et al., 2006). As a consequence of oxidative stress, LPO may occur from the attack of cellular polyunsaturated fatty acids by ROS, causing membrane destabilisation and disintegration. Also, LPO reaction originates malondialdehyde (MDA), known for causing protein synthesis inhibition, DNA adducts formation and for being genotoxic, mutagenic and carcinogenic (Melegari et al., 2012).

In shellfish exposed to PST, an increase of ROS was observed by several authors, depicting a species-specific response of the antioxidant system when considering Manila clam (*Ruditapes philippinarum*), mussels (*Mytilus galloprovincialis*) and scallops (*Patinopecten yessoensis*) feeding on *Alexandrium tamarense* (Abi-Khalil et al., 2017; Cao et al., 2018; Choi et al., 2006; Qiu et al., 2013). Mussels presented a faster response, taking advantage of CAT, SOD and GPx to reduce ROS, while scallops presented a slower response (Qiu et al., 2013). Also, PST exposure induced LPO and DNA damage in shellfish, namely in oysters (*Crassostrea gigas*) and scallops (*Chlamys farreri*) (Abi-Khalil et al., 2017; Cao et al., 2018; Choi et al., 2006; Qiu et al., 2013).

As mentioned above, shellfish have to deal with abiotic environmental changes, such as the increase of seawater temperature, acidification, and to recurrent exposure to HABs. Exposure to environmental drivers, either individually or combined, causes significant impacts in shellfish. While warming effects can be ambivalent depending on oxygen content and food availability, the balance between these conditions is difficult to occur, and adverse outcomes as behavioural and metabolic changes, growth rates reduction and increased xenobiotic accumulation are often reported (Anestis et al., 2007; Coppola et al., 2017; Filgueira et al., 2016). Acidification affects shellfish by compromising the structure and growth of the shell and altering several physiological and metabolic processes, from thermal stress tolerance to growth rates (Duarte et al., 2014; Griffith and Gobler, 2020; Nikinmaa and Anttila, 2015).

Despite the growing awareness for non-linear responses, and additive, synergistic or antagonistic interactions when exposure to multiple stressors occurs, limited data is available regarding the combine effects of warming and acidification in shellfish (Hu et al., 2015; Nardi et al., 2017; Wu et al., 2016). Even less is known when a third stressor, such as HABs, is added to warming and acidification exposure.

Therefore, the present study aims to assess the organ-specific (gills and hepatopancreas) modulation of the antioxidant system and the eventual induction of LPO and DNA damage in mussels *Mytilus galloprovincialis* exposed to toxic dinoflagellate *Gymnodinium catenatum* under two relevant climate change drivers, ocean warming and acidification, as well as addressing the follow-up of the responses in the post-exposure period.

2. Material and methods

2.1. Mussels collection and acclimation

One hundred and forty immature mussels *Mytilus galloprovincialis* (53.8 ± 6.2 mm shell length) were harvested from the Aveiro Lagoon (Portugal) in July 2016, during a period of no PSP toxicity in shellfish (Braga et al., 2018). Mussels were cleaned from macro-algae and epibionts, and placed in four 150-L tanks, under the conditions described in Braga et al. (2018).

Each tank simulated an environmental scenario of current conditions of temperature and pH (CC: 19 °C; pH 8.0), warming (W: 24 °C; pH 8.0), acidification (A: 19 °C; pH 7.6) and warming and acidification combined (WA: 24 °C; pH 7.6) (Fig. 5.1). These treatments correspond to the current environmental conditions and scenarios predicted by the Intergovernmental Panel on Climate Change (IPCC, 2013). Each tank was subdivided into 6 sections to allow an experimental design encompassing toxin-exposed and control groups in triplicate (2 × 3 = 6).

Mussels were allowed to gradually adjust to the new conditions, increasing 1 °C and decreasing 0.1 pH unit per day, and then were let to acclimate for 21 days. Temperature and pH levels were automatically adjusted whenever needed, through automatic seawater refrigeration systems, submerged digital heaters and a computerised pH control system, as described in Braga et al. (2018).

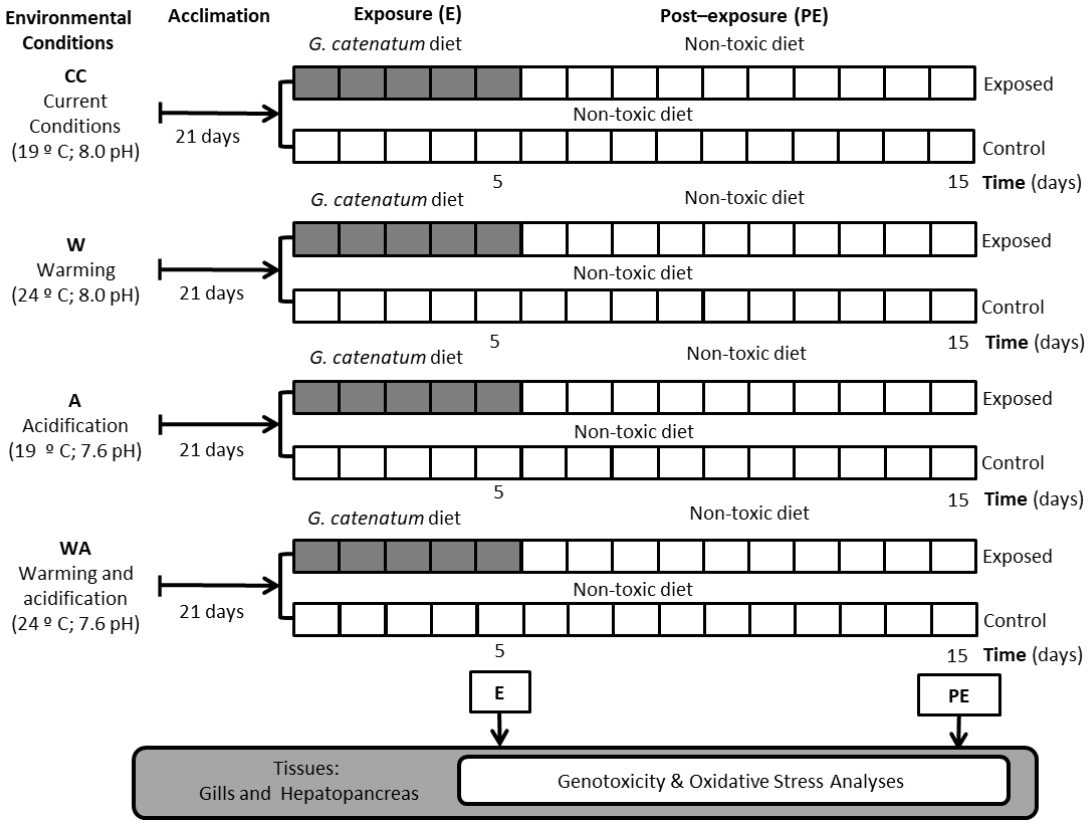

Fig. 5.1 Design of mussel feeding experiment, with toxic *Gymnodinium catenatum* diet (in grey) and non-toxic diet - *Tetraselmis sp*. (in white), under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH). In the timescale, the numbers 5 and 15 identify the sampling days, on the fifth day of exposure to toxic algae (E) and on the tenth day of post-exposure (PE), respectively.

The following abiotic conditions were maintained in the tanks: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) salinity = 35.7 ± 0.4 ‰; iii) photoperiod 12 h light : 12 h dark. Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Ammonia, nitrite and nitrate levels were daily checked using colourimetric tests (Tropic Marin, USA), and kept below detectable levels with daily water changes, except nitrates, which were kept below 2.0 mg L^{-1}. Seawater total alkalinity was also weekly measured in every tank, spectrophotometrically at 595 nm, following a protocol described by Sarazin et al. (1999) and the combination of total alkalinity and pH was used to calculate carbonate system parameters. A summary of seawater parameters is reported in Supplementary material (Appendix D: Table S1 and Fig. S1). During acclimation, the mussels were fed with

200,000 cells per day of the freeze-dried *Tetraselmis* sp., a non-toxic diet (Necton, Olhão, Portugal).

2.2. Gymnodiniun catenatum cultivation

The strain IO-13-04 of *G. catenatum*, isolated in 2005 in the NW Portuguese coast, was obtained from the Lisbon University (ALISU) algae culture collection. Cells were cultivated as in Braga et al. (2018). Briefly, cells were grown in filtered and autoclaved seawater (30 ‰ salinity) and enriched with GSe medium (Doblin et al., 1999), at 18 °C with a 12 h light : 12 h dark cycle under fluorescent lights. Cells were concentrated (10 µm mesh sieve) and harvested at a density of approximately 2.5×10^6 cells per litre.

The toxin concentrations were determined in algae cell culture as described in Braga et al. (2018), whose toxin profile included the sulfocarbamoyl toxins C1+2 (with a concentration of 40.80 fmol.cell^{-1}, corresponding to 95.7 % of the toxin molar fraction) and GTX5 (1.01 fmol.cell^{-1}, 2.4 %), and the decarbamoyl toxins dcNeo (0.42 fmol.cell^{-1}, 1.0 %), dcGTX2+3 (0.31 fmol.cell^{-1}, 0.7 %) and dcSTX (0.1 fmol.cell^{-1}, 0.2 %).

2.3. Exposure of mussels to toxic dinoflagellates

In the exposed groups, mussels were fed with approximately 91 000 *G. catenatum* cells per day per mussel, for 5 days. At the end of the exposure period, the feeding was changed to a non-toxic diet (*Tetraselmis* sp., as described for acclimation), which lasted for 10 days to allow toxin elimination and the recovery of the eventual effects. Mussels from the control groups were fed as described above for the acclimation period.

On the fifth day of exposure to toxic dinoflagellates *G. catenatum*, mussels from the exposed and control groups were harvested *per* condition to assess oxidative stress and genetic damage. The same sampling procedure was repeated on day 15, at the end of the post-exposure period (10 days). The exposure period and the algal concentration was chosen in order to simulate a natural bloom and to ensure that the toxin accumulation in mussels reached the regulatory limits of 800 µg of STX equivalents per kg of shellfish.

The toxin concentrations in the mussels (whole soft tissues) at days 5 and 15 are presented in table 5.1, as described in Braga et al. (2018).

Table 5.1 PSP toxicity (µg STX eq. kg^{-1}, mean ± SD) determined in mussels (whole soft tissues) exposed to toxic *Gymnodinium catenatum* for 5 days (E) followed by a non-toxic diet during the 10 days (PE), under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH), adapted from Braga et al. (2018)

Environmental Conditions	**STX concentration** (µg STX eq.kg^{-1})	
	Exposure (E)	**Post-exposure (PE)**
CC (19 °C; 8.0 pH)	1494 ± 202	414 ± 140
W (24 °C; 8.0 pH)	662 ± 23	261 ± 7
A (19 °C; 7.6 pH)	761 ± 63	268 ± 71
WA (24 °C; 7.6 pH)	856 ± 61	272 ± 99

2.4. Evaluation of oxidative stress and genetic damage

2.4.1. Mussel tissues sampling and preparation

Seven mussels were harvested in each sampling moment for antioxidants, LPO and genetic damage assessment. The gills and hepatopancreas were excised from each specimen and divided into two subsamples: one for oxidative stress analyses, which was immediately frozen in liquid nitrogen and stored at - 80 °C until analyses. The other subsample used for genetic damage evaluation was slowly frozen in an antifreeze solution of PBS and DMSO (9:1, v:v) and kept in thermal insulation material, initially at -20°C for 1 week and then at -80 °C until analyses.

2.4.2. Oxidative stress analyses

The oxidative stress parameters were analysed following the procedures previously described by Marques et al. (2017, 2016). The tissues were homogenised and divided into two aliquots: for post-mitochondrial supernatant (PMS) and the LPO sample preparation.

The PMS fraction was obtained by centrifugation, while LPO aliquot was obtained by adding butylated hydroxytoluene (BHT) and phosphate buffer to the PMS fraction to prevent oxidation (Bird and Draper, 1984; Ohkawa et al., 1979; Wilhelm Filho et al., 2001a, 2001b). Both PMS and LPO aliquots were stored at -80 °C until further analyses.

The total protein content was determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard the results were expressed in mg mL^{-1}.

Catalase (CAT) activity was determined following Claiborne (1985) method, with Marques et al. (2016) modifications. The assay mixture was adjusted for 5 µL of PMS samples. The absorbance variation was measured in a microplate reader (SpectraMax 190), at 240 nm. The CAT activity was calculated in µmol H_2O_2 consumed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 43.5 M^{-1} cm^{-1}.

The superoxide dismutase (SOD) activity was assessed using a Ransod kit (Randox Laboratories Ltd., UK). SOD activity was detected in the microplate reader at 505 nm, and the results were expressed as SOD units mg $protein^{-1}$.

Glutathione reductase (GR) activity was determined following Cribb et al. (1989) with Marques et al. (2016) modifications. The enzyme activity was determined by measuring the oxidation of NADPH at 340 nm in the microplate reader and calculated as nmol NADPH oxidised min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 × 10^3 M^{-1} cm^{-1}.

Glutathione peroxidase (GPx) activity was determined according to Claiborne (1985) and modified as in Athar and Iqbal (1998) as described in (Marques et al., 2017). NADPH oxidation was recorded in the microplate reader at 340 nm. GPx activity was calculated in nmol NADPH oxidised min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 × 10^3 M^{-1} cm^{-1}.

Glutathione-S-transferase (GST) activity was determined following the Habig et al. (1974) method as described in (Marques et al., 2017). The absorbance variation was recorded in the microplate reader at 340 nm. GST activity was calculated as nmol CDNB conjugate formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 9.6 × 10^3 M^{-1} cm^{-1} (Bird and Draper, 1984).

The total glutathione content (GSHt) was determined following Baker et al. (1990) and Tietze (1969). Proteins were precipitated, centrifuged, and GSHt was analysed in the

supernatant as in (Marques et al., 2017). The GSHt content was assessed through the formation of 5-thio-2-nitrobenzoic acid (TNB), measured in the microplate reader at 415 nm. The results were express as nmol of TNB formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 14.1×10^3 M^{-1} cm^{-1}.

Lipid peroxidation (LPO) was determined through thiobarbituric acid reactive substances (TBARS) quantification as in Ohkawa et al. (1979) and Bird and Draper (1984) adapted by Wilhelm Filho et al. (2001a, 2001b). Briefly, protein determination was performed on the aliquot prepared for LPO determination after homogenisation. LPO was then determined as described in (Marques et al., 2017). The absorbance was measured at 535 nm in the microplate reader. LPO was expressed in nmol of TBARS formed mg $protein^{-1}$ using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.4.3. Genetic damage evaluation

Genotoxicity caused by PST and the altered environmental conditions was assessed by the comet assay. The alkaline version of this methodology was performed following Collins (2004) procedures with Guilherme et al. (2010) modifications. The tissues were cut (gills two times and hepatopancreas one time) in a PBS bath (pH 7.4; 0,01 M). 1 mL of cell suspension in PBS was collected in each sample and centrifuged at 1,500 rpm, at 4 °C, for 5 min. The supernatant was then discarded (990 µL) and the pellet resuspended in a new PBS (1 mL). Again, the cell suspension was centrifuged and 990 µL of the supernatant discarded. To the remaining 20 µL of cell suspension, was added 70 µL of 1 % agarose LMP (in PBS). From this solution, two mini-gels, with 6 µL of cell suspension, were placed onto an agarose NMP pre-coated slide and refrigerated for 5 min at 4 °C. In order to improve the assay output, each slide contained four samples, in a system of eight gels per slide with two replicates per sample

The slides were immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1 % Triton X-100, and 10 % of DMSO, pH 10) at 4 °C, for at least 1 h, in the dark. The slides were then moved to the electrophoresis tank (Sub-Cell® GT, Bio-Rad), immersed in the electrophoresis solution, for 20 min, to alkaline treatment. The DNA migration was performed for 15 min, at a fixed voltage of 25 V, a current of 300 mA (power supply PowerPac™, Bio-Rad), which results in 0.7 V cm^{-1} (achieved by adjusting the total volume

of buffer). The slides were then neutralised in PBS for 10 min, followed by 10 min in distilled water and 10 min in ethanol (100 %) at 4 °C, and left to dry at room temperature overnight. For nucleoids counting and DNA damage evaluation, the slides were stained with ethidium bromide (20 μg mL^{-1}) and observed using a Leica DMLS fluorescence microscope (**×** 400 magnification).

Visual classification of nucleoids into five comet classes, according to the tail intensity and length (0 - no tail; 4 - almost all DNA in tail), was performed to quantify DNA damage. The Genetic Damage Indicator (GDI) was calculated according to the formula:

$$\mathrm{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

Where *i* is the number of each defined class (ranging within 0-4).

GDI values were expressed as "arbitrary unit" in a range of 0–400 per 100 scored nucleoids (average value for the two mini-gels observed per sample).

2.5. Statistical analysis

Statistica 7.0 software was used for statistical analysis. The three sets of data, antioxidants, lipid peroxidation and genetic damage, were tested for outliers through the Grubbs Statistic Test, normality through the Shapiro-Wilks test, and homogeneity of variance through Levene's Test, to assess statistical demands. When the statistical demands were not satisfied, the data were transformed. The different sets of data were analysed only within each sampling moment (Exposure or Post-exposure). A two-way ANOVA followed by Tukey HSD test as post-hoc comparison tests were applied to assess the effects of each factor and the interaction of the factors in mussels' responses. The factors considered were "toxins exposure" (mussels exposed to toxic algae and negative control), and "environmental condition" (mussels under four different conditions, CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH). Statistical differences were considered significant at $p < 0.05$ (Zar, 1996).

3. Results

3.1. Oxidative stress responses of mussels exposed to Gymnodinium catenatum under different conditions of temperature and pH

3.1.1. Antioxidant system modulation: gills responses

Mussels with a diet restricted to non-toxic algae (*Tetraselmis* sp.) showed no significant responses of the gills antioxidant system on day 5 when comparing the different environmental conditions tested (CC, W, A and WA) (Fig. 5.2 and Table S2). However, on day 15, a significant increase of GPx activity was observed in mussels kept under W, A, and WA when compared to CC, as well as an increase of GSHt content in mussels under the combined effect of warming and acidification (WA) when compared to W.

Analysing the effects of exposure to the toxic algae *G. catenatum* (through statistical comparisons with the unexposed group) within each environmental scenario, significant changes are noticeable (Fig. 5.2 and Table S2). At the peak of toxin accumulation (day 5), a decrease of SOD activity and GSHt content, as well as induction of GPx activity, were observed in mussels maintained under the current conditions (CC). Similarly, a decrease of SOD and induction of GPx activity was observed in mussels under warming conditions (W). However, an inversion of GSHt content variation was registered, being significantly augmented in mussels maintained in W. Mussel gills were particularly impacted under the acidification conditions (A), showing induction of CAT, SOD and GPx activity, as well as GSHt content increase. Responses of mussels to the toxic algae under the combined effect of warming and acidification (WA) were similar to CC, with a reduction of SOD activity and GSHt content, coupled with induction of GPx activity.

Following toxin exposure, the antioxidant responses of mussels at the end of 10 days feeding on *Tetraselmis* sp. (post-exposure period) were limited to induction of GPx activity under CC and its reduction under WA.

