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Marine toxins in bivalves: accumulation, kinetics and subcellular responses

Dissertação para obtenção do Grau de Doutor em Ambiente

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“It is good to have an end to journey toward; but it is the journey that matters, in the end.”—Ernest Hemingway

To my Grandparents

Luzinha and Álvaro

Preface

This dissertation is submitted as partial fulfillment of the requirements for the Doctoral Degree in Environmental Sciences and includes the results of my PhD study carried out from September 2010 to August of 2014 in the Faculty of Sciences and Technology, New University of Lisbon and in the Portuguese Institute of Sea and Atmosphere, Division of Environmental Oceanography and Bioprospection.

I hereby declare that, as the first author of four manuscripts, I provided the major contribution to the research and experimental work developed, to the interpretation of results, and to the preparation of the manuscripts submitted during the PhD study. Also a relevant contribution was given in an article where I appear as second author. The copyright of the publications was transferred to the publishers, and these articles are reproduced with their permission and subject to copyright restrictions imposed by them.

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Gratitude is due to my parents, Pedro and Francisco for their continuous support and encouragement. I also thank to my friends for their support, particularly in the final stages of this PhD.

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Abstract

Toxicity of bivalves by paralytic shellfish toxins is a worldwide problem, with severe effects in humans, and in production and harvest of bivalves. To address this issue several studies have been performed in this thesis. Laboratory experiments with the clam *Ruditapes decussatus* fed with *Gymnodinium catenatum* cells allowed an estimate of uptake and depuration rates of individual paralytic shellfish toxins. Approximately 95% of C1+2 and 85% of B1, the major toxins produced by *G. catenatum* cells supplied to the clams, were converted into other toxins or lost in solution. An example is dcSTX, the only toxin quantified in feaces produced by clams. Studies on the nutrient pool, phytoplankton assemblages, and mussel toxicity in a eutrophic coastal lagoon and adjacent coastal area displaying frequent upwelling episodes and bivalve toxicity pointed to longer and more acute bivalve toxicity episodes in the lagoon. This was interpreted as the effect of changes in the nutrient pool, promoting the abundance of toxic cells imported from the coastal water. The partitioning of toxins among sub-cellular fractions in digestive glands of cockles (*Cerastoderma edule*) exposed to a *G. catenatum* bloom and under post-bloom conditions indicated changes of organelle toxin profiles after exposure, despite high affinity of toxins to the insoluble cellular fraction. A review of the toxicity caused by *G. catenatum* in mussels (*Mytilus* spp.), cockles (*C. edule)*, wedge clams (*Donax trunculus)* and surf clams (*Spisula solida)* from the Portuguese areas was executed for the period 1994-2012. An irregular multi-annual variation of toxicity episodes was registered, including a prolonged period (1996-2004) of low toxicity by PSTs. In other years, a seasonal signal was found in autumn/early winter. Connectivity of toxicity episodes among three estuarine systems, and between a coastal lagoon and the adjacent coastal area, was identified. Toxin profiles in 405 composite samples of mussels, cockles, wedge clams and surf clams pointed to changes between bivalves of low and high toxicity, mirroring toxin biotransformation after blooms. Biotransformation seems to be faster in *S. solida* due to the prevalence of decarbamoyl derivates independently of the toxicity value. **Keywords:** Paralytic shellfish toxins; Bivalves; Kinetics; Subcellular partitioning; Biotransformation