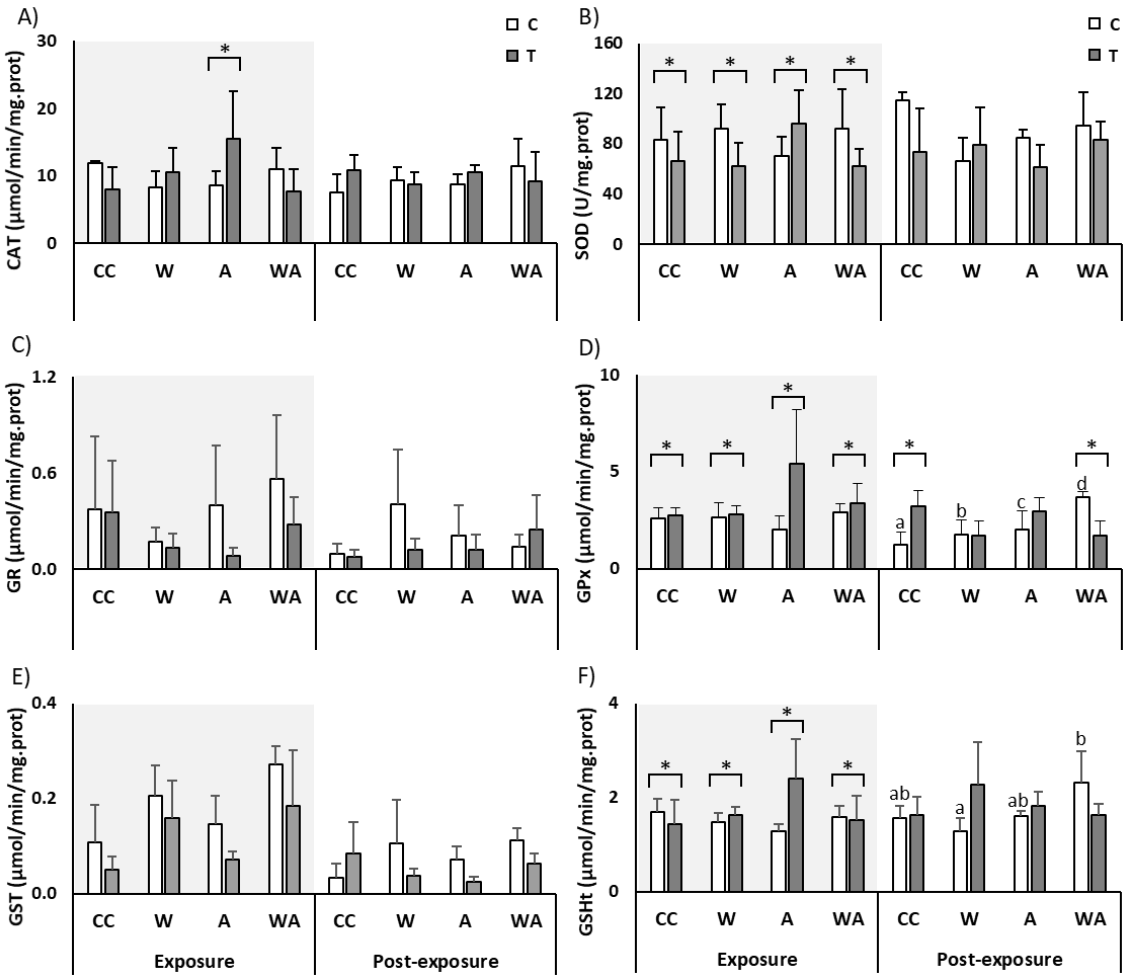

Fig. 5.2. Antioxidant responses (mean ± SD) in gills of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Parameters include: A) catalase (CAT), B) superoxide dismutase (SOD), C) glutathione reductase (GR), D) glutathione peroxidase (GPx), E) glutathione-S-transferase (GST) activities, and F) total glutathione content (GSHt). Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis sp.*) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

3.1.2. Antioxidant system modulation: hepatopancreas responses

Mussels maintained under different environmental conditions (CC, W, A and WA) did not show significant responses of the antioxidant system on day 5, when the diet was constituted by non-toxic algae (*Tetraselmis* sp.). An exception was the increase of GST activity observed under WA when compared with the mussels kept under A (Fig. 5.3 and Table S3). However, on day 15, CAT activity was significantly reduced in mussels under warming (W) and the combined effect of warming and acidification (WA). In contrast, an increase in CAT activity was observed for mussels under acidification (A) conditions, always in comparison with CC. A reduction of GPx activity was also observed in mussels under acidification (A) conditions comparing to warming (W).

When *G. catenatum* was introduced into mussels' diet, significant changes were observed for two parameters (Fig. 5.3 and Table S3). Increasing GR activity and GSHt content were observed in mussels (toxin-exposed *vs* unexposed) under CC, while under the remaining conditions (W, A and WA), the only parameter affected was GR activity, which decreased in W and increased in A and WA. After switching the diet to non-toxic algae (*Tetraselmis* sp.), which corresponded to the post-exposure period, the antioxidant responses of mussels were limited to induction of GPx activity in CC and its reduction under the remaining environmental conditions.

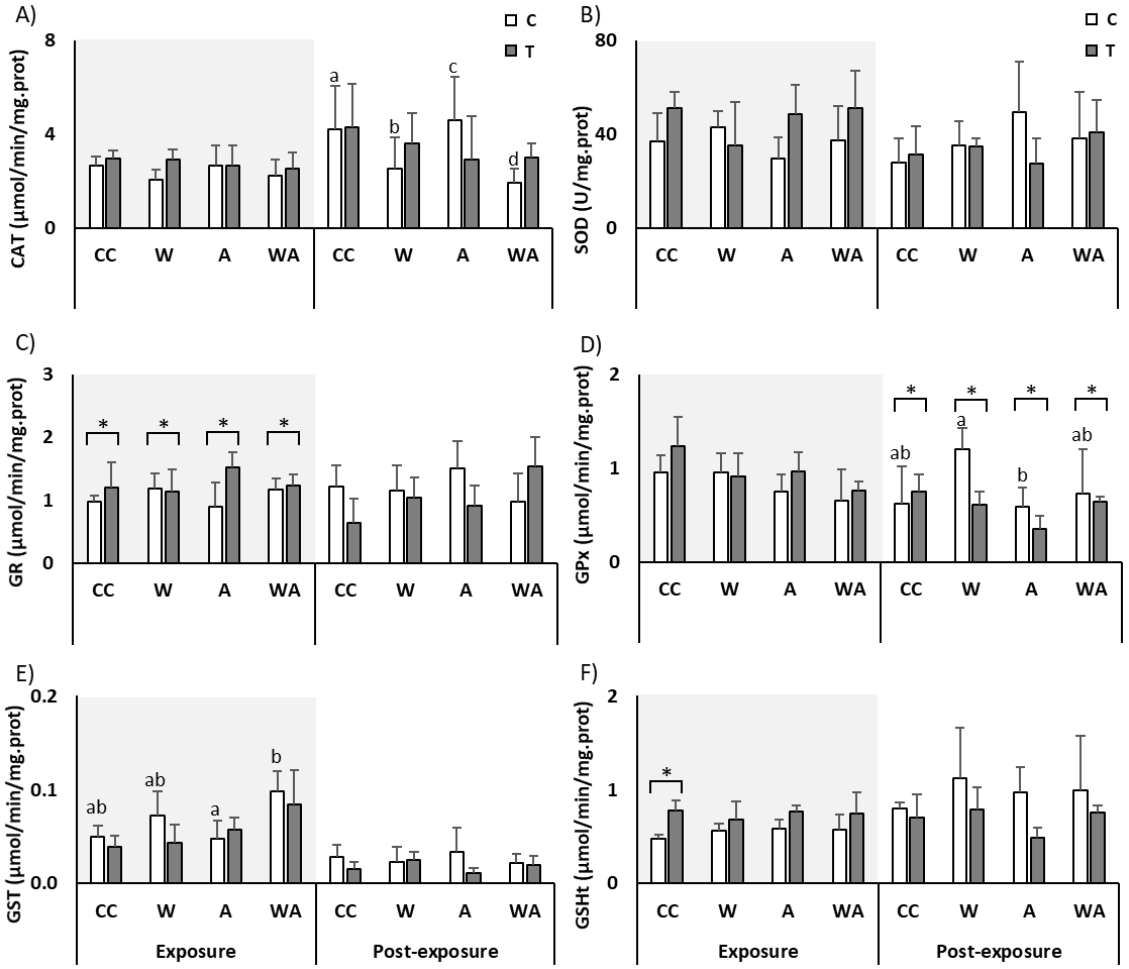

Fig. 5.3 Antioxidant responses (mean ± SD) in hepatopancreas of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Parameters include: A) catalase (CAT), B) superoxide dismutase (SOD), C) glutathione reductase (GR), D) glutathione peroxidase (GPx), E) glutathione-S-transferase (GST) activities, and F) total glutathione content (GSHt). Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis sp*.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

3.1.3. Peroxidative damage induction: LPO in gills

Mussels fed on non-toxic algae revealed no significant alterations on LPO levels when comparing the different environmental conditions on day 5, with the exception of an LPO decrease observed in group W comparing to CC (Fig. 5.4 and Table S4). However, on day 15 LPO increased in all altered conditions tested (W, A, and WA) *versus* CC, with particularly high levels being observed in mussels under W and A (also significantly higher than WA).

In the case of mussels exposed to *G. catenatum*, a significant increase of LPO values was observed in animals maintained under the combined effect of warming and acidification (WA) (Fig. 5.4 and Table S4). After replacing toxic diet by non-toxic algae, a significant increase in the LPO level was measured in mussels under CC conditions, while mussels maintained under the remaining environmental conditions (W, A, and WA) presented significantly lower values.

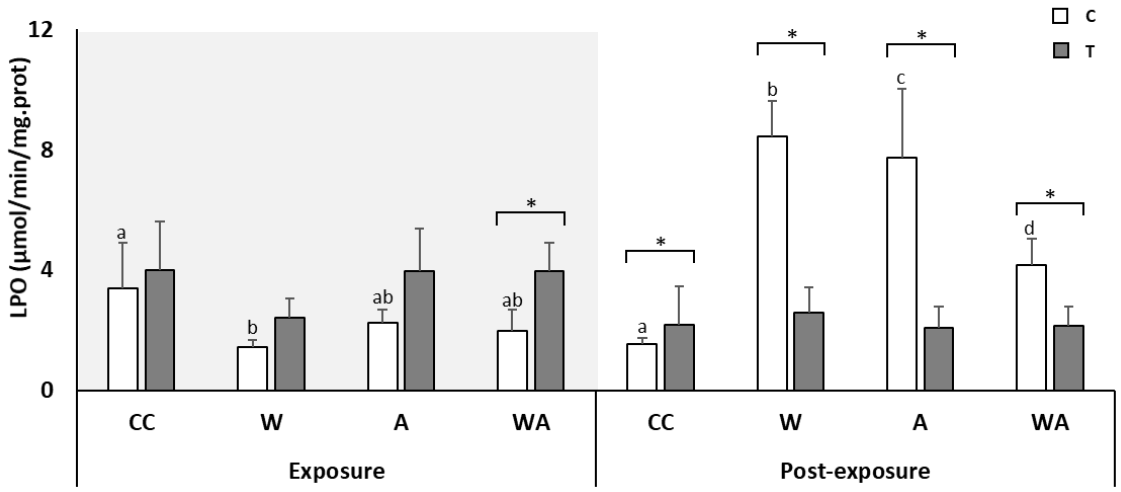

Fig. 5.4 Lipid peroxidation damage (LPO) (mean ± SD) in gills of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis sp.*) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

3.1.4. *Peroxidative damage induction:* LPO in hepatopancreas

When non-toxic algae constituted the diet, no significant changes in LPO levels were observed at any environmental condition (CC, W, A or WA) tested (Fig. 5.5 and Table S5). Also, no significant changes in LPO were observed in mussels exposed to *G. catenatum* in comparison with those unexposed (Fig. 5.5 and Table S5), in any condition.

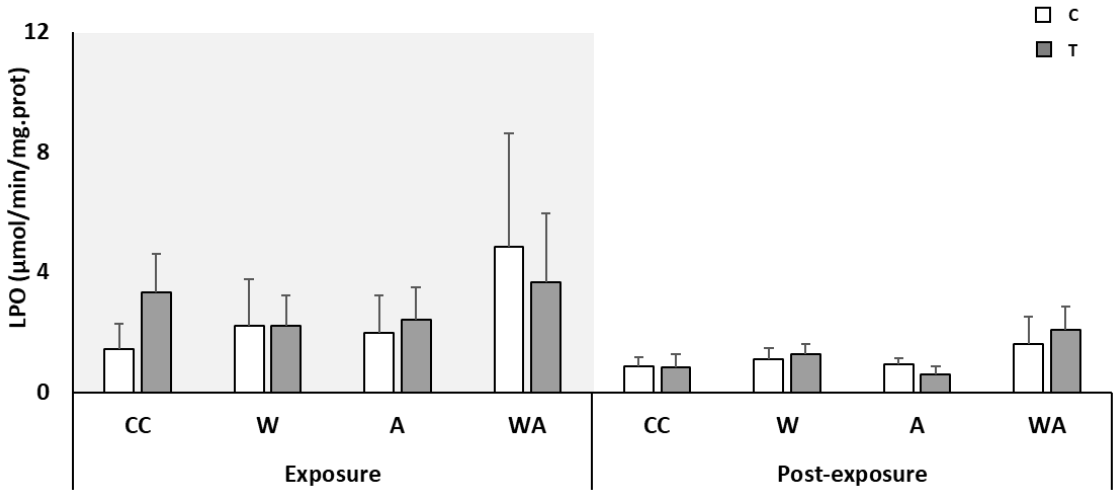

Fig. 5.5 Lipid peroxidation damage (LPO) (mean ± SD) in hepatopancreas of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis sp.*) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

3.2. Genetic damage in mussels exposed to Gymnodinium catenatum under different conditions of temperature and pH

3.2.1. *DNA damage induction* in gills

Mussels fed on non-toxic algae did not display significant changes in the genetic damage indicator (GDI) at any of the environmental condition that were tested (CC, W, A or WA) (Fig. 5.6 and Table S6). However, when the toxic dinoflagellate *G. catenatum* was introduced into mussels' diet, significantly higher values of GDI were observed in gills in all the environmental conditions tested, except for CC (Fig. 5.6 and Table S6). After replacing the toxic diet by non-toxic algae, simulating an elimination stage after an algae bloom, the GDI measured in the gills did not present significant differences in this experiment.

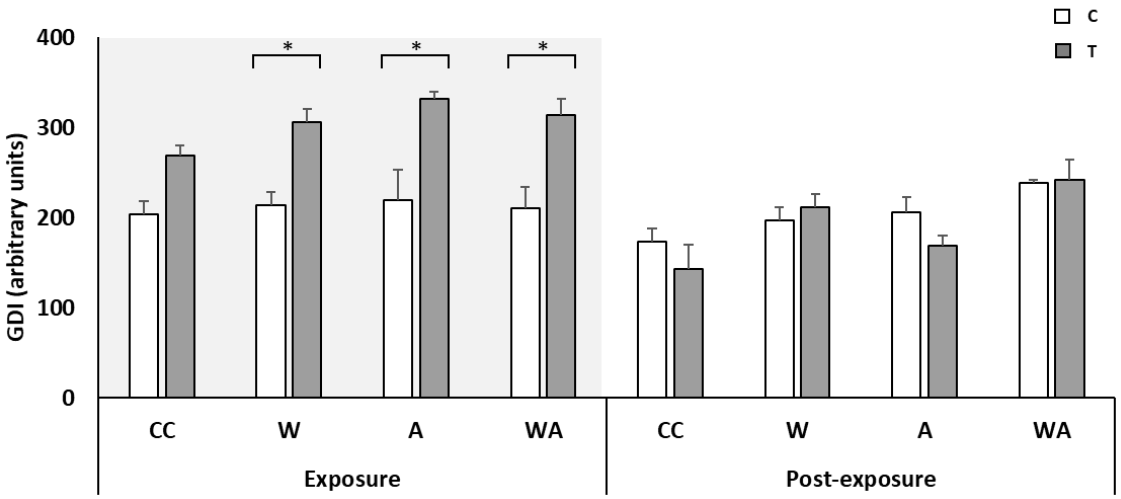

Fig. 5.6 Genetic Damage Indicator (GDI) (mean ± SD, expressed as arbitrary units) in gills of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis sp.*) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

3.2.2. DNA damage induction in hepatopancreas

Analysing the GDI levels in mussels fed on non-toxic algae according to the environmental scenario (CC, W, A or WA), significant differences were observed between all the conditions tested, on day 5, with mussels under A presenting the highest GDI (Fig.5.7 and Table S7). At the end of the experimental period, the highest GDI value was observed in mussels under WA, though all the altered conditions displayed higher DNA damage than CC.

Exposure to *G. catenatum* also produced significant changes in the hepatopancreas GDI, disclosing increased values in all the environmental conditions tested on day 5 (Fig. 5.7 and Table S7). On the recovery period, only mussels under WA presented significant differences, with an increase in the GDI value.

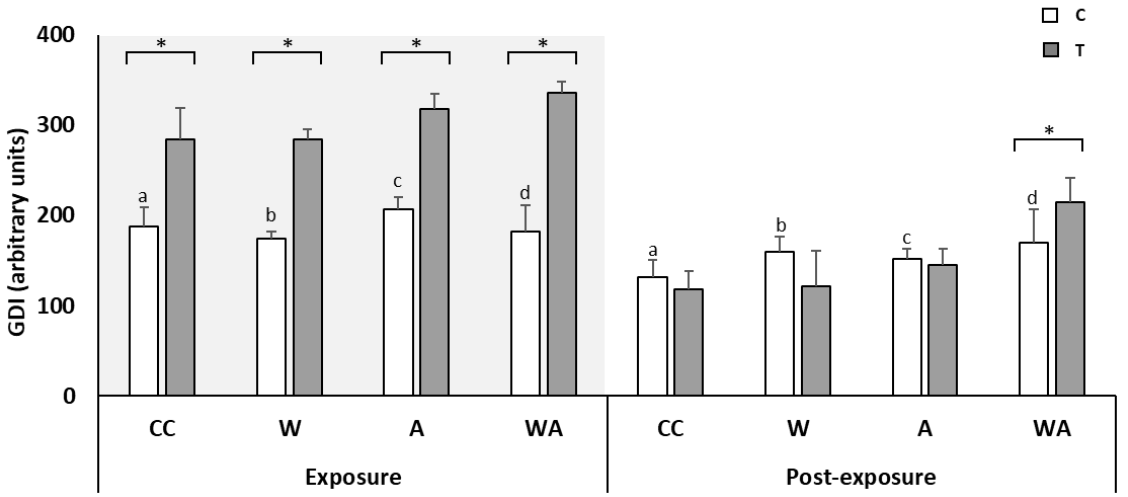

Fig. 5.7 Genetic Damage Indicator (GDI) (mean ± SD, expressed as arbitrary units) in hepatopancreas of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis sp*.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

4. Discussion

4.1. DNA damage and oxidative stress responses of mussels exposed to toxic algae Gymnodinium catenatum under the current conditions of seawater temperature and pH

The present work evaluated the biochemical and cytogenetic responses in two key organs, viz. gills and hepatopancreas, of mussels exposed to the toxic dinoflagellate *Gymnodinium catenatum*. Both gills and hepatopancreas antioxidant systems were modulated at the end of 5 days simulating the exposure to a toxic algal bloom.

Gills showed ambivalent responses of the antioxidant system, which, together with the absence of LPO and GDI increments, suggests a low risk of paralytic shellfish toxins

(PST). Gills are the first organ in contact with the toxic dinoflagellates, but toxins may only reach gills after systemic circulation or due to eventual cell lysis of *G. catenatum*. By this reason, the amount of toxins reaching the gills on day 5 were not high enough to substantially induce ROS production, promote changes on antioxidant activities (CAT, GR, and GST), and induce damage in key molecules like lipids and DNA.

On the other hand, the observed decrease in SOD activity and GSHt content may be an early sign of toxicity, as the induction of ROS and antioxidant responses is a relatively rapid phenomenon (Qiu et al., 2013). The antioxidant depletion reported could be related to SOD protein degradation, particularly when the presence of PST may compromise enzyme and co-factors renewal (Mat et al., 2013; Mello et al., 2013). As for GSHt, the overall responses of the glutathione redox cycle (GPx and GR) suggest the occurrence of active removal of GSSG from the cells (Fabioux et al., 2015; Kaplowitz, 1981; Soldatov et al., 2007). Also, GSH sequestration through the formation of PST-GSH conjugates may be occurring, as GSH is closely related to the PST biotransformation (Sakamoto et al., 2000; Sato et al., 2000). The increase of LPO detected 10 days after the diet change, disclosing a late effect, cannot be dissociated from the exposure route (toxin reaches the gills mainly systemically). It can also be a consequence of the above-hypothesised impairments occurring at the peak of exposure, affecting SOD action and glutathione redox cycle. Therefore, the statement that, under the current environmental conditions (CC), *G. catenatum* toxins do not possess a pro-oxidant potential on gill cells must be assumed cautiously. The hepatopancreas, which is the main toxins storage organ, showed an effective capacity to deal with PSTs. The observed increase of GSHt content can be related with the need for ROS elimination and with toxin metabolism and biotransformation. In addition, no LPO was detected throughout the experiment suggesting the hepatopancreas ability to cope with pro-oxidant challenges associated with PST.

Nevertheless, an increase in DNA damage was observed in the hepatopancreas at day-5 of the exposure period. The possible adaptation of this organ to cope with PST by increasing toxins biotransformation and elimination via glutathione-dependent pathways (as denunciated by the GSHt increase) does not seem enough to protect DNA integrity under high PST concentrations. In fact, the increasing damage detected in the DNA could be related to the glutathione-dependent elimination pathways. Several *in*

vitro studies indicated that GSH might be involved in shellfish transformation of PST analogues (Sakamoto et al., 2000; Sato et al., 2000). These studies reported a spontaneous reaction of several STX analogues after incubation with GSH, under heavy heating conditions (70 °C), resulting in chemically simpler compounds.

The role of GSH is usually related to the increase of hydrosolubility of xenobiotics, enhancing their elimination and decreasing their toxicity (Kaplowitz, 1981). In the case of PSTs, the structurally less complex compounds, products of toxins transformation, such as dcSTX and STX, present higher toxic potential. It is thus possible that the observed increase of DNA damage is related to the increase in glutathione content and lower elimination of the most potent PST. However, it is important to note that at the end of the experiment, when most of the toxins were eliminated, GDI decreased to control levels, pointing out the activation of DNA repair processes, through base excision repair (BER) and nucleotide excision repair (NER) mechanisms, and/or damaged cells turnover.

Exposure to the toxic algae seems to cause more damage in the DNA than in the lipids, as no significant variation was observed in LPO. This lack of LPO increase in the hepatopancreas, combined with low responsiveness of the antioxidant system, suggests that the DNA integrity loss, was not of oxidative origin and other mechanisms independent of ROS and oxidative stress may be causing the damage in the genetic material. Not much is known on how the toxins react with shellfish DNA, but, recently, oysters exposed to PST showed that DNA fragmentation associated apoptosis is a caspase-dependent process rather than an oxidative phenomenon (Abi-Khalil et al., 2017).

Gills and hepatopancreas seem to have different responses/vulnerabilities and mechanisms to cope with PST. This idea is in line with the differential PST metabolism theory proposed by Jaime et al. (2007) to justify distinct PST biotransformation in several tissues/organs of shellfish species.

4.2. Interference of warming and acidification on DNA damage and oxidative stress responses of mussels to toxic algae Gymnodinium catenatum

Several studies have been carried out to assess the impact of environmental changes in shellfish. Most of these studies were focused on a single stressor, such as the temperature, pH or salinity (Anestis et al., 2007; Múgica et al., 2015b; Nikinmaa and Anttila, 2015; Parker et al., 2013), and few approaches covered multiple factors or tried to understand their combined effects in bivalves (Freitas et al., 2017; Hu et al., 2015; Matozzo et al., 2013). In the present work, the effects of exposure to the toxic dinoflagellate *G. catenatum* were studied under scenarios of warming (W) and acidification (A), and with the two factors combined (warming and acidification - WA).

4.2.1. Modulation of mussels' DNA integrity and oxidative stress endpoints by warming and/or acidification

Mussels kept under warming (W) conditions and feeding on non-toxic algae only reflected alterations in the antioxidant system responses at the end of the experimental period. Despite some previous studies indicating that increased temperature affects mussels metabolic of mechanisms (Anestis et al., 2010, 2007), the results of this study are in accordance with data obtained by Kamel et al. (2012) showing no significant different antioxidant enzymes responses. It should be highlighted that a significant GDI reduction in hepatopancreas was observed at the peak of toxin accumulation (day 5) and the opposite variation at the end of the post-exposure period (day 15), suggesting an early activation of DNA repair mechanisms and their exhaustion with the continuation of the stimulus.

The effect of an acidified environment on the antioxidant system of mussels has been under debate, and while, some authors reported the induction of the activity of antioxidant enzymes (Hu et al., 2015; Matozzo et al., 2013), others suggested unaltered activities (Freitas et al., 2017; Nardi et al., 2017). In this study, a similar response was found in mussels under acidification (A) and W conditions, as no effects were detected in the antioxidant system. Regarding LPO, acidification seems to cause a delayed effect in the gills, but not affecting hepatopancreas, revealing a tissue-dependent response. However, acidification caused a GDI increase in hepatopancreas, suggesting that the

DNA repair machinery is unable to respond effectively to the stressor, which may lead to degradation of the population genetic information.

Under the combined effect of warming and acidification (WA), significant differences were only detected at the end of the experiment. Results obtained in the present work are in line with those reported by Hu et al. (2015) and Matozzo et al. (2013) where the antioxidant system response seems to increase with shellfish exposure to a higher temperature and lower pH levels, and where the significant interactions between the effects of the stressors were detected for some of the parameters tested, namely GPx. On the other hand, some studies are presenting contrasting information, with an opposite response in GPx modulation in the gills (Nardi et al., 2017), highlighting the need for more studies considering the organ-specific impacts of exposure to multiple stressors.

4.2.2. Effects of exposure to toxic algae under warming conditions

Environmental changes and their effects on marine organisms are a complex issue that results from the interaction of multiple factors, that may lead to either decreased (antagonism) or increased (synergism or potentiation) toxicity (Duarte et al., 2014; Lischka et al., 2011). The most purposive approach to shed light on the combined impact of toxins and warming, elucidating causality relationships and hypothetical factor interactions, goes through comparing the effects of both stressors acting simultaneously with those resulting from isolated exposures (see Supplementary material, Appendix D, Table S8, for a summary of all interactions). Hence, in what concerns gills and addressing first the antioxidant responses, it is clear that no relevant differences emerged (no interactions) as a result of the combination of factors (toxic algae exposure and warming). The exception was GPx at the PE, when an antagonistic interaction of both factors was evident, neutralising the activity elevation.

Regarding LPO in gills, the interpretation is more complex. No effect was detected in mussels fed with toxic algae under W, as was observed for CC, suggesting a dominance of the toxin-triggered processes. However, an antagonistic interaction (with mutual interference) was detected at the end of the experiment, when an LPO reduction was

measured, which contrast with induction of LPO measured when factors were evaluated individually.

Concerning genetic damage, the occurrence of a synergistic interaction on day 5, when simultaneous exposure induced DNA damage, must be highlighted. Indeed, it is important to recognise that while no interactions were detected for the antioxidant responses, the increase of GDI values was parallel to the increase in GSHt content in mussels gills exposed to the toxic algae. This increase in GDI levels is an environmentally relevant finding, highlighting the increased risk of mussels exposed to the interaction of harmful algal blooms and warming.

On the other hand, no signs of increased risk resulting from the concomitant exposure to toxins and warming were observed from the LPO analysis in hepatopancreas. Overall, deeming the damage indicators LPO and GDI, gills showed to be more sensitive to the interaction of environmental factors than the hepatopancreas, reinforcing the previous findings and stating the existence of organ-specific response patterns.

4.2.3. Effects of exposure to toxic algae under acidification conditions

Mussels under the combined impact of toxic algae and acidification showed relevant differences on the gills' antioxidant responses when compared to the effects observed in mussels exposed to non-toxic algae under the acidification treatment (Table S8). The interactions detected for these parameters while measurable do not fit the typified patterns (*i.e*., antagonism, synergism and potentiation). In terms of LPO, which is indicative of damage mainly on cellular membranes, an antagonistic effect was found at the end of the experiment in gills. This antagonistic response is related to the activation of repair mechanisms to minimise the membrane damage (Fabioux et al., 2015; Guilherme et al., 2008b). However, these repair mechanisms may be specific for LPO damage as no clear sign of apoptosis, as a reduction of DGI, was observed. At the peak of toxin exposure (day 5), a significant increase in DGI was observed under acidification, indicating a genotoxicity potentiation under these conditions.

It is noteworthy that the simultaneous increase of GSHt and GDI previously found in the hepatopancreas of mussels exposed to *G. catenatum* under CC was also observed in

the gills of mussels exposed to the toxic algae under warming and under acidification conditions. This result emphasises the existence of a possible relation between the role of GSHt in toxins elimination and the increased DNA damage, as suggested above, reinforcing the idea that glutathione-dependent elimination pathways may increase the risk to shellfish when PSP analogues are concerned (Sakamoto et al., 2000; Sato et al., 2000).

The only significant response in hepatopancreas after 5 days of exposure to the toxic algae under acidification was GR activity, which seems to be modulated by the toxin presence since it was similar to the response obtained in mussels exposed to the toxins under CC. Also, the genetic damage was clearly modulated by the toxin presence with the increase of GDI. In this case, an underlying addictive effect may be occurring since mussels exposed to acidification and toxins independently already revealed a significant increase in genetic damage.

4.2.4. Effects of exposure to toxic algae under combined conditions of warming and acidification

The combined effect of exposure to the three stressors (W + A + Toxin) did not originate any relevant interaction in the antioxidant response system of gills at the peak of toxic algae exposure. The main differences in the gills' response profile were observed for LPO and GDI. A significant increase in both damages was found after exposure to toxic algae, warming and acidification due to the stressors' interaction. This interaction profile, probably, fits better on the additivity then on synergism and reveals that under combined exposure to warming and acidification mussels' gills are particularly vulnerable to PST. These responses changed at the end of the post-exposure period when only LPO exhibited interaction effects. Interestingly, a pattern was perceptible, revealing that after 10 days of toxin elimination, under any of the altered environmental conditions (W, A or WA), there was an antagonistic effect in the gills, presented as a decrease of LPO levels. This pattern may be associated with LPO damage repair, though the lack of DNA damage and the reduced response exhibited by the antioxidant system pointed out the cell renewal as a possible cause to this decrease. The late effects detected in LPO may be related with the need of repairing the DNA damage observed at

the peak of toxin accumulation, or it may be related with toxin-triggering apoptosis mechanisms (Abi-Khalil et al., 2017, 2016). Again, the reduced antioxidant response coupled with the LPO and DNA damage modulation suggests that the PST affected the gills cells through several pathways.

In the hepatopancreas, the responses showed a clear organ-specific pattern. An antagonistic response was observed for GSHt on day 5. Despite the increase in the co-factor content of mussels fed with toxic algae under CC, no response was observed under the combined effect of exposure to toxic algae and WA. These results emphasise the complexity of the interaction effects and highlight the need for a better understanding of how GSH and PST react with each other. The involvement of GSH in the toxin metabolism and biotransformation may also be related to the toxin elimination (Braga et al. 2018). Therefore, studies analysing the individual GSH and GSSG contents with the assessment of a potential formation of PST-GSH conjugates under different environmental conditions may be essential to understand the toxins effects and the elimination process in mussels.

While the antioxidant system responses were modulated by the environmental conditions during exposure, the results observed for GDI levels, support a toxin-triggered process dominance, as increased damage was observed in mussels under CC and in mussels under WA when the toxic algae were introduced.

Notwithstanding, at the end of the post-exposure period, an antagonistic response was observed in the antioxidant system. The GPx activity, which decreased in mussels under WA, was induced in mussels under CC. GPx activity decrease may have been related either with enzyme exhaustion, due to a continuing stimulus; or with recovery to basal levels, due to reduction of the stressor, since during post-exposure toxin elimination occurred in the mussels (Braga et al., 2018). The latter is, however, the most likely hypothesis since GPx exhaustion would lead to a significant increase in LPO damage, not detected in the data (Fabioux et al., 2015; Guilherme et al., 2008b).

An increase in ROS content could have explained the increase of GDI levels occurring at this stage. However, as referred above, the data analysis does not point to an increase in ROS, on the contrary, as no LPO was not observed, and in the antioxidant system, only GPx was altered, ROS seems to be decreasing in the cells. Therefore, this study reveals that under combined warming and acidification, mussels' capacity for recovery and

repair of DNA damage is compromised. Nevertheless, further studies are needed to understand whether the impairment of DNA recovery capacity is due to the reduction of DNA repair mechanisms, or due to a decrease in cell turnover and apoptosis. The inhibition of repair enzymes has been described in mussels for other xenobiotics, such as Cd, which interferes in the final ligation step of BER, leading to the accumulation of DNA breaks in mussels (Emmanouil et al., 2007; Lynn et al., 1997). So, PST interference on BER and NER mechanisms should not be discarded, especially when these toxins are known to cause enzyme inhibition or transcription modulation (Mat et al., 2013; Mello et al., 2013).

Overall, the integrity of the genetic information was seriously compromised with the synergetic effects caused by the exposure of mussels to *G. catenatum* under projected conditions of warming and acidification.

5. Conclusions

To the best of our knowledge, this is the first study addressing the biochemical and cytogenetic responses of mussels *Mytilus galloprovincialis,* exposed to the toxin-producing dinoflagellate *Gymnodinium catenatum*, under four environmental conditions simulating current and predicted environmental conditions of seawater warming and acidification.

Overall, exposure to *Gymnodinium catenatum* under CC caused an active modulation of the antioxidant systems in both organs, which have different responses/vulnerabilities and mechanisms to cope with the paralytic shellfish toxins (PST). Under CC, the toxins systemic distribution in gills seems to contribute to a low risk of incurring genetic damage from PST exposure. In contrast, hepatopancreas, which seems to be better adapted to cope with the pro-oxidant challenges, was more prone to genetic damage, suggesting that PST originate non-oxidative damage in this organ cells.

The different responses in mussels exposed to PSTs under one single environmental condition (W or A) and those observed under combined environmental conditions (WA) highlights the importance and need for more studies focusing on the interaction of exposure to multiple stressors. Under altered environmental conditions (W, A or WA),

toxin accumulation caused an increase in the genetic damage both in gills and hepatopancreas, with the non-correspondent response of LPO and the antioxidant system s, reinforcing that non-oxidative DNA damage might be occurring. Living under WA conditions increased mussels vulnerability to PST, with a dominance of toxin-triggered processes and the DNA damage increase, compromising the integrity of the genetic information and the mussels' DNA repair capacity.

Finally, this study highlights the role of defence mechanisms protecting shellfish from marine biotoxins, such as saxitoxin, remarking that exposure to multiple stressors predicted in a climate change scenario coupled with the increasing harmful algal blooms potentiates the risk to shellfish *Mytilus galloprovincialis* populations.

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Chapter 6

In vitro bioaccessibility of the marine biotoxin okadaic acid in shellfish

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In vitro bioaccessibility of the marine biotoxin okadaic acid in shellfish

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Abstract

Okadaic acid (OA) and their derivatives are marine toxins responsible for the human diarrhetic shellfish poisoning (DSP). To date the amount of toxins ingested in food has been considered equal to the amount of toxins available for uptake by the human body. In this study, the OA fraction released from the food matrix into the digestive fluids (bioaccessibility) was assessed using a static *in vitro* digestion model. Naturally contaminated mussels (*Mytilus galloprovincialis*) and donax clams (*Donax sp*.), collected from the Portuguese coast, containing OA and dinophysistoxin-3 (DTX3) were used in this study. Bioaccessibility of OA total content was 88 % and 75 % in mussels and donax clams, respectively. Conversion of DTX3 into its parent compound was verified during the simulated digestive process and no degradation of these toxins was found during the process. This is the first study assessing the bioaccessibility of OA-group toxins in naturally contaminated seafood. This study provides relevant new data that can improve and lead to more accurate food safety risk assessment studies concerning these toxins.

Keywords: Diarrhetic shellfish poisoning, okadaic acid, dinophysistoxins, toxicity, mussels, clams

1. Introduction

Diarrhetic shellfish poisoning (DSP) is a human food-borne illness characterized by gastrointestinal disorders with acute symptoms including diarrhea, nausea, abdominal pain and vomiting. DSP is caused by the ingestion of shellfish containing okadaic acid (OA) and its derivatives, which are the most frequent and abundant marine toxins regularly found in shellfish from southern European coastal areas (Vale et al., 2008). Marine toxins are produced by specific phytoplankton species (e.g. dinoflagellates) and accumulate to great extent in filter-feeding bivalves, such as mussels, clams, oysters and cockles. In order to protect consumers and minimize the risks of acute intoxications, shellfish cannot be harvested and marketed in EU countries when OA-group toxins levels exceed 160 µg OA equivalents per kg of shellfish meat (Regulation (CE) n^{o} 853/2004).

OA and its isomeric compounds, dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2) are polyether carboxylic acids and heat stable lipophilic toxins (Marr et al., 1992). Biotransformation of these compounds in shellfish tissues commonly results in a complex mixture of 7-*O*-acyl esters derivatives, collectively referred as dinophysistoxin-3 (DTX3) (Vale and Sampayo, 2002a). OA and its related toxins are potent serine/threonine protein phosphatase inhibitors leading to alterations in several intracellular processes and causing severe mucosal damages in the intestinal tract (Matias et al., 1999). Moreover, OA has been considered as a potent tumor promoter on mouse skin (Suganuma et al., 1988).

So far, quantitative data on OA toxicokinetics in humans is scarce. Most studies regarding the distribution and dynamics of ingested OA have been performed with rodents. Among these studies, most have focused on the acute toxicity following injection of the toxic compound directly in the abdominal or peritoneal cavity in mice. This is the case of the toxicity equivalence factors (TEF) for OA, DTX1 and DTX2, estimated based on intra peritoneal injection of toxins (Aune et al., 2007; EFSA, 2008). From the few studies involving oral exposure to OA, it is important to highlight that when mice are exposed to OA concentrations of 50 µg kg^{-1} only 59 % of the toxin is accumulated in the gastrointestinal tract (Matias et al., 1999). Yet, OA standard dissolved in water and methanol was orally administered by these authors instead of a naturally contaminated food matrix. The difference between the amount of toxin

ingested and assimilated depends on several processes, including the bioaccessibility, i.e. the release of compounds from the food matrix during the digestive process that can potentially be absorbed by the gut epithelia (Metian et al., 2009; Versantvoort et al., 2005).

To the best of our knowledge, bioaccessibility of OA-group toxins has not been studied so far. Despite the concentrations of toxins in shellfish for human consumption is subjected to routine monitoring programs all over the world, determining the quantity of toxins released from the food matrix during the digestive process is still poorly understood. Bioaccessibility provides more accurate data for the evaluation of human health risk assessment to food safety authorities (Cardoso et al., 2015). Due to the great improvement of *in vitro* methodologies that simulate the human digestion by mimicking the complexity of the real process (Guerra et al., 2012), it is now possible to evaluate the bioaccessibility of toxins in seafood. Bioaccessibility studies in shellfish has been profusely explored as far as essential trace elements (e.g. selenium, manganese, copper and zinc) (Calatayud et al., 2012; He and Wang, 2013; Laird and Chan, 2013; Marval-León et al., 2014) and chemical contaminants from both natural and anthropogenic sources are concerned (e.g. polycyclic aromatic hydrocarbons (PAHs) and toxic metals) (Amiard et al., 2008; Cano-Sancho et al., 2015; Gao and Wang, 2014; He et al., 2015; Intawongse et al., 2012; Leufroy et al., 2012; Maulvault et al., 2011; Yu et al., 2011). In this context, the aim of the present study was to assess the bioaccessibility of OA-group toxins in two of the main shellfish species (mussels *Mytilus galloprovincialis* and donax clams *Donax* sp.) widely consumed in Southern Europe and recurrently contaminated with DSP toxins.