Resumo

A toxicidade em bivalves devido a toxinas marinhas paralisantes é um problema mundial, com efeitos severos no Homem e na produção e apanha de bivalves. De modo a aprofundar esta temática foram realizados vários estudos. Os estudos em laboratório com fornecimento de células de *Gymnodinium catenatum* a amêijoas (*Ruditapes decussatus*) permitiram estimar taxas de captação e depuração de toxinas marinhas paralisantes. Aproximadamente 95% de C1+2 e 85% de B1, as toxinas maioritárias produzidas pela alga tóxica, foram convertidas em outras toxinas ou perdidas em solução. A toxina dcSTX é um exemplo, tendo sido a única toxina quantificada nas fezes produzidas pelas amêijoas. Os estudos sobre nutrientes, espécies de fitoplâncton e toxicidade em mexilhões, provenientes de uma lagoa eutrófica costeira e da zona costeira adjacente, apontaram para a ocorrência de episódios mais intensos e prolongados na lagoa. Estas ocorrências foram interpretadas como o efeito das alterações nas razões de nutrientes promovendo a abundância de células tóxicas importadas da zona costeira. A partição de toxinas nas frações sub-celulares das glândulas digestivas do berbigão *Cerastoderma edule* exposto a um florescimento de *G. catenatum* e sob condições de pós-florescimento, indicaram a alteração dos perfis de toxinas nos organelos após a exposição, apesar da elevada afinidade para a fração insolúvel. Foi realizada uma revisão dos dados de toxicidade paralisante (1994-2012) em mexilhão (*Mytilus* spp.), berbigão (*C. edule*), conquilha (*Donax trunculus*) e amêijoa-branca (*Spisula solida*) da costa portuguesa, tendo-se verificado uma variação plurianual dos episódios, com picos no outono/início de inverno, incluindo um período prolongado (1996-2004) de reduzida toxicidade. Foi identificada conectividade de episódios entre três sistemas estuarinos e entre uma lagoa costeira e zona costeira adjacente. Os perfis de toxinas em 405 amostras de mexilhão, berbigão, conquilha e amêijoa-branca apresentaram diferenças entre amostras de toxicidade reduzida e elevada, refletindo a biotransformação de toxinas após os florescimentos. Esta biotransformação aparenta ser mais rápida na *S. solida* devido à prevalência dos compostos decarbamoilados independentemente do valor de toxicidade. **Palavras-chave:** Toxinas marinhas paralisantes; Bivalves; Cinética; Partição-subcelular; Biotransformação

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Acronyms and Abbreviations

ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
ASP	Amnesic shellfish poisoning
AZP	Azaspiracid shellfish poisoning
CFP	Ciguatera fish poisoning
CRM	Certified reference material
dcGTX	Decarbamoylgonyautoxin
dcNEO	Decarbamoylneosaxitoxin
dcSTX	Decarbamoylsaxitoxin
DIN	Dissolved inorganic nitrogen
DO	Dissolved oxygen
doGTX	13-deoxy-decarbamoylgonyautoxin
DON	Dissolved organic nitrogen
DOP	Dissolved organic phosphorus
doSTX	13-deoxy-decarbamoylsaxitoxin
DSP	Diarrhetic shellfish poisoning
DST	Diarrheic shellfish toxin
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GTX	Gonyautoxin
HAB	Harmful algal bloom
IPCC	Intergovernmental Panel of Climate Change
LC-FLD	Liquid chromatography with fluorescence detection
LD_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
LOD	Limit of detection

MSFD	Marine Strategy Framework Directive
MU	Mouse unit
NEO	Neosaxitoxin
NSP	Neurologic shellfish poisoning
OA	Okadaic acid
PCA	Principal component analysis
POM	Particulate organic matter
PSP	Paralytic shellfish poisoning
RL	Regulatory limit
SPE	Solid phase extraction
SPE- COOH	SPE - ion exchange cartridge with carboxylic acidsiliane
STX	Saxitoxin
TEF	Toxicity equivalence factor
WFD	Water Framework Directive

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1. Introduction

1.1. Marine toxins

Marine toxins are chemical compounds naturally produced by microalgae or bacteria. Their chemical structure may be included in the classes of alkaloids, amino acids, carboxylic acids, and linear, stair and cyclic polyethers (Yasumoto, 2001) (Table 1.1).

Of the approximately 4,000 phytoplankton species documented to date only 200 of them have a high proliferation rate under specific circumstances (Zingone and Enevoldsen, 2000; Masó and Garcés, 2006). Those circumstances are not fully understood, although specific climatic and hydrographical conditions have been pointed out as key factors to algal blooms (Sellner et al., 2003; Moore et al., 2008). Of the 200 species, approximately 80 species, belonging either to dinoflagellates or diatoms, are natural producers of marine toxins (Zingone and Enevoldsen, 2000). During a toxin-producer algal bloom, toxins mainly associated with the phytoplankton cells may be ingested by shellfish (both molluscs and crustaceans), and planktivorous fish, leading to responses at organism and sub-cellular level and death under extreme episodes (White et al., 1981; Bricelj et al., 1991; Scholin et al., 2000; Deeds et al., 2008). Suspension-feeding mollusc bivalves are the principal vectors for the transfer of several major groups of toxins, due to their ability to pump large volumes of seawater and to concentrate toxins without massive mortality. Consumption of bivalves and other marine organisms containing high toxin concentrations may lead to human health problems (Wang, 2008; Gerssen et al., 2010) (Figure 1.1). Indeed, the deleterious effects of toxins in humans have been given the names paralytic, diarrhetic, neurotoxic and amnesic shellfish poisoning (PSP, DSP, NSP and ASP, respectively) to the corresponding toxins. Syndromes are caused by toxins mainly synthesized by dinoflagellates, although ASP is known to be associated with diatoms.