2. Materials and methods

2.1. Collection of shellfish and sample preparation:

Five samples of naturally contaminated mussels (*Mytilus galloprovincialis*) and donax clams (*Donax spp.*) were collected between April and May 2014 in shellfish producing areas of the Southern Portuguese coast. Each sample was composed by at least 30 individuals. Samples were obtained through the Portuguese Monitoring Program for Marine Biotoxins carried out by the Portuguese Institute for the Sea and

Atmosphere (IPMA). Bivalves were opened, removed from the shell, washed with running water to remove any residue (e.g. sand), properly drained, homogenized with a blender and stored at -20 ˚C until further analysis.

2.2. In vitro human digestion model

2.2.1. Reagents

Inorganic and organic components used to prepare the digestion fluids were the following: KCl (Merck, 99.5 % m/v), KSCN (Sigma, P2713), NaH_2PO_4 (Merck, 99.5 % m/v), Na_2SO_4 (Merck, 90 % m/v), NaCl (Merck, 99.5 % m/v), $NaHCO_3$ (Merck, 99.5 % m/v), $CaCl_2.2H_2O$ (Sigma, C3881), (Merck, 99.5 % m/v), Na_2SO_4 (Merck, 90 % m/v), NaCl (Merck, 99.5 %) m/v, NaHCO3 (Merck, 99.5 % m/v), NH_4Cl (Riedel-de Haën, 99.5 % m/v, KH_2PO_4 (Merck, 99.5 %), $MgCl_2$ (Riedel-de Haën, 99.5 % m/v), HCl (Merck, 37 % m/v), urea (Sigma, U5128), glucose (Sigma, G5400), glucuronic acid (Sigma, G5269), D-(+)-Glucosamine hydrochloride (Sigma, G4875), uric acid (Sigma, U2625), albumin from bovine serum (Sigma, A7906), α-amylase, from Aspergillus oryzae (Sigma, 86250), mucin from porcine stomach (Sigma, M2378), pepsin from porcine stomach mucosa (Sigma, P7125), lipase from porcine pancreas, type II (Sigma, L3126), pancreatin from porcine pancreas (Sigma, P8096), trypsin from porcine pancreas (Sigma, T6567), α-chymotrypsin from bovine pancreas (Sigma, C4129) and bile porcine extract (Sigma, B8631).

2.2.2. Digestion protocol

The OA bioaccessibility was assessed using a static *in vitro* human digestion protocol adapted from Versantvoort et al. (2005). The simulated gastro-intestinal digestion was performed in three different phases: oral, gastric and intestinal, using the following simulated digestion fluids: saliva, gastric juice, duodenal juice and bile. A 1.5 g aliquot of each shellfish sample was *in vitro* digested in duplicate. Samples were digested using a rotator tube mixer (25 rpm; LSCI, Portugal) at 37 ºC throughout the entire experiment (see experimental setup in Fig. 6.1). Each sample was digested with the following sequential digestion phases in the same tube: oral (4 mL of saliva fluid, 5 min, pH 7.0 ± 0.2), stomach (8 mL of gastric fluid, 2 hrs, pH 2.0 ± 0.2) and intestinal (8 mL of duodenal

juice, 4 mL of bile juice, 2 hrs, pH 7.0 ± 0.2) (see details in Table 6.1). In each digestion phase, the pH was adjusted before the respective digestion step.

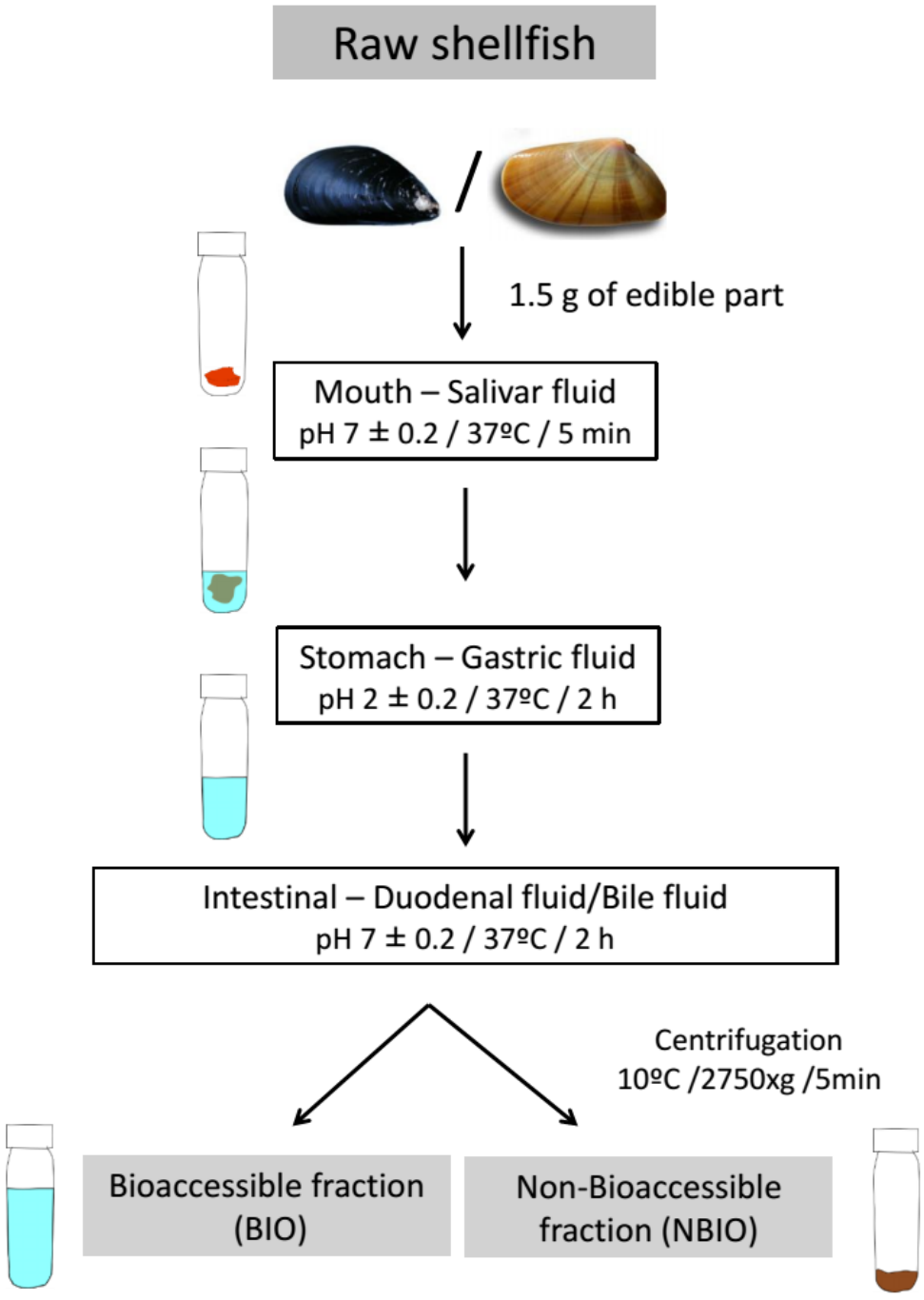

Fig. 6.1 Scheme of the *in vitro* digestion protocol used to access the OA-group toxins bioaccessibility.

At the end, samples were kept at 4 ºC for 10 minutes to stop the digestion and centrifuged at 2,750 × *g* during 10 min at 10 ºC to separate the bioaccessible fraction (BIO) from the sample residues (non-bioaccessible fraction – NBIO).

Table 6.1 Composition of simulated digestion fluids used for the *in vitro* digestion protocol. The volumes (mL) and mass (mg) are calculated for a volume of 100 mL for each simulated digestion fluid.

		Oral phase	**Gastric phase**	**Intestinal phase**	
		Saliva	**Gastric juice**	**Duodenal juice**	**Bile**
	Components (stock concentration)	**Volume of stock (mL)**	**Volume of stock (mL)**	**Volume of stock (mL)**	**Volume of stock (mL)**
Inorganic and organic components	KCl (89,6 g/L)	2.0	1.8	1.2	0.8
	KSCN (20 g/L)	2.0	-	-	-
	NaH_2PO_4 (88,8 g/L)	2.0	0.6	-	-
	$NaSO_4$ (57 g/L)	2.0	-	-	-
	NaCl (175,3 g/L)	0.4	3.1	8.0	6.0
	$NaHCO_3$ (84,7 g/L)	4.0	-	1.33*	13.6
	$CaCl_2.2H_2O$ (22,2 g/L)	-	3.6	0.144*	0.080*
	NH_4Cl (30,6 g/L)	-	2.0	-	-
	KH_2PO_4 (8 g/L)	-	-	2.0	-
	$MgCl_2$ (5 g/L)	-	-	2.0	-
	Urea (25 g/L)	1.6	0.7	0.8	2.0
	Glucuronic acid (2g/L)	-	2.0	-	-
	Glucose (65 g/L)	-	2.0	-	-
	Glucoseamine hydrochloride (33 g/L)	-	2.0	-	-
	Bioactive component	**Mass of stock (mg)**	**Mass of stock (mg)**	**Mass of stock (mg)**	**Mass of stock (mg)**
Bioactive components	α-amilase	180	-	-	-
	Uric acid	3	-	-	-
	Mucin	5	-	-	-
	BSA	-	200	200	360
	Pepsin	-	500	-	-
	Pancreatin	-	600	1800	-
	Lipase	-	-	300	-
	Trypsin	-	-	3.2	-
	α-chymotrypsin	-	-	34.8	-
	Bile	-	-	-	6000

* volume added to each *in vitro* digestion reaction (mixture of simulated digestion fluid and food) – as precipitation may occur.

2.2.3. Digestion efficiency

To confirm the *in vitro* digestion efficiency, total protein levels were determined in the shellfish before digestion, and in the bioaccessible (BIO) and non-bioaccessible (NBIO) fractions using a nitrogen analyzer (FP-528 DSP, LECO, St. Joseph, USA; limit of detection = 0.84 mg N). The calibration standard curve was performed with EDTA following the methodology described by Saint-Denis and Goupy (2004).

Protein recovery (%) was defined as the following ratio: (BIO + NBIO) × 100/BD, where BIO + NBIO are the sum of protein levels detected in the bioaccessible (BIO) and non-bioaccessible (NBIO) fractions, and BD is the amount of protein detected in shellfish before digestion. Protein recovery (%) obtained in mussels and donax clams after the digestion process was 101.25 ± 10.67 and 106.25 ± 13.81, respectively.

The protein levels in the bioaccessible fraction (%) were calculated as the following ratio: BIO × 100/ (BIO + NBIO). In contrast, the protein levels in the non-bioaccessible fraction (%) were calculated as the following ratio: NBIO × 100/ (BIO + NBIO). Protein levels (%) in the bioaccessible fraction were 85.2 ± 12.3 (mussel) and 87.3 ± 4.7 (clam), respectively.

2.3. Toxins analysis

2.3.1. Reagents

All reagents used for toxins extraction and analysis were of analytical grade or higher. Methanol (p.a. ≥ 99.8 %) was obtained from Sigma-Aldrich; Methanol (LC-MS grade), Acetonitrile (LC-MS grade) and Ammonium formate (≥ 99 % purity) were obtained from Fluka; Formic acid (98 % p.a.) from Roth; Hydrochloric acid (37 %) from Panreac; and Sodium hydroxide (≥ 99 % p.a.) was obtained from Merck. Water was purified using a Milli-Q 185 Plus system from Millipore. Certified reference standard solutions of Okadaic Acid (CRM-OA-c), Dinophysistoxin 1 (CRM-DTX1) and Dinophysistoxin 2 (CRM-DTX2) were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council (NRC, Canada).

2.3.2. Toxins extraction

Extraction of OA-group toxins, hydrolysis and their determination via liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection was carried out following the Standardised Operating Procedure of the European Reference Laboratory for Marine Biotoxins (EURLMB) for the determination of marine lipophilic biotoxins in bivalve mollusks (EURLMB, 2015). Briefly, a 2 g aliquot of shellfish meat homogenate and the non digested sample residues (NBIO) were extracted with 9.0 mL MeOH by vortexing for 2 min. After centrifugation at 2,000 × g for 10 min, the resulting supernatant was transferred to a new 30 mL centrifuge tube. The remaining tissue was re-extracted with 9.0 mL MeOH and homogenized with a Polytron mixer (Kinematica, Switzerland). After centrifugation, supernatants were combined and the volume adjusted to 20 mL with MeOH. An aliquot of this extract was filtered through a 0.2 µm syringe filter and 10 µL injected to the LC-MS/MS system for determination of free OA, DTX1 and DTX2.

An alkaline hydrolysis was performed to determine the total content of OA-group of toxins by converting the 7-O-acyl ester derivatives (DTX3) into their parent toxin. The hydrolysis was started by adding 313 µL of 2.5M NaOH to a 2.5 mL aliquot of the methanolic extract in a test tube, which was homogenized for 30 seconds in the vortex and heated at 76 ˚C for 40 min in a heating block. The sample was let to cool down until reaching room temperature and neutralized with 313 µL of 2.5M HCl. The sample was vortex for 30 seconds and an aliquot was filtered through 0.2 µm syringe filter and 10 µL injected to the LC-MS/MS system.

Toxins were extracted from a 3.0 mL aliquot of digestive juice samples (bioaccessible fraction – BIO) with 5.0 mL dichloromethane (CH2Cl2). After vortexing for 1 min the sample was centrifuged (2,000 × g for 2 min) and the aqueous phase discarded. The organic phase was evaporated to dryness at 40 ˚C under reduced pressure in an evaporation system (RapidVap, Labconco, USA). The extract was re-constituted in 3 mL MeOH. An aliquot was filtered and injected to the LC-MS/MS system and another aliquot was hydrolyzed before injected to chromatographic system, in order to determine the free and total content of OA-group of toxins, respectively. The CH2Cl2 extraction that

constitutes a modification to the method demonstrated acceptable recovery (93 ± 7 %) after spiking experiments with OA.

2.3.3. Toxins determination by LC-MS/MS

The LC-MS/MS equipment consisted of a Dionex Ultimate 3000 LC system (Thermo Scientific) coupled to a triple quadrupole mass spectrometer TSQ Quantum Access Max (Thermo Scientific) equipped with a heated electrospray ionisation source HESI-II. The chromatographic separation was performed using an Accucore reverse phase-mass spectrometry (RP-MS) column (150 × 2.1 mm, 2.6 µm), protected with a guard column Accucore RP-MS (10 × 2.1, 2.6 µm). The mobile phase A was water with 2 mM ammonium formate and 50 mM formic acid, and the mobile phase B was 95 % acetonitrile with 2 mM ammonium formate and 50 mM formic acid. An elution gradient at a flow rate of 0.4 mL min-1 was used as follows: 0-1 min, gradient from 50 - 0 % eluent A; 1-4 min 0 % eluent A; 4-5 min gradient 0 – 50 % eluent A; 5-6 min, 50 % eluent A. The detection was carried out in Multiple Reaction Monitoring (MRM) acquisition mode. Two MRM transitions were monitored in negative polarity: m/z 803>255 for OA and DTX2 quantification and m/z 803>563 for OA and DTX2 confirmation; m/z 817>255 for DTX1 quantification and m/z 817>563 for DTX1 confirmation. Calibration standard solutions included the following concentration levels: 2.0, 7.1, 20.3, 40.6, 68.0 ng mL-1. The limit of detection was 0.5 ng OA mL-1.

The toxicity equivalent factors stated by EFSA (2008) were used for calculation of OA-group toxins in terms of okadaic acid equivalents: OA = 1, DTX1 = 1, DTX2 = 0.6. Although toxicity of DTX3 is considered lower, the TEF values for DTX3 are equal to those of the corresponding parent toxins (OA, DTX1, and DTX2) (EFSA, 2008).

2.4. Statistics

Data analysis was performed using the statistical program SigmaPlot Version 10.0. One-way ANOVA followed by Tukey HSD test were performed to test significant differences between the toxin in the food matrix (BD), bioaccessible (BIO) and non-bioaccessible (N-BIO) fractions. Data were previously tested for normality and

homogeneity of variance by the Kolmogorov-Smirnov test and the Levene Median test. The t-Student's test was performed to test significant differences between two groups of samples, the toxin in the food matrix, and the total digested toxin (the some of bioaccessible and non-bioaccessible fractions). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Toxin analysis of the shellfish samples

Toxicity levels varied from 402 to 1,530 OA equiv. kg^{-1} in mussel samples and from 231 to 395 OA equiv. kg^{-1} in donax clams (Table 6.2). Only OA and its ester derivatives (DTX3) were detected. While mussels were characterized by a predominance of OA, donax clams showed higher content of DTX3.

Table 6.2 Okadaic acid (OA) and dinophysistoxin-3 (DTX3) in mussel (*Mytilus galloprovincialis*) and donax clam (*Donax sp.*) samples collected in the Portuguese coast.

Species	**Harvest date**	**OA equiv. ($\mu g.kg^{-1}$)**	**Toxin Molar Fraction (%)**	
			OA	**DTX3**
Mussel	Mar-14	1,530	58.4	41.6
	Apr-14	402	67.4	32.6
	Apr-14	613	76.8	23.2
	Apr-14	764	60.1	39.9
	May-14	473	90.5	9.5
Donax clam	Apr-14	231	31.7	68.3
	Apr-14	310	37.7	62.3
	Apr-14	322	24.3	75.7
	May-14	229	39.6	60.4
	May-14	395	29.5	70.5

3.2. Okadaic acid bioaccessibility

High percentages of bioaccessible OA were found in both shellfish species. OA bioaccessibility estimated with this in vitro model revealed that 87.7 ± 6.2 % and 75.3 ± 9.4 % of OA total content is released into the digestive juice from mussels and donax clam matrices, respectively (Fig. 6.2). While a tendency for lower bioaccessibility of OA might be associated with donax clams, no significant differences were assigned between species ($p > 0.05$). The remaining amount of toxins, not released during the digestive process into the gastrointestinal tract, was found in sample non-digestible residues (N-BIO) (data not shown).

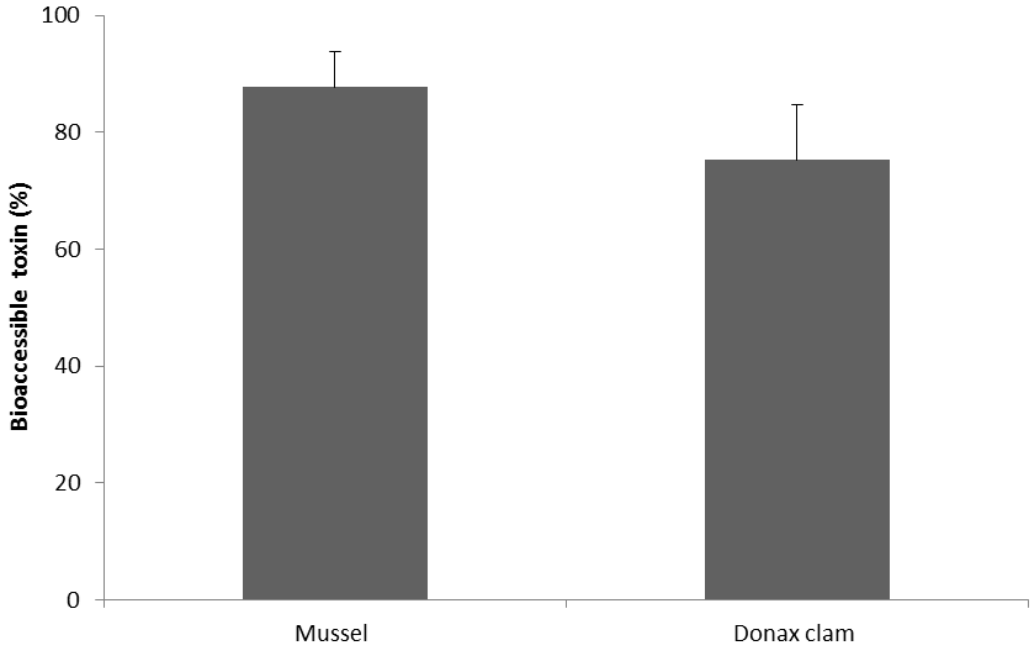

Fig. 6.2 : Bioaccessibility (%, mean ± SD) of OA total content in mussel (Mytilus galloprovincialis) and donax clam (Donax sp.) samples

In terms of molar fraction, a marked modification of the toxins profile was observed between raw shellfish meat and the digested samples in both species (Fig. 6.3). A significant reduction of DTX3 ($p < 0.05$) and a slight increase of OA were observed in the bioaccessible fraction. The lower levels of DTX3 in this fraction seem to indicate that these derivatives are not released from the food matrix. However, the small amounts of toxins detected in the non-digestible fraction were mostly composed by OA, suggesting

that OA ester derivatives undergo an enzymatic conversion into OA in the digestive juice (Fig. 6.3).

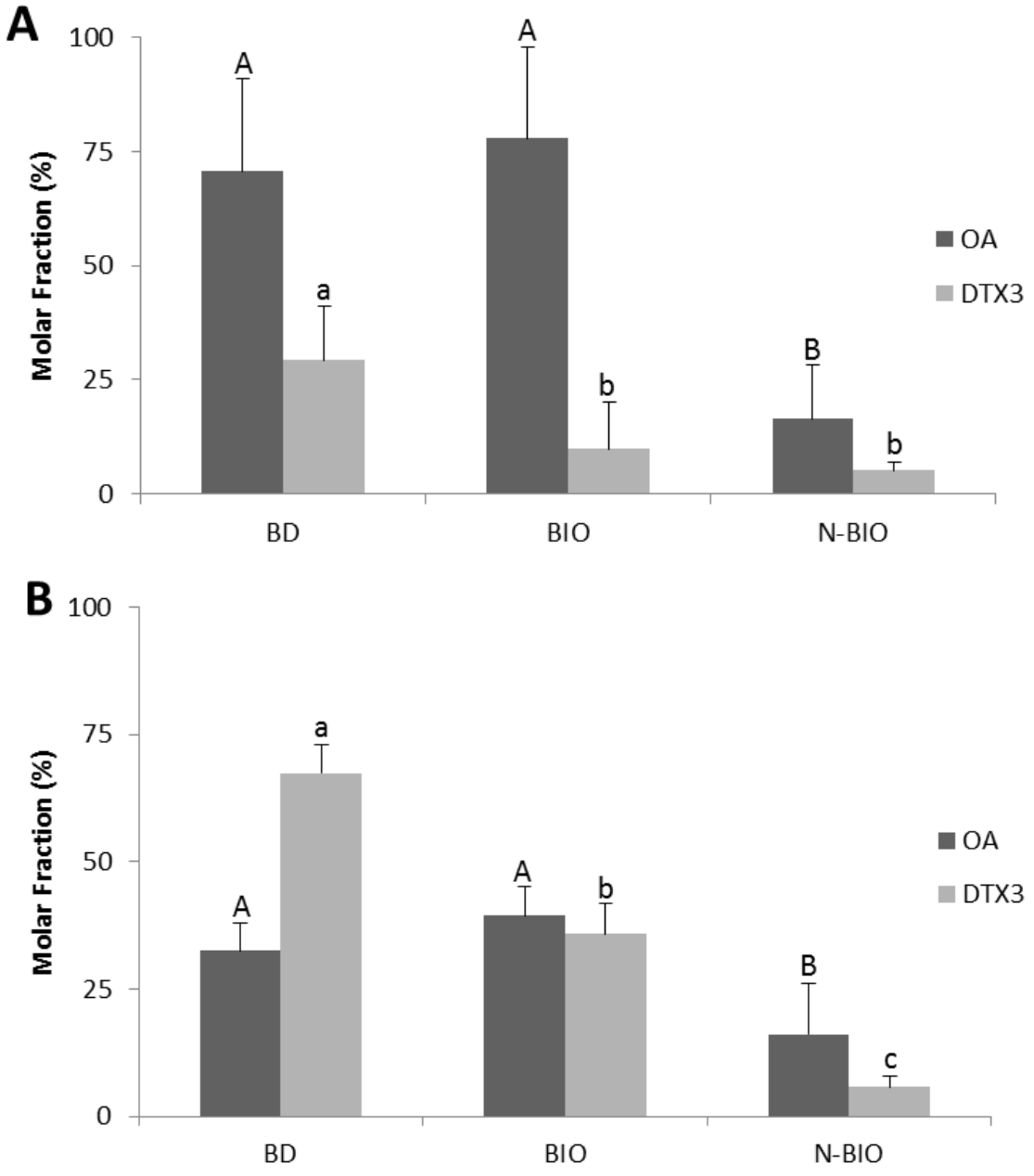

Fig. 6.3 Profile of OA-group toxins (%, mean ± SD) in raw shellfish matrix (BD) and in the bioaccessible (BIO) and non-bioaccessible (N-BIO) fractions for: A) mussels; and B) donax clams. Different upper case letters represent significant differences in OA levels ($p < 0.05$) and different lower case letters represent significant differences in DTX3 levels ($p < 0.05$).

The degradation of toxins was not observed through the digestive process, since no significant differences ($p > 0.05$) were observed between the total amount of OA-group toxins in the shellfish seafood matrix and the sum of these toxins found in the bioaccessible fraction (BIO) and in residues after digestion (NBIO).

4. Discussion

To the best of our knowledge, this study is the first study to assess the bioaccessibility of OA-group toxins in seafood. The bioaccessibility determined by the *in vitro* digestion model indicate that most OA and derivatives are released from either mussels and donax clams matrices. Although the release of toxins from the food into the digestive fluids was observed, the bioaccessibility of OA and DTX3 cannot be individually determined due to the lack to analytical standards for the various compounds that are collectively referred as DTX3. The profile of toxins, in terms of molar fraction, revealed that the bioaccessible fraction was composed by higher levels of free OA and significantly lower levels of DTX3 than the food matrix, indicating that these esters derivatives are converted into OA in the gastrointestinal tract. Since it is not possible to quantitatively measure the effectiveness of this conversion due to the lack of standards, the bioaccessibility percentage estimated in this study corresponds to the OA total content, i.e. free OA plus DTX3. The conversion of DTX3 into OA during the *in vitro* digestion process is also supported by the fact that degradation or loss of toxins was not observed. No significant differences in OA total content were registered between the food matrix and the sum of toxins in the bioaccessible and non-bioaccessible fractions. Hydrolysis of DTX3 in the human digestive tract by lipases and other enzymes has been previously suggested (Doucet et al., 2007). Although DTX3 is considered a weak inhibitor of serine/threonine protein phosphatases 2A (Hallegraeff et al., 1995), its rapid hydrolysis in the digestive fluids, where it is converted into its parent toxin, has been suggested as the main reason for human disease outbreaks after the ingestion of seafood contaminated with OA esters (Torgersen et al., 2005; Vale and Sampayo, 2002b). Human gastro-intestinal micro-organisms may also play an important role in toxins transformation. Despite the existence of few studies suggesting that bacteria do not contribute to the esterification of OA in bivalves hepatopancreas (Rossignoli et al.,

2011), human gastro-intestinal flora is known to play an active role in the conversion of several compounds (Liard et al., 2013). Then, it is plausible to inquiry whether the micro-intestinal flora is able to influence DSP toxins biotransformation.

Contrarily to most bivalve species, mussels and donax clams contain higher amounts of free forms of DSP toxins (Rossignoli et al., 2011; Vale and Sampayo, 2002a), which make these species as preferred food matrices to study the bioaccessibility of OA-group toxins. On the other hand, the higher proportion of toxin free forms in both bivalve species is responsible for their low depuration rates (Rossignoli et al., 2011). Indeed, raft production of mussels and wild catch of donax clams are recurrently affected by long closure periods to harvesting due to harmful algal blooms (Vale et al., 2008). Despite the differences in bioaccessibility percentages between both bivalve species were not statistically significant, a tendency for lower toxins bioaccessibility was observed in clams compared to mussels. Lower bioaccessibility rates associated with a certain species indicate that consumers are less exposed to toxins. Such differences might be associated to the higher accumulation of DTX3 in clams compared to mussels, where OA is the predominant toxin accumulated. Therefore, confirming that the release of DSP toxins during digestion is less effective from donax clams tissues is a relevant finding for risk assessment studies. A decrease of human risk with the ingestion donax clam compared to mussels may avoid the current long closer periods for harvesting donax clam in Southern Europe.

To date, in risk assessment, the bioavailability, i.e. the amount of contaminant that reaches systemic circulation, has been considered equal to the amount of OA present in the food matrix, but as reported in this study bioaccessibility is below 100 % in both shellfish species highly affected by OA contamination in Southern Europe. Moreover, the absorption of purified OA through the human gut barrier has been studied using *in vitro* intestinal Caco-2 cell models showing a poor permeability to the intestinal tract (Ehlers et al., 2011; Fernández et al., 2014). On the other hand, it is important to consider that DSP induces gastrointestinal epithelial cells injuries and severe mucosal damages in the intestinal tract (Matias et al., 1999). Therefore, the toxins released from the food matrix may exert their toxic effects in the cells of digestive tract before entering the blood stream.

In conclusion, risk assessment based solely on DSP toxins occurrence in seafood can conduct to an overestimation of the exposure and lead to regulatory measures more conservative than taking into account the amount of toxins that can be absorbed by the intestinal epithelia (bioaccessibility). Unfortunately, the impact of results obtained in this study on regulatory limit values is hampered by the greater uncertainties inherent with the current dietary intake estimates for consumers and extrapolation of toxicity reference values from experimental animal models to humans. Therefore, there is a need for methods, as is the static *in vitro* digestion, an excellent tool to assess the OA bioaccessibility, that provides relevant information to enable food safety authorities to perform more realistic and accurate risk assessment studies.

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Chapter 7

General Discussion and Conclusions

General Discussion and Conclusions

1. General discussion

The importance of shellfish as an ecological and socio-economic asset was highlighted several times throughout this work. Both aspects of shellfish harvesting can be extensively impaired by external factors, either biotic or abiotic, natural or anthropogenic, giving emphasis to the need for adequate management of wild populations and farmed productions (Blanchet et al., 2019; Chiesa et al., 2011; Filgueira et al., 2016; Karatayev et al., 2009; Mcneely and Schutyser, 2003; Sousa et al., 2009; Wijsman et al., 2018).

To promote a healthy balance between the ecological and socio-economic interests, scientific knowledge on shellfish species, namely on how they react and cope with external stressors, must be potentiated. Embodying this requirement, the effect of several stressors and issues directly affecting shellfish and their habitats were investigated in this thesis. The accumulation and elimination dynamics of marine biotoxins in native *vs.* invasive species, the role of shellfish as vectors of marine biotoxins into humans, including effects of exposure to harmful algal blooms (HABs) under climate change scenarios, were the main focus of the studies here reported.

Recalling, three primary goals were pursued during the elaboration of these studies: i) To determine whether native and invasive species cope with exposure to HABs differently, providing new insights on species-specificities and ecosystem functioning fragilities in the presence of invasive species and HABs; ii) To evaluate how commercially valuable shellfish species cope with simultaneous exposure to several climate change drivers and HABs, providing knowledge on toxicokinetics, physiological and genotoxic responses; iii) To assess marine biotoxins bioaccessibility, contributing for new information liable of being used in health risk assessments.

1.1. Exposure to HABs: Do native and invasive species deal with exposure to HABs differently?

Two congener clam species *Ruditapes decussatus* (native) and *R. philippinarum* (non-indigenous) were chosen to respond to the first main objective. These species have similarities in environmental requirements, frequently coexisting in the same habitat and being exposed to HABs in the same frequency and intensity (Freitas et al., 2012; Lopes et al., 2018; Velez et al., 2015c, 2015b).

To answer the first key-question, three operational objectives were established: i) Assessment of toxicity and bioaccumulation patterns of marine toxins in naturally contaminated specimens of both bivalve species living in sympatry; ii) Comparative evaluation, of toxin accumulation and elimination patterns following exposure to the dinoflagellate *Prorocentrum lima* (producer of OA-group toxins) under controlled laboratory conditions; iii) Comparative evaluation of the susceptibility to DNA damage in counterpoint with DNA damage repair capacity following exposure to the dinoflagellate *P. lima*.

These three objectives and the main question behind them were assessed in chapters 2 and 3 using a strategy based on field observations and experiments under laboratory-controlled conditions. DSP, ASP and PSP toxicity data of clam species living in sympatry in Aveiro lagoon were obtained from IPMA's Official Control of Marine Biotoxins in shellfish. To complement data from the Official Control, additional analyses were performed to detect emerging toxins, namely tetrodotoxin (TTX) and β-Methylamino-l-alanine (BMAA). Results showed OA-group toxins as the most frequent toxin group found in the clams, and a clear pattern of higher toxicity was observed in native clams, *R. decussatus* (Chapter 2 - Braga et al., 2017). Laboratory experiments under controlled conditions confirmed these observations (Chapter 3 - Braga et al. submitted).

The invasive species seems to be better prepared to deal with exposure to algal blooms of OA-toxins producers, presenting lower thresholds for triggering toxin biotransformation and elimination. They also presented a higher capacity to cope with the toxins at a genotoxicity level. At the concentrations tested, it was clear that native clams presented an increase in genetic damage with repair mechanisms triggered since

the early stage of exposure, while the invasive clam did not show an increase in DNA damage and the repair mechanisms were lately triggered after the changing to non-toxic diet (Chapter 3 - Braga et al. *submitted*). Therefore, the answer is "Yes, *R. decussatus* and *R. philippinarum,* as native and invasive species, differently cope with exposure to HABs".

One can say that differences are inherent the fact of being two species and not related with the categorization as native or invasive. However, as reported above, these clams are congener species, with very similar morphological traits and environmental requirements, coexisting in the same habitat and competing for the same resources (Freitas et al., 2012; Karatayev et al., 2009; Lopes et al., 2018; Moreira et al., 2012; Ricciardi and Rasmussen, 1998; Velez et al., 2015c, 2015b). Moreover, they also present similarities when it comes to physiological parameters, for example, the filtration rate ranges (Nakamura, 2001).

According to the findings here reported, it is reasonable to propose that recurrent exposure to HABs may play a significant role in the increasing distribution areas of *R. philippinarum*, as it may impair *R. decussatus* reproduction and competition for space within the habitat.

Exposure to toxic algae caused a significant increase in DNA damage, in DNA repair mechanisms triggered and damaged cells elimination in *R. decussatus*. Following the general idea that DNA repair mechanisms increase energy consumption in cells (Szabo et al., 1996), it is then possible to propose that *R. decussatus* expends more energy while coping with marine biotoxins than *R. philippinarum*. A redistribution of the energy budget that may significantly reduce the amount of energy available for somatic growth and reproduction (Delgado and Pérez Camacho, 2007).

It is also vital to address that recurrent exposure to marine biotoxins, in particular OA-group toxins, may cause errors in the DNA repair processes, possibly unbalancing, even more, the energy budget. When the repair process is completed and the DNA strands corrected, the repair machinery must receive a signal through the PP1 and PP2A to end the repair and be disassembled to allow for chromatin condensation and a normal cell function. However, exposure to OA-group toxins is going to impair this process inhibiting PP1 and PP2A activity and preventing this machinery dephosphorylation and disassemble (González-Romero et al., 2012; Herman et al.,

2002). Consequently, as no signal to end the process is given to the repair machinery a continuous expenditure of energy may result in a defective DNA repair, and eventually lead to apoptosis (González-Romero et al., 2012; Herman et al., 2002).

Reproduction rates may be also compromised by recurrent exposure to marine biotoxins and unbalance of the energy budget, delaying gametes production or cause them to be reabsorbed (Delgado and Pérez Camacho, 2007). Toxins may cause the gametes to became infeasible. Gametes, particularly spermatozoa, are not able to repair DNA damage (Fernández-Díez et al., 2016; Marçal et al., 2020), compromising the genome integrity and undermining, not only, the complex process that is external fertilization is shellfish, but also, offspring survival rates, due to transmissible damage (Mai et al., 2013; Marçal et al., 2020).

This information is not only an important step for understanding how exposure to marine biotoxins affect dynamics between native and invasive species, but it is also of high importance for the shellfish industry. However, it cannot be forgotten that results are valid for the toxins currently monitored. The toxins monitored in the Portuguese coast are undoubtedly the most frequent and present a significant threat to consumers, but it is essential to have in mind that unmonitored toxins may also have significant impacts on humans and represent an unassessed risk for consumers and public health of local communities (Banack et al., 2007; Banack and Murch, 2009; Bradley and Mash, 2009; P. A. Cox et al., 2005; Murch et al., 2004; Noguchi and Arakawa, 2008; Pablo et al., 2009).

1.2. How do bivalve species cope with exposure to HABs and climate change drivers?

The occurrence of HABs and marine biotoxins are one of the many factors affecting coastal areas and shellfish. Several parameters from anthropogenic pollution to environmental changes affect bivalves, compromising their viability as fisheries resource and as native populations (Filgueira et al., 2016; González and Puntarulo, 2016; Hégaret et al., 2007a; Moore et al., 2008; Wijsman et al., 2018). Nonetheless, the effects of combined exposure to multiple stressors, independently of type or origin, is yet poorly understood (Duarte et al., 2014; Freitas et al., 2017; Lischka et al., 2011; Matozzo et al., 2013). Changes in environmental conditions, such as increase of seawater temperature

and acidification are nowadays major concerns when it comes to species maintenance and survival. Recent studies indicate that warming and acidification have an important effect in HABs, increasing their frequency, intensity and dispersion (Anderson et al., 2002; Edwards et al., 2006; Hallegraeff, 2010, 1993; Moore et al., 2008; Sellner et al., 2003).

Regarding the second major goal pursued in this study, two operational objectives were established to answer this question: i) Assessment of the individual and combined effects of climate change drivers (warming and acidification) on toxins accumulation and elimination dynamics in mussels *Mytilus galloprovincialis* exposed to the toxin-producing dinoflagellate *Gymnodinium catenatum*; ii) Assessment of the organ-specific (gills and hepatopancreas) antioxidant response, oxidative and genotoxic damage in *M. galloprovincialis* exposed to *G. catenatum* under individual and combined climate change drivers.

These operational objectives and the main question behind them were assessed in chapters 4 and 5. For this part of the work, a different bivalve species and a different HABs producing algae were selected. The bivalve species used was the mussel *M. galloprovincialis*, which is commonly used as indicator species for marine biotoxins occurrence, has a wide distribution range and high commercial interest. Mussels were exposed and acclimated to 4 different environmental conditions: CC) current conditions (19 °C; pH 8.0); W) warming (24 °C; pH 8.0); A) acidification (19 °C; pH 7.6); WA) warming plus acidification (24 °C; pH 7.6). After acclimation, mussels were fed with *G. catenatum* cells.

This study demonstrated that exposure to HAB-species such as *G. catenatum* under altered environmental conditions causes significant changes in the amounts of toxins accumulated, reducing it significantly under WA. Nevertheless, it also showed that toxin elimination was significantly slower under WA conditions, suggesting that simultaneous exposure to warming and acidification conduce to lower toxicity and longer elimination periods (Chapter 4 - Braga et al., 2018).

Although it was not intention of this study to investigate processes to potentiate toxins elimination, it shed light on mechanisms favouring elimination of PSTs. Results indicate that mussels maintained under acidification conditions have higher elimination rates. Acidified environments may be a possible way to promote toxin elimination in

mussels and other shellfish species (Chapter 4 - Braga et al., 2018). However, further investigation is needed. It will be important to reach a balance between toxin elimination rates, mussels well-being and organoleptic characteristics.

The second part of the experiment assessed the oxidative stress responses and DNA damage occurring in mussels exposed to the toxic algae under the 4 environmental conditions tested. Relevant results were obtained focusing on antioxidant system, LPO and DNA damage responses of two organs (gills and digestive gland). A low risk of damage was found for the gills, while for hepatopancreas, despite a reduced antioxidant response and no LPO, an increased risk for DNA damage was observed when exposed to PSTs. Overall, when relating the antioxidant response system, the lipid peroxidation and the DNA damage, the results indicated that the DNA damage detected in the mussels was not caused by ROS or oxidative stress pointing out to PSTs causing DNA damage by other mechanisms.

When mussels were simultaneously exposed to HABs and altered climate conditions, a complex and almost unpredictable response caused by the stressors' interaction was revealed. No clear interactions of stressors effects were detected in the antioxidant responses in gills under warming and PSTs, but antagonistic responses for LPO and a synergetic response in DNA damage were observed if compared to mussels exposed to PSTs under current conditions. In hepatopancreas, the simultaneous exposure to these stressors did not reveal any significant effect of warming and PSTs interaction. On the other hand, acidification induced stressor effects interactions in antioxidant responses (although not fitting in the typified patterns), in LPO (antagonistic response) and DNA damage (potentiation) when compared with mussels exposed to PSTs under current conditions (Chapter 5 - Braga et al., 2020).

The combination of warming and acidification revealed an increase of mussel's vulnerability to PSTs. Not only potentiated the DNA damage sustained by the mussels during the exposure period, but it also compromised the recovery of this damage after the 10 days of toxin elimination (Chapter 5 - Braga et al., 2020). Throughout this study, a strong relation between PSTs and GSHt content was observed. The results suggested a possible glutathione-dependent elimination pathway with the potential formation of PST-GSHt conjugates. The involvement of GSH in the PSTs metabolism, biotransformation and elimination, seems to come at a high cost to mussels. The

increase in GSHt content seems to be related to the increase in DNA damage. Overall, results reveal that exposure to this particular combination of stressors increases the mussel's vulnerability to PSTs, promoting toxin dominated processes and compromising DNA integrity by increasing the occurrence of damage and preventing their repair (Chapter 5 - Braga et al., 2020).

As an answer to the question "How bivalve species cope with simultaneous exposure to several climate change drivers and harmful algal blooms?" the results showed a significant impact of altered environmental conditions in shellfish. The increase of HABs frequency coupled with lower elimination rates can have significant socio-economic impacts in the shellfish harvesting, possibly jeopardising mussels farming. Previous studies suggested that species-specific responses to HABs and environmental changes may occur. Therefore, information considering different shellfish species is required. Also, other biotoxins, as BMAA, TTX and CTX, should be included in this studies, as the increase of emerging marine biotoxins is expected to occur under altered environmental conditions (Chateau-Degat et al., 2005; Hallegraeff, 2010; Moore et al., 2008).

However, we cannot forget that gradual exposure to combined altered environmental conditions may have a significant impact on shellfish populations, and different responses may occur when mussels co-evolve with the environmental changes. Notwithstanding, the data collected reveal at least two possible issues for mussels: the first at organism level, the DNA damage caused by the new conditions to the mussels, increasing genetic disorders, introducing malformations, diseases, reduced grow-rates, reducing fertility and reproduction rates. The second, at a population level, as the compromised DNA may lead to less viable descendants, reducing recruitment and jeopardising the population survival.

1.3. What is the amount of biotoxins available to be absorbed by the human body after digestion?

Considering a scenario where HABs frequency, intensity and distribution are expected to increase, as well as the demand for shellfish as a protein source, will increase, a balance between resources availability, environmental conditions, consumers demand and human health safety has to be reached. Until now the data

available regarding toxin transference from mussels to consumers were based on information from bioassays performed in small mammals by abdominal or peritoneal injection (Suzuki et al., 1996; Yasumoto et al., 1978) and then extrapolated to humans. The need for a more accurate source of information on how human digestion extracts and transforms toxins after shellfish consumption arise. These data are needed for the elaboration of human health risk assessments by the safety authorities, leading to the last main goal approached in this thesis.

One operational objective was established to respond to this goal: i) Determination of OA-group toxins bioaccessibility in two commercially important shellfish species naturally contaminated, mussels *M. galloprovincialis* and clams *Donax* sp.

Considering the high incidence of OA-group toxins in Portugal and Southern Europe, these compounds were selected for the study. Hence, a group of naturally contaminated mussels and donax clams, containing high amounts of OA-group toxins, was subjected to an *in vitro* digestion protocol. The OA-group toxins content in parental and esterified conformation was analysed in the initial food matrix and both the bioaccessible and the non-bioaccessible fractions resultant from the digestion protocol. These analyses assessed the amount of toxins extracted from the matrix food and available to be assimilated by the gut epithelial, *i.e.*, bioaccessibility (Metian et al., 2009; Versantvoort et al., 2005).

The *in vitro* digestion revealed that 88 % and 75 % of the initial toxin content in mussels and donax clams, respectively, is available to be absorbed. Digestion also altered the proportions of parental/esterified toxin conformations, increasing the toxin on the parental conformation, which suggests the conversion of ester derivatives into the parental conformation (Chapter 6 - Braga et al., 2016).

So, the answer to the question “What is the amount of biotoxins available to be absorbed by the human body after digestion?” is for OA-group toxins near 88 % in mussels and 75 % in donax clams. These results point to an overestimation of the exposure when risk assessments are based only in the OA-group toxins concentration in the shellfish and data extrapolation from animal models. Regarding the differences between mussels and donax clams, the data suggest that bioaccessibility may be even lower in shellfish with the toxin profile dominated by esters, which was investigated elsewhere (Manita et al., 2017).

2. Future perspectives

The studies carried out in this thesis allowed to answer all the initial questions proposed leading to significant findings in different areas. At the same time, new questions have emerged:

1) With the expected increase in HABs frequency, intensity and distribution, a regular survey of emerging marine biotoxins, as BMAA, TTX and CTX should be considered to monitor their presence and detect possible increases in their concentrations, to prevent possible intoxications and other health issues.

2) While the main results point to a pessimistic scenario for the shellfish industry regarding biotoxins accumulation in the long run, a possible mechanism to promote PSP toxins elimination was found under acidified conditions. Similar exposure experiments should be carried out with other shellfish species and other marine biotoxins, not only to assess the impact of warming and acidification in the biotoxins toxicokinetics but also to determine if exposure to altered conditions may promote the elimination of other biotoxins (DA and OA-group).

3) It can be extremely interesting to further investigate the relation between PSTs and GSH. This study proposes the occurrence of PST-conjugates, but confirmation and identification of these compounds are still needed and may be of extreme importance for understanding toxin elimination in mussels. It is also important to confirm the relation detected in chapter 5 between the increase of GSHt and DNA damage in the presence of PSTs, as it demonstrates that GSH role in xenobiotics eliminations does not always result in less toxic compounds, a generalised idea.

4) Considering that bioaccessibility (the amount of toxin available to be absorbed by the human body) is lower than the amount present in the initial foodstuffs and the bioavailability (the amount of toxin that is absorbed by the human body and reaches the bloodstream) can be even lower, a current overestimation of the risk can be excessively protecting consumers. The application of more accurate data in risk assessment studies may represent a significant reduction in the number of precautionary days that shellfish harvesting is banned.

3. Conclusions

From the ecological to the more anthropocentric point of view, several issues were investigated, bridging part of the information gap regarding marine biotoxins accumulation and their effects in bivalves. All the questions initially raised were answered:

- "*Do native and invasive species deal with exposure to HABs differently?* "

Yes, the invasive species is less prone to accumulate toxins and suffer from genotoxicity.

- "*How do bivalve species cope with exposure to HABs and climate change drivers?*"

Bivalves were significantly impacted by simultaneous exposure to toxic algae and altered environmental conditions. Altered environmental conditions affected biotoxins toxicokinetics, by decreasing accumulation but prolonging the elimination periods, what can be translated into important socio-economic impacts and jeopardise bivalves farming. The combined exposure to altered environmental conditions and toxic algae originates different responses in mussels, highlighting the occurrence of interaction effects. Simultaneous exposure induced an increase in DNA damage, compromising the genetic information integrity and endangering mussel populations.

- "What is the amount of biotoxins available to be absorbed by the human body after digestion?"

The quantity of biotoxins recovered after *in vitro* digestion only amounted near 80 % of the initial toxin content in mussels, indicating that an overestimation of risk may be occurring when basing Risk Assessments studies solely on the initial toxin content present in shellfish. The *in vitro* digestion revealed to be a good tool for obtaining more accurate data regarding toxin transference from bivalves to consumer.

Overall, throughout this thesis, several areas of study were approached, focusing on increasing knowledge about impacts of biotoxins in bivalves and how these animals cope whit their presence and effects. The information collected is relevant for a more comprehensive approach for shellfish production management in terms of biotoxins occurrence and accumulation. The complexity of the relationships established between

algae and shellfish species, with an increasing number and type of toxic compounds, and coupled with the impacts of external factors such as warming, acidification, salinity or even anthropogenic pollution, points to the need for an effective environmental survey. This monitoring program should be capable of performing continuous assessment of biotic and abiotic parameters, and address anthropocentric requirements, in order to obtain a healthier balance between environmental and socio-economic interests.

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Annexes

Annex Index:

Annex A:

Supplementary data from Chapter 2 - Native (*Ruditapes decussatus*) and non-indigenous (*R. philippinarum*) shellfish species living in sympatry: comparison of Regulated and Non-Regulated biotoxins accumulation.

Appendix A.1: Supplementary Tables

Table S1. DSP (µg OA eq. kg^{-1}), ASP (mg DA kg^{-1}) and PSP (µg STX eq. kg^{-1}) in native clam (*R. decussatus*) and non-indigenous clam (*R. philippinarum*), harvested in Ria de Aveiro, Portugal, between January 2014 and December 2016 (NA - Not Analized; NQ - Not Quantified; ND - Not Detected; * - Outliers).

Date (dd-mm-yyyy)	Location	*R. decussatus*			*R. philippinarum*		
		OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}	OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}
13/01/2014	RIAV1	40	NA	NA	22	NA	NA
21/04/2014	RIAV1	304	NA	NA	229	NA	NA
28/04/2014	RIAV3	92	NA	NA	70	NA	NA
07/05/2014	RIAV2	143	NA	NA	111	NA	NA
13/05/2014	RIAV1	62	NA	NA	83	NA	NA
13/05/2014	RIAV2	15	NA	NA	42	NA	NA
19/05/2014	RIAV3	53	NA	NA	81	NA	NA
02/06/2014	RIAV1	282	NA	NA	280	NA	ND
02/06/2014	RIAV3	* 334	NA	NA	* 185	NA	NA
03/06/2014	RIAV2	181	NA	NA	72	NA	NA
11/06/2014	RIAV1	NA	NA	NA	19	22.4	NA
11/06/2014	RIAV2	NA	NA	NA	18	7.8	NA
17/06/2014	RIAV1	478	2.5	NA	482	2.6	NA
17/06/2014	RIAV3	NA	NA	NA	476	2.5	NA
24/06/2014	RIAV1	87	NA	NA	94	NA	NA
24/06/2014	RIAV2	* 779	NA	NA	* 193	NA	NA
01/07/2014	RIAV1	74	NA	NA	109	NA	NA
01/07/2014	RIAV2	149	NA	NA	116	NA	NA
08/07/2014	RIAV2	60	NA	NA	97	NA	NA

Table S1. DSP (µg OA eq. kg^{-1}), ASP (mg DA kg^{-1}) and PSP (µg STX eq. kg^{-1}) in native clam (*R. decussatus*) and non-indigenous clam (*R. philippinarum*), harvested in Ria de Aveiro, Portugal, between January 2014 and December 2016 (NA - Not Analized; NQ - Not Quantified; ND - Not Detected; * - Outliers). (Cont.)

Date (dd-mm-yyyy)	Location	*R. decussatus*			*R. philippinarum*		
		OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}	OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}
29/07/2014	RIAV1	106	NA	NA	118	NA	NA
29/07/2014	RIAV3	82	NA	NA	104	NA	NA
12/08/2014	RIAV2	185	NA	NA	127	NA	NA
19/08/2014	RIAV2	194	NA	NA	171	NA	NA
26/08/2014	RIAV2	182	NA	NA	113	NA	NA
26/08/2014	RIAV3	* 104	NA	ND	* 234	NA	NA
15/09/2014	RIAV1	* 198	NA	NA	* 69	NA	NA
15/09/2014	RIAV2	277	NA	NA	167	NA	NA
15/09/2014	RIAV3	253	NA	NA	247	NA	NA
29/09/2014	RIAV2	61	NA	NA	76	NA	NA
23/03/2015	RIAV3	31.0	NA	ND	32.0	NA	ND
13/04/2015	RIAV1	NA	3.8	NA	NA	NQ	NA
14/04/2015	RIAV2	23.0	3.4	NA	ND	ND	NA
21/04/2015	RIAV3	* 220	NA	NA	* 33	NA	NA
27/04/2015	RIAV2	174	NA	NA	135	NA	NA
28/04/2015	RIAV1	90	NA	NA	40	NA	NA
18/05/2015	RIAV1	66	NA	NA	88	NA	NA
19/05/2015	RIAV2	81	NA	ND	50	NA	ND
25/05/2015	RIAV3	31	NA	NA	31	NA	NA
03/06/2015	RIAV4	49	NA	NA	38	NA	NA
22/06/2015	RIAV1	52	NA	NA	80	NA	NA

Table S1. DSP (µg OA eq. kg^{-1}), ASP (mg DA kg^{-1}) and PSP (µg STX eq. kg^{-1}) in native clam (*R. decussatus*) and non-indigenous clam (*R. philippinarum*), harvested in Ria de Aveiro, Portugal, between January 2014 and December 2016 (NA - Not Analized; NQ - Not Quantified; ND - Not Detected; * - Outliers). (Cont.)

Date (dd-mm-yyyy)	Location	*R. decussatus*			*R. philippinarum*		
		OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}	OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}
22/06/2015	RIAV2	113	NA	NA	86	NA	NA
30/06/2015	RIAV3	ND	NA	NA	27	NA	NA
27/07/2015	RIAV1	97	NA	NA	ND	NA	NA
03/08/2015	RIAV2	* 201	NA	NA	* 31	NA	NA
10/08/2015	RIAV1	156	NA	NA	120	NA	NA
10/08/2015	RIAV2	133	NA	NA	90	NA	NA
10/08/2015	RIAV3	154	NA	NA	66	NA	NA
17/08/2015	RIAV3	ND	NA	NA	70	NA	NA
24/08/2015	RIAV4	86	NA	ND	NQ	NA	NA
08/09/2015	RIAV3	* 209	NA	NA	* 21	NA	NA
14/09/2015	RIAV1	91	NA	NA	104	NA	NA
04/04/2016	RIAV2	89	NA	NA	34	NA	NA
18/04/2016	RIAV3	* 376	NA	NA	* 80	NA	NA
18/04/2016	RIAV1	118	NA	NA	143	NA	NA
23/05/2016	RIAV1	65	NA	NA	NQ	NA	NA
13/06/2016	RIAV3	212	NA	NA	197	NA	NA
20/06/2016	RIAV3	NQ	NA	NA	59	NA	NA
20/06/2016	RIAV4	NQ	NA	NA	33	NA	NA
05/07/2016	RIAV3	NQ	NA	NA	40	NA	NA
05/09/2016	RIAV3	34	NA	NA	73	NA	NA
12/09/2016	RIAV2	NQ	NA	NA	NQ	NA	NA

Table S1. DSP (µg OA eq. kg^{-1}), ASP (mg DA kg^{-1}) and PSP (µg STX eq. kg^{-1}) in native clam (*R. decussatus*) and non-indigenous clam (*R. philippinarum*), harvested in Ria de Aveiro, Portugal, between January 2014 and December 2016 (NA - Not Analized; NQ - Not Quantified; ND - Not Detected; * - Outliers). (Cont.)

Date (dd-mm-yyyy)	Location	*R. decussatus*			*R. philippinarum*		
		OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}	OA eq. kg^{-1}	ASP mg. kg^{-1}	STX eq. kg^{-1}
13/09/2016	RIAV4	66	NA	NA	74	NA	NA
13/10/2016	RIAV2	89	NA	NA	NQ	NA	NA
18/10/2016	RIAV3	ND	NA	NA	130	NA	NA
18/10/2016	RIAV4	* 349	NA	NA	* 166	NA	NA
08/11/2016	RIAV4	145	NA	NA	200	NA	ND
06/12/2016	RIAV4	45	NA	262	NQ	NA	NQ
12/12/2016	RIAV1	NA	NA	439	NA	NA	ND
12/12/2016	RIAV2	NA	NA	NQ	NA	NA	NQ
19/12/2016	RIAV2	NA	NA	2330.7	NA	NA	190.6
20/12/2016	RIAV4	NQ	NA	150.7	NQ	NA	NQ
27/12/2016	RIAV4	NQ	NA	771.3	NQ	NA	265

Table S2. Profile of OA-group toxins determined in the native clam (*R. decussatus*) and the non-indigenous clam (*R. philippinarum*), harvested in Ria de Aveiro, Portugal during 2015.

Date (dd-mm-yyyy)	Location	*R. decussatus*				*R. philippinarum*			
		OA Free (%)	DTX2 Free (%)	OA Esters (%)	DTX2 Esters (%)	OA Free (%)	DTX2 Free (%)	OA Esters (%)	DTX2 Esters (%)
21/04/2015	RIAV3	8 %	0 %	92 %	0 %	0 %	0 %	100 %	0 %
27/04/2015	RIAV2	12 %	0 %	88 %	0 %	20 %	0 %	80 %	0 %
28/04/2015	RIAV1	0 %	0 %	100 %	0 %	0 %	0 %	49 %	51 %
22/06/2015	RIAV2	0 %	0 %	100 %	0 %	19 %	0 %	81 %	0 %
03/08/2015	RIAV2	0 %	0 %	75 %	25 %	0 %	0 %	53 %	47 %
10/08/2015	RIAV1	0 %	0 %	72 %	28 %	0 %	0 %	66 %	34 %
10/08/2015	RIAV2	0 %	0 %	66 %	34 %	17 %	0 %	43 %	40 %
10/08/2015	RIAV3	0 %	0 %	69 %	31 %	0 %	0 %	67 %	33 %
08/09/2015	RIAV3	0 %	0 %	68 %	32 %	0 %	0 %	100 %	0 %
14/09/2015	RIAV1	0 %	0 %	74 %	26 %	0 %	0 %	75 %	25 %

Table S3. BMAA molar fraction (protein bound BMAA and Total Soluble BMAA, in %) in the native clam (*R. decussatus*) and the non-indigenous clam (*R. philippinarum*), harvested in Ria de Aveiro, Portugal during 2015.

Date (dd-mm-yyyy)	Location	*R. decussatus*		*R. philippinarum*	
		Total Soluble BMAA (%)	Protein-BMAA (%)	Total Soluble BMAA (%)	Protein-BMAA (%)
21/04/2015	RIAV3	88 %	12 %	94 %	6 %
27/04/2015	RIAV2	43 %	57 %	14 %	86 %
28/04/2015	RIAV1	28 %	72 %	38 %	62 %
22/06/2015	RIAV2	66 %	34 %	49 %	51 %
03/08/2015	RIAV2	85 %	15 %	53 %	47 %
10/08/2015	RIAV1	51 %	49 %	71 %	29 %
10/08/2015	RIAV2	61 %	39 %	29 %	71 %
10/08/2015	RIAV3	19 %	81 %	5 %	95 %
08/09/2015	RIAV3	22 %	78 %	90 %	10 %
14/09/2015	RIAV1	25 %	75 %	13 %	87 %

Annex B:

Supplementary data from Chapter 3 - Invasive clams (*Ruditapes philippinarum*) are better equipped to deal with harmful algal blooms than native species (*R. decussatus*): evidence of species-specific toxicokinetics and DNA vulnerability.

Appendix B.1: Supplementary Tables

Table S1 LC conditions and Mobile Phases used in lipophilic toxins analysis via Agilent 1260 Infinity LC system coupled to a Sciex Qtrap 4500 mass spectrometer.

LC conditions	
Column	XBridge BEH C18 2.5 µm 2.1 × 50 mm Column XP (Waters, Ireland)
Flow	0.3 mL/min
Column temp.	25 ºC
Injection volume	10 µl

Gradient		
Time (min)	**Mobile Phase A (%)** (Water; 2 mM ammonium formate; 50 mM formic acid)	**Mobile Phase B (%)** (95 % acetonitrile; 2 mM ammonium formate ; 50 mM formic acid)
0	50	50
1	0	100
4	50	50
5	50	50
6	50	50
6.5	90	10
9	90	10

Ion source	
Courtain gas (CUR)	25.0
Collision gas (CAD)	High
Ion transfer voltage (IS)	-1500.0
Temperature (TEM)	500.0
Ion source gas 1 (GS1)	50.0
Ion source gas 2 (GS2)	50.0
Entrance potencial	-10.0

MS parameters					
Compound	**Q1 Mass (Da)**	**Q3 Mass (Da)**	**DP (volts)**	**CE (volts)**	**CXP (volts)**
OA/DTX-2	803.358	255.000	-210.000	-64.000	-9.000
OA/DTX-2	803.258	112.700	-210.000	-91.000	-5.000
DTX-1	817.380	255.000	-230.000	-62.000	-9.000
DTX-1	817.380	112.700	-230.000	-102.000	-11.000

Table S2. Summary of the Two-way ANOVA relative to the Genetic Damage Indicator (GDI) measured in *R. decussatus* and *R. philippinarum* exposed for 5 days (Exposure) to the toxic algae *P. lima* (Toxins exposure, control and exposure groups) and after 5 days of toxin elimination (Post - exposure).

		Exposure				Post-Exposure			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
R. decussatus	**Intercept**	1	21.54	5.40E+11	0.00	1	1.05	125.13	0.00
	Time	1	0.00	2.34	0.14	1	0.06	7.61	0.01
	Toxins	1	0.00	21.44	0.00	1	0.00	0.47	0.50
	Time*Tox.	1	0.00	0.00	0.97	1	0.21	25.26	0.00
	Error	18	0.00			19	0.01		
R. philippinarum	**Intercept**	1	7.39E+05	2.27E+03	0.00	1	6.04E+05	829.56	0.00
	Time	1	1.73E+03	5.31	0.03	1	4088.26	5.62	0.03
	Toxins	1	1.37E+03	4.19	0.05	1	1183.73	1.63	0.22
	Time*Tox.	1	1.41E+03	4.34	0.05	1	736.59	1.01	0.33
	Error	21	3.26E+02			21	727.66		

Table S3. Summary of the Two-way ANOVA relative to the Genetic Damage Indicator plus the Net Enzyme-Sensitive Sites (GDI+NSS) for the enzyme EndoIII in *R. decussatus* *R. philippinarum* exposed for 5 days (Exposure) to the toxic algae *P. lima* (Toxins exposure, control and exposure groups) and after 5 days of toxin elimination (Post - exposure).

		Exposure				Post-Exposure			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
R. decussatus	**Intercept**	1	7.99E+12	739.85	0.00	1	7.03E+03	68.25	0.00
	Time	1	2.21E+08	0.02	0.89	1	167.18	1.62	0.22
	Toxins	1	1.40E+10	1.30	0.27	1	2.43	0.02	0.88
	Time*Tox.	1	1.47E+07	0.00	0.97	1	421.09	4.09	0.06
	Error	20	1.08E+10			19	102.95		
R. philippinarum	**Intercept**	1	1.26E+06	6522.33	0.00	1	1.18E+06	3908.44	0.00
	Time	1	426.31	2.21	0.15	1	716.89	2.37	0.14
	Toxins	1	1128.98	5.86	0.03	1	4.74	0.02	0.90
	Time*Tox.	1	89.05	0.46	0.50	1	1824.37	6.02	0.02
	Error	20	192.59			20	302.80		

Table S4. Summary of the Two-way ANOVA relative to the Net Enzyme-Sensitive Sites (NSS) for the enzyme EndoIII in *R. decussatus R. philippinarum* exposed for 5 days (Exposure) to the toxic algae *P. lima* (Toxins exposure, control and exposure groups) and after 5 days of toxin elimination (Post - exposure).

		Exposure				Post-Exposure			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
R. decussatus	**Intercept**	1	25712.84	105.76	0.00	1	37126.55	68.79	0.00
	Time	1	1.64	0.01	0.94	1	361.86	0.67	0.43
	Toxins	1	5483.96	22.56	0.00	1	3880.45	7.19	0.02
	Time*Tox.	1	572.16	2.35	0.14	1	800.39	1.48	0.24
	Error	16	243.13			15	539.69		
R. philippinarum	**Intercept**	1	9351.93	74.43	0.00	1	306.93	1213.86	0.00
	Time	1	9.71	0.08	0.78	1	0.28	1.12	0.31
	Toxins	1	28.24	0.22	0.64	1	0.09	0.34	0.57
	Time*Tox.	1	3060.73	24.36	0.00	1	0.02	0.10	0.76
	Error	16	125.64			17	0.25		

Table S5. Summary of the Two-way ANOVA relative to the Genetic Damage Indicator plus the Net Enzyme-Sensitive Sites (GDI+NSS) for the enzyme FPG in *R. decussatus R. philippinarum* exposed for 5 days (Exposure) to the toxic algae *P. lima* (Toxins exposure, control and exposure groups) and after 5 days of toxin elimination (Post - exposure).

		Exposure				Post-Exposure			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
R. decussatus	**Intercept**	1	1.15E+06	5302.29	0.00	1	1.06E+13	1420.15	0.00
	Time	1	6094.42	28.14	0.00	1	1.35E+11	18.07	0.00
	Toxins	1	396.29	1.83	0.19	1	7.24E+10	9.71	0.01
	Time*Tox.	1	25.30	0.12	0.74	1	2.86E+09	0.38	0.54
	Error	21	216.59			21	7.46E+09		
R. philippinarum	**Intercept**	1	1.05E+06	3186.36	0.00	1	9.91E+05	3972.14	0.00
	Time	1	561.23	1.71	0.20	1	59.11	0.24	0.63
	Toxins	1	140.56	0.43	0.52	1	33.81	0.14	0.72
	Time*Tox.	1	57.43	0.17	0.68	1	126.79	0.51	0.48
	Error	22	328.73			21	249.50		

Table S6. Summary of the Two-way ANOVA relative to the Net Enzyme-Sensitive Sites (NSS) for the enzyme FPG in *R. decussatus R. philippinarum* exposed for 5 days (Exposure) to the toxic algae *P. lima* (Toxins exposure, control and exposure groups) and after 5 days of toxin elimination (Post - exposure).

		Exposure				Post-Exposure			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
R. decussatus	**Intercept**	1	1640.98	96.55	0.00	1	181.89	564.74	0.00
	Time	1	92.02	5.41	0.04	1	2.91	9.04	0.01
	Toxins	1	135.60	7.98	0.01	1	0.01	0.03	0.86
	Time*Tox.	1	1.47	0.09	0.77	1	0.20	0.63	0.44
	Error	14	17.00			15	0.32		
R. philippinarum	**Intercept**	1	23344.98	38.39	0.00	1	47774.87	56.73	0.00
	Time	1	1586.21	2.61	0.12	1	2826.10	3.36	0.08
	Toxins	1	92.46	0.15	0.70	1	2114.14	2.51	0.13
	Time*Tox.	1	228.40	0.38	0.55	1	1109.00	1.32	0.27
	Error	19	608.11			19	842.22		

Table S7. Summary of the Two-way ANOVA relative to the BER in *R. decussatus R. philippinarum* exposed for 5 days (Exposure) to the toxic algae *P. lima* (Toxins exposure, control and exposure groups) and after 5 days of toxin elimination (Post - exposure).

		Exposure				Post-Exposure			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
R. decussatus	**Intercept**	1	7350.00	642.39	0.00	1	183.61	3222.87	0.00
	Time	1	32.67	2.86	0.11	1	1.80	31.61	0.00
	Toxins	1	864.00	75.51	0.00	1	1.84	32.28	0.00
	Time*Tox.	1	24.00	2.10	0.16	1	0.21	3.66	0.07
	Error	20	11.44			20	0.06		
R. philippinarum	**Intercept**	1	259.42	4431.20	0.00	1	43332.68	144.92	0.00
	Time	1	2.56	43.65	0.00	1	7115.48	23.80	0.00
	Toxins	1	0.22	3.79	0.06	1	3219.89	10.77	0.00
	Time*Tox.	1	0.09	1.52	0.23	1	1311.91	4.39	0.05
	Error	24	0.06			20	299.01		

Appendix B.2: Supplementary Figures

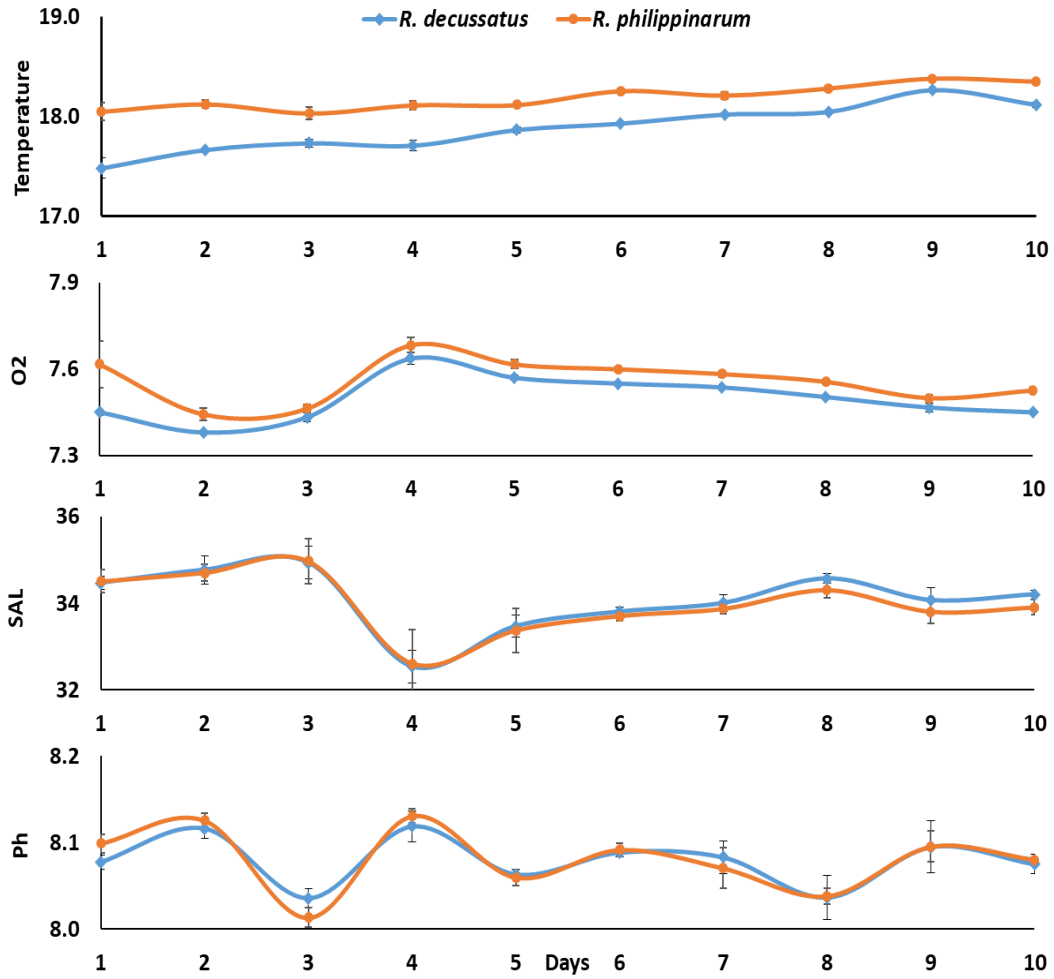


Fig. S1. Daily measurements (mean ± SD) of Temperature (ºC), pH, O_2 (mg L^{-1}) and Salinity in the tanks where *R. decussatus* and *R. philippinarum* were maintained during the 5 days of Exposure to the toxic algae *P. lima* and the 5 days of Post – exposure after for toxin elimination.

Annex C:

Supplementary data from Chapter 4 - Combined effects of warming and acidification on accumulation and elimination dynamics of paralytic shellfish toxins in mussels *Mytilus galloprovincialis*.

Appendix C.1: Supplementary Tables

Table S1. Sea water characteristics (mean ± SD) used to maintain *M. galloprovincialis* during the experiment. Salinity (‰), Temperature (ºC), pH (in pH units), total alkalinity (TA, mmol/kgSW), total CO2 (TCO2, mmol/kgSW), pCO2 (matm), HCO3 (mmol/kgSW), Ω Ara.

	Exposure temperature			
	19º C		24º C	
	Normocapnia	Hypercapnia	Normocapnia	Hypercapnia
Salinity (‰)	35.4 ± 0.3	35.4 ± 0.3	36.0 ± 0.3	35.8 ± 0.3
t (ºC)	19.5 ± 0.8	19.4 ± 0.4	24.3 ± 0.6	24.6 ± 0.6
pH (Chosen Scale)	8.1 ± 0.1	7.6 ± 0.0	8.1 ± 0.1	7.6 ± 0.0
TA (mmol/kgSW)	2806.3 ± 426.4	2688.2 ± 139.0	2566.0 ± 157.2	2310.0 ± 270.4
TCO2 (mmol/kgSW)	2482.6 ± 316.7	2623.4 ± 104.5	2206.0 ± 32.8	2224.0 ± 240.4
pCO2 (matm)	392.2 ± 6.2	1491.8 ± 93.4	351.7 ± 84.2	1259.6 ± 101.0
HCO3 (mmol/kgSW)	2202.4 ± 254.7	2485.5 ± 96.3	1910.5 ± 24.1	2094.6 ± 226.3
Ω Ara	4.1 ± 1.0	1.4 ± 0.2	4.5 ± 0.9	1.5 ± 0.2

Appendix B.2: Supplementary Figures

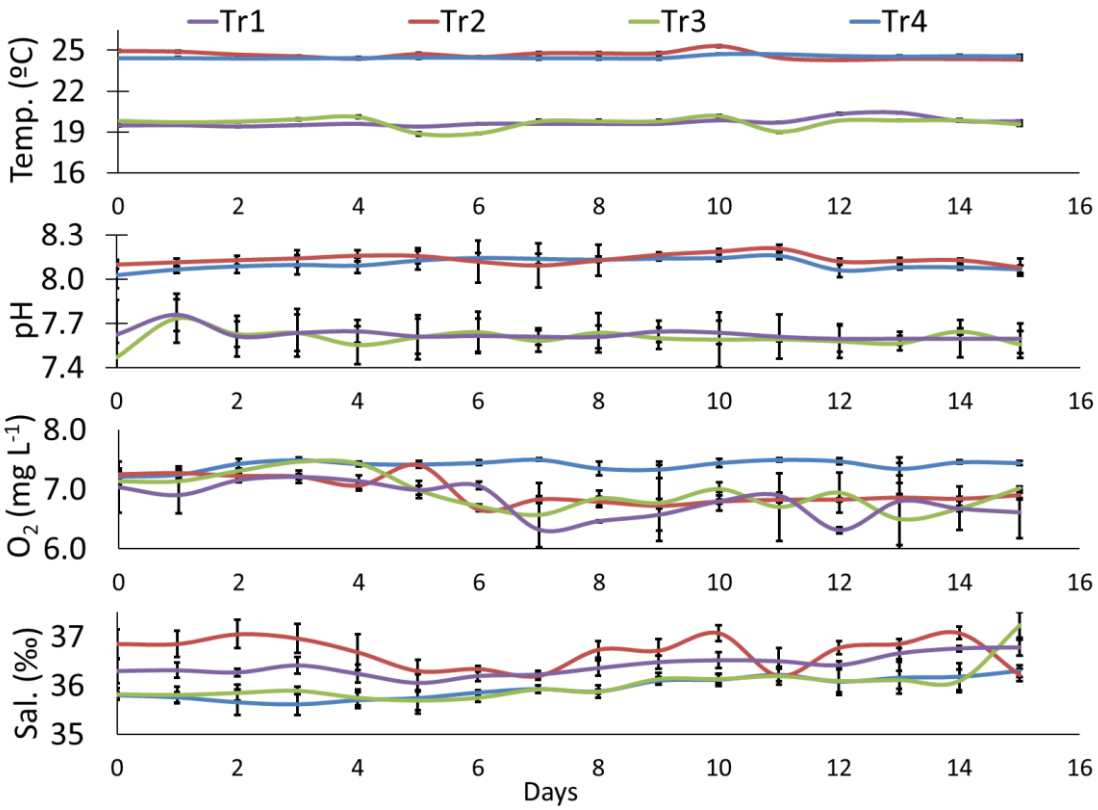
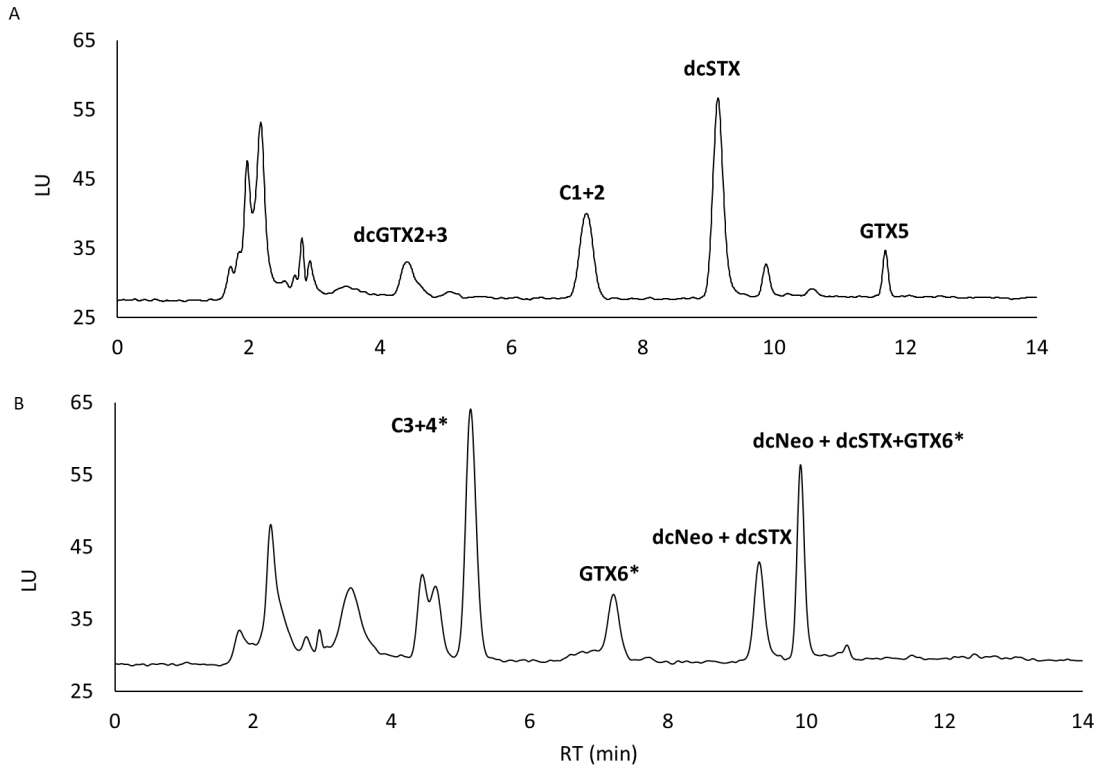


Fig. S1. Daily measurements (mean ± SD) of water temperature (ºC), pH, O2 (mg L-1) and salinity (‰) in the tanks simulating the four environmental conditions (CC: 19 ºC and 8.0 pH; W: 24 ºC and 8.0 pH; A: 19 ºC and 7.6 pH; WA: 24 ºC and 7.6 pH).



* identity of this compound is presumed from literature and not from analytical standards

Fig. S2. HPLC–FLD chromatogram of peroxide (A) and periodate (B) oxidation of SPE-C18 cleaned-up extract of mussels (*Mytilus galloprovincialis*) fed with *Gymnodinium cateantum* during 5 days under actual conditions (CC).

Annex D:

Supplementary data from Chapter 5 - DNA damage and oxidative stress responses of mussels *Mytilus galloprovincialis* to paralytic shellfish toxins under warming and acidification conditions – elucidation on the organ-specificity.

Appendix D.1: Supplementary Tables

Table S1. Seawater characteristics (mean ± SD) used to maintain *M. galloprovincialis* during the experiment. Salinity (‰), temperature (ºC), pH, total alkalinity (TA, mmol/kgSW), total CO2 (TCO2, mmol/kgSW), carbon dioxide partial pressure (pCO2, matm), bicarbonate ion concentration (HCO3, mmol/kgSW), calcite saturation states (Ω Cal), and aragonite saturation states (Ω Ara).

	Environmental Conditions			
	19º C		**24º C**	
	Normocapnia (CC)	**Hypercapnia (A)**	**Normocapnia (W)**	**Hypercapnia (WA)**
Salinity (‰)	35.4 ± 0.3	35.4 ± 0.3	36.0 ± 0.3	35.8 ± 0.3
Temperature (^{o}C)	19.5 ± 0.8	19.4 ± 0.4	24.3 ± 0.6	24.6 ± 0.6
pH	8.1 ± 0.1	7.6 ± 0.0	8.1 ± 0.1	7.6 ± 0.0
TA (mmol/kgSW)	2806.3 ± 426.4	2688.2 ± 139.0	2566.0 ± 157.2	2310.0 ± 270.4
TCO_2 (mmol/kgSW)	2482.6 ± 316.7	2623.4 ± 104.5	2206.0 ± 32.8	2224.0 ± 240.4
pCO_2 (matm)	392.2 ± 6.2	1491.8 ± 93.4	351.7 ± 84.2	1259.6 ± 101.0
HCO_3 (mmol/kgSW)	2202.4 ± 254.7	2485.5 ± 96.3	1910.5 ± 24.1	2094.6 ± 226.3
Ω Cal	6.32 ± 1.53	2.12 ± 0.25	6.75 ± 1.40	2.23 ± 0.26
Ω Ara	4.1 ± 1.0	1.4 ± 0.2	4.5 ± 0.9	1.5 ± 0.2

Table S2. Summary of the Two-way ANOVA relative to the antioxidant responses of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), total glutathione content (GSHt), measured in gills of mussels exposed for 5 days (Exposure) to the toxic algae *G. catenatum* (Toxins exposure, control and exposed groups) and after 10 days of toxin elimination (Post-exposure) under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH).

Tissue: Gills		**Exposure (E)**				**Post-exposure (PE)**			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
CAT	**Intercept**	1	5759.8	259.944	0.000	1	23843.8	146.010	0.000
	Environmental	3	42.1	1.902	0.142	3	77.4	0.474	0.702
	Toxins exposure	1	30.1	1.360	0.249	1	438.3	2.684	0.110
	Environ.*Tox.	3	86.0	3.882	0.015	3	308.7	1.890	0.148
	Error	48	22.2			37	163.3		
SOD	**Intercept**	1	306197.1	601.417	0.000	1	647.4	280.386	0.000
	Environmental	3	183.3	0.360	0.782	3	15.5	6.718	0.001
	Toxins exposure	1	2089.2	4.104	0.049	1	0.0	0.003	0.959
	Environ.*Tox.	3	2275.1	4.469	0.008	3	5.6	2.431	0.083
	Error	43	509.1			32	2.3		
GR	**Intercept**	**1**	148.3	230.466	0.000	1	195.4	191.629	0.000
	Environmental	**3**	1.0	1.612	0.202	3	1.3	1.315	0.285
	Toxins exposure	**1**	2.1	3.216	0.080	1	1.3	1.296	0.263
	Environ.*Tox.	**3**	0.3	0.414	0.744	3	0.8	0.775	0.516
	Error	**40**	0.6			34	1.0		
GPx	**Intercept**	**1**	1.4	255.547	0.000	1	252.2	449.530	0.000
	Environmental	**3**	0.0	0.585	0.628	3	2.1	3.757	0.018
	Toxins exposure	**1**	0.0	8.496	0.006	1	0.5	0.978	0.328
	Environ.*Tox.	**3**	0.0	4.008	0.014	3	7.9	13.994	0.000
	Error	**40**	0.0			41	0.6		
GST	**Intercept**	**1**	0.3	218.596	0.000	1	540.4	929.481	0.000
	Environmental	**3**	0.0	3.768	0.018	3	0.8	1.357	0.271
	Toxins exposure	**1**	0.0	0.373	0.545	1	0.0	0.005	0.946
	Environ.*Tox.	**3**	0.0	1.066	0.374	3	1.4	2.372	0.086
	Error	**42**	0.0			36	0.6		
GSHt	**Intercept**	**1**	0.3	1239.235	0.000	1	22236.2	153.151	0.000
	Environmental	**3**	0.0	0.324	0.808	3	169.8	1.169	0.334
	Toxins exposure	**1**	0.0	7.944	0.008	1	278.7	1.919	0.174
	Environ.*Tox.	**3**	0.0	5.878	0.002	3	499.7	3.442	0.026
	Error	**37**	0.0			37	145.2		

Table S3. Summary of the Two-way ANOVA relative to the antioxidant responses of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), total glutathione content (GSHt), measured in hepatopancreas of mussels exposed for 5 days (Exposure) to the toxic algae *G. catenatum* (Toxins exposure, control and exposure groups) and after 10 days of toxin elimination (Post-exposure) under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH).

Tissue: Hepatopancreas		**Exposure (E)**				**Post-exposure (PE)**			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**P**
	Intercept	1	317.9	502.771	0.000	1	947.3	419.650	0.000
	Environmental	3	0.4	0.689	0.564	3	8.0	3.532	0.022
CAT	**Toxins exposure**	1	1.5	2.340	0.134	1	0.7	0.297	0.588
	Environ.*Tox.	3	0.4	0.590	0.625	3	8.5	3.750	0.017
	Error	40	0.6			48	2.3		
	Intercept	1	48.3	777.486	0.000	1	577.9	3686.953	0.000
	Environmental	3	0.0	0.610	0.613	3	0.2	1.075	0.371
SOD	**Toxins exposure**	1	0.4	6.521	0.015	1	0.1	0.505	0.481
	Environ.*Tox.	3	0.1	1.010	0.400	3	0.4	2.402	0.082
	Error	35	0.1			40	0.2		
	Intercept	**1**	56.0	886.456	0.000	1	59.2	390.030	0.000
	Environmental	**3**	0.0	0.388	0.762	3	0.2	1.607	0.203
GR	**Toxins exposure**	**1**	0.5	7.268	0.010	1	0.4	2.346	0.134
	Environ.*Tox.	**3**	0.2	3.619	0.020	3	0.9	5.671	0.003
	Error	**48**	0.1			39	0.2		
	Intercept	**1**	36.5	675.396	0.000	1	12.4	237.855	0.000
	Environmental	**3**	0.3	5.227	0.004	3	0.1	1.286	0.294
GPx	**Toxins exposure**	**1**	0.2	4.003	0.053	1	1.1	21.622	0.000
	Environ.*Tox.	**3**	0.1	1.044	0.384	3	0.5	9.524	0.000
	Error	**38**	0.1			37	0.1		
	Intercept	**1**	395.7	2837.302	0.000	1	32.84	19680.208	0.000
	Environmental	**3**	1.1	7.672	0.000	3	0.00	0.286	0.835
GST	**Toxins exposure**	**1**	0.5	3.634	0.064	1	0.01	3.307	0.078
	Environ.*Tox.	**3**	0.3	2.431	0.079	3	0.00	1.429	0.251
	Error	**40**	0.1			35	0.00		
	Intercept	**1**	296.9	2090274.208	0.000	1	1.8	2.312	0.138
	Environmental	**3**	0.0	0.425	0.736	3	0.4	0.452	0.717
GSHt	**Toxins exposure**	**1**	0.0	27.156	0.000	1	2.7	3.499	0.070
	Environ.*Tox.	**3**	0.0	0.496	0.687	3	0.7	0.958	0.424
	Error	**48**	0.0			33	0.8		

Table S4. Summary of the Two-way ANOVA relative to the lipid peroxidation damage (LPO) measured in gills of mussels exposed for 5 days (Exposure) to the toxic algae *G. catenatum* (Toxins exposure, control and exposure groups) and after 10 days of toxin elimination (Post-exposure) under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH).

Tissue: Gills		**Exposure (E)**				**Post-exposure (PE)**			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
	Intercept	1	40.9	365.185	0.000	1	12.2	414.247	0.000
	Environmental	3	0.7	6.433	0.001	3	0.1	2.886	0.045
LPO	**Toxins exposure**	1	2.6	23.050	0.000	1	0.6	21.203	0.000
	Environ.*Tox.	3	0.1	1.198	0.324	3	0.2	7.586	0.000
	Error	36	0.1			48	0.0		

Table S5. Summary of the Two-way ANOVA relative to the lipid peroxidation damage (LPO) measured in hepatopancreas of mussels exposed for 5 days (Exposure) to the toxic algae *G. catenatum* (Toxins exposure, control and exposure groups) and after 10 days of toxin elimination (Post-exposure) under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH).

Tissue: Hepatopancreas		**Exposure (E)**				**Post-exposure (PE)**			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**P**	**Degr. of Freedom**	**MS**	**F**	**p**
	Intercept	1	**114.7**	**254.469**	**0.000**	**1**	0.0	0.050	0.824
	Environmental	3	**1.6**	**3.445**	**0.024**	**3**	1.7	9.787	0.000
LPO	**Toxins exposure**	1	**0.1**	**0.179**	**0.674**	**1**	0.0	0.020	0.888
	Environ.*Tox.	3	**0.5**	**1.187**	**0.325**	**3**	0.4	2.168	0.107
	Error	48	**0.5**			**39**	0.2		

Table S6. Summary of the Two-way ANOVA relative to the Genetic Damage Indicator (GDI) measured in gills of mussels exposed for 5 days (Exposure) to the toxic algae *G. catenatum* (Toxins exposure, control and exposure groups) and after 10 days of toxin elimination (Post-exposure) under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH).

Tissue: Gills		**Exposure (E)**				**Post-exposure (PE)**			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**p**	**Degr. of Freedom**	**MS**	**F**	**P**
DNA Damage	**Intercept**	1	316.4	379.280	0.000	1	374.8	140.630	0.000
	Environmental	3	5.3	6.328	0.003	3	19.7	7.395	0.001
	Toxins exposure	1	71.0	85.078	0.000	1	0.0	0.005	0.942
	Environ.*Tox.	3	2.1	2.564	0.079	3	0.7	0.256	0.856
	Error	23	0.8			28	2.7		

Table S7. Summary of the Two-way ANOVA relative to the Genetic Damage Indicator (GDI) measured in hepatopancreas of mussels exposed for 5 days (Exposure) to the toxic algae *G. catenatum* (Toxins exposure, control and exposure groups) and after 10 days of toxin elimination (Post-exposure) under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH).

Tissue: Hepatopancreas		**Exposure (E)**				**Post-exposure (PE)**			
Source of variation		**Degr. of Freedom**	**MS**	**F**	**p**	**Degr. of Freedom**	**MS**	**F**	**P**
DNA Damage	**Intercept**	1	32891.4	922.586	0.000	1	1140199.7	1749.858	0.000
	Environmental	3	220.8	6.194	0.001	3	10859.4	16.666	0.000
	Toxins exposure	1	7781.5	218.266	0.000	1	114.1	0.175	0.678
	Environ.*Tox.	3	139.0	3.899	0.015	3	3857.9	5.921	0.002
	Error	42	35.7			42	651.6		

Table S8. Synopsis of the antioxidant responses, lipid peroxidation damage (LPO) and Genetic Damage Indicator (GDI) measured in gills and hepatopancreas of mussels exposed for 5 days (Exposure) to the toxic algae *G. catenatum* (Toxin exposure: T) and after 10 days of toxin elimination (Post-exposure) under four environmental conditions (Environmental conditions, CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH)

	Gills Exposure							
	CAT	SOD	GR	GPx	GST	GSHt	LPO	GDI
T+CC		↓		↑		↓		
W							↓	
T+W		↓		↑		↑		↑
A								
T+A	↑	↑		↑		↑		↑
WA								
T+WA		↓		↑		↓	↑	↑

	Hepatopancreas Exposure							
	CAT	SOD	GR	GPx	GST	GSHt	LPO	GDI
T+CC			↑			↑		↑
W								↓
T+W			↓					↑
A								↑
T+A			↑					↑
WA								↓
T+WA			↑					↑

	Gills Post-exposure							
	CAT	SOD	GR	GPx	GST	GSHt	LPO	GDI
T+CC				↑			↑	
W				↑			↑	
T+W							↓	
A				↑			↑	
T+A							↓	
WA				↑			↑	
T+WA				↓			↓	

	Hepatopancreas Post-exposure							
	CAT	SOD	GR	GPx	GST	GSHt	LPO	GDI
T+CC				↑				
W	↓							↑
T+W				↓				
A	↑							↑
T+A				↓				
WA	↓							↑
T+WA				↓				↑

Where: Black arrows (↑ &↓) represent adaptive responses (not always identifiable a favourable or unfavourable); Green arrows (↓) represent improved conditions; Red arrows (↑) represent adverse effects; Green background cells (■) identifies Synergism; Orange background cells (■) identifies Antagonism; Yellow background cells (■) identifies Potentiation; Blue background cells (■) identifies Additivity.

Appendix D.2: Supplementary Figures

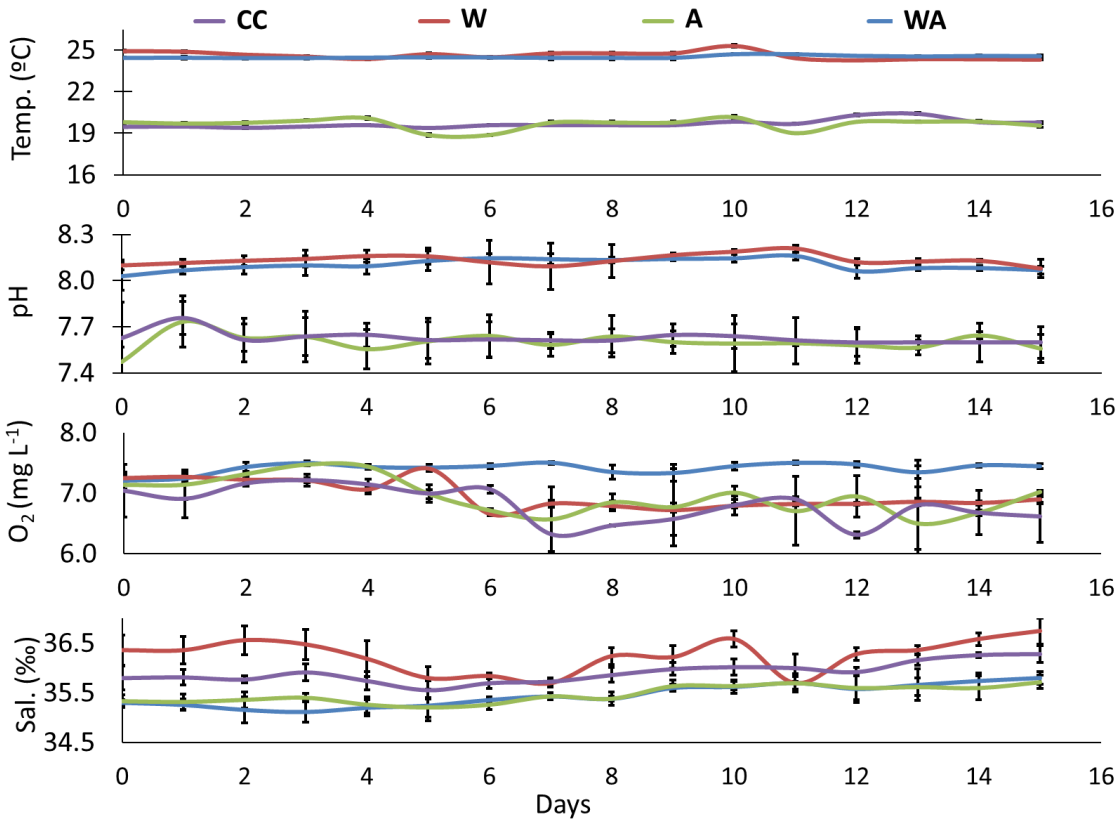


Fig. S1. Daily measurements (mean ± SD) of Temperature (ºC), pH, O2 (mg L-1) and Salinity in the tanks simulating the four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH).

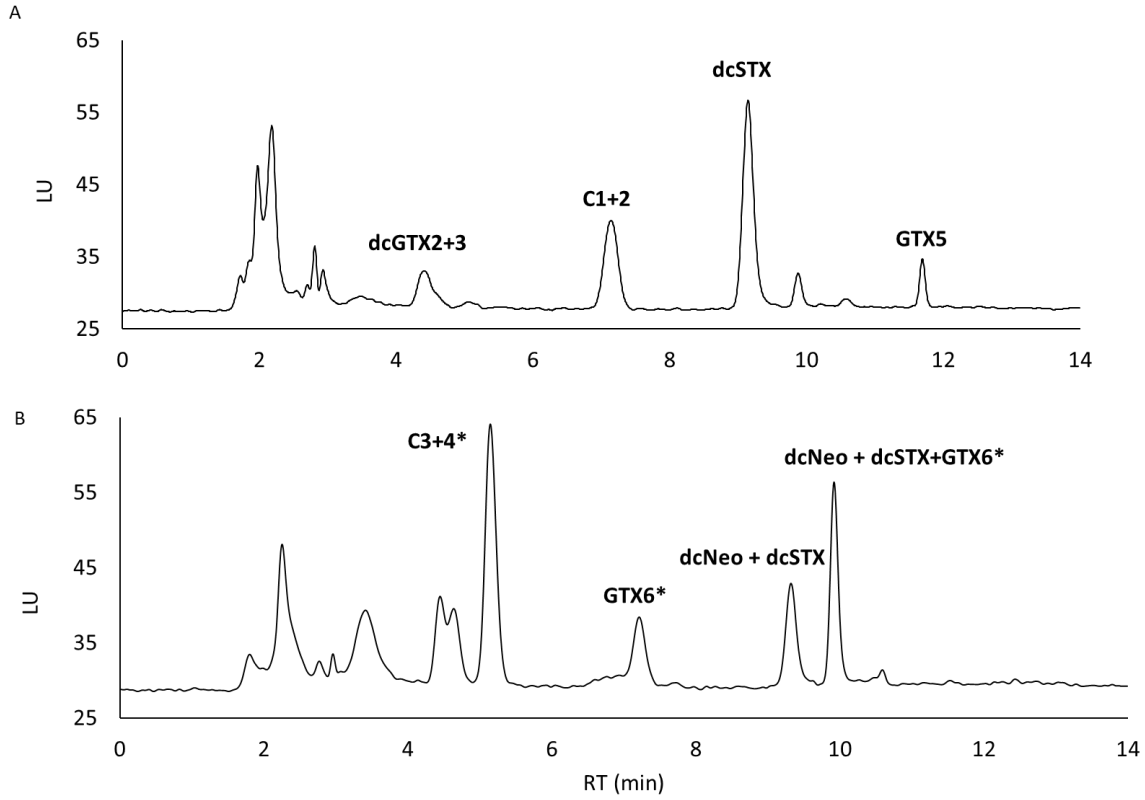


Fig.S2. Illustrative LC-FLD chromatogram of PSP toxins profile in the Portuguese Coast obtained from whole soft tissue of mussel samples after (A) peroxide and (B) periodate oxidations.