Table 1.1. Groups of marine toxins, chemical classes and examples of structures**.**

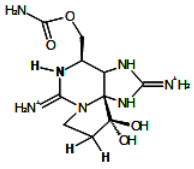
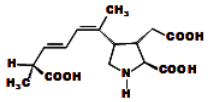
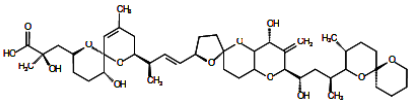
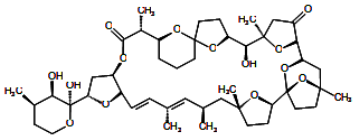
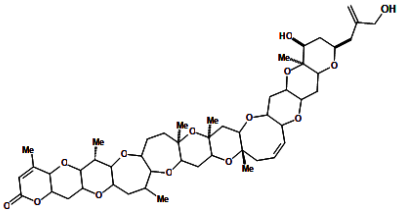
Toxin group	Class	Structure (e.g.)
Saxitoxins	Alkaloids	
Domoic acid	Aminoacids	
Azaspiracids	Linear polyethers	
Pectenotoxins	Cyclic polyethers	
Ciguatoxins	Stair polyethers	

Table 1.2 gives examples of phytoplankton species producers of marine toxins, corresponding to human syndromes and primary vectors.

Table 1.2. Marine toxin groups and their phytoplankton species producers (adapted from Gerssen et al., 2010).

Toxin group	Syndrome	Primary vector	Genus	Species	References
Saxitoxins	PSP	Bivalves	*Alexandrium*	*angustitabulatum, catenella,*	Beppu et al., 2008
				fundyense, lusitanium, minutum	Martin et al., 2009
				tamarense, tamiyavanichii	MacKenzie et al., 2004
			Gymnodinium	*catenatum*	Oshima et al., 1987
			Pyrodinium	*bahamense*	Usup et al, 2012
Domoic acid	ASP	Bivalves	*Pseudo-nitzchia*	*australis, calliantha, cuspidata, delicatissima,*	Bates and Trainer, 2006
				pseudodelicatissima,	
				multiseries, fraudulenta, multistriata,	
				pugens, seriata,galaxiae, turgidula	
Brevetoxins	NSP	Bivalves	*Karenia*	*brevis, brevisulcata, mikimotoi, selliformis, papilionacea*	Lansberg and Flewelling, 2009
			Chatonella	*cf. Verruculosa*	Watkings et al., 2008
Okadaic acid,	DSP	Bivalves	*Phalacroma*	*rotundatum*	Caroppo et al., 1999
dinophysistoxins			*Prorocentrum*	*lima, arenarium, belizeanium, concavum,*	Nascimento et al., 2005
and pectenotoxins			*Dinophysis*	*acuminata, acuta, caudata, arenarium, fortii, mitra,*	Draisci et al., 1996; MacKenzie et al.,
				norvergica, ovum, rotundata, sacculus, tripos	2005; Kamiyama and Suzuki, 2009
Yessotoxins	-	Bivalves	*Protoceratium*	*reticulatum*	Loader et al., 2007
			Lingulodinium	*polyedrum*	Paz et al., 2004
			Gonyaulax	*spinifera*	Rhodes et al., 2006
Azaspiracids	AZP	Bivalves	*Azadinium*	*Spinosum*	Tillmann et al., 2009
Spirolides	-	Bivalves	*Alexandrium*	*ostenfeldii, peruvianum*	Cembella et al., 2000; Touzet et al., 2008
Gymnodimines	-	Bivalves	*Karenia*	*selliforme*	Miles et al., 2003
			Gymnodinium	*mikimotoi*	Seki et al., 1995
Ciguatoxins	CFP	Fish	*Gambierdiscus*	*australes, pacificus, plynesiensis, toxicus, yasumotoi*	Litaker et al., 2010
Palytoxins	Palytoxin poisoning	Fish	*Ostreopsis*	*Siamensis*	Ciminiello et al., 2013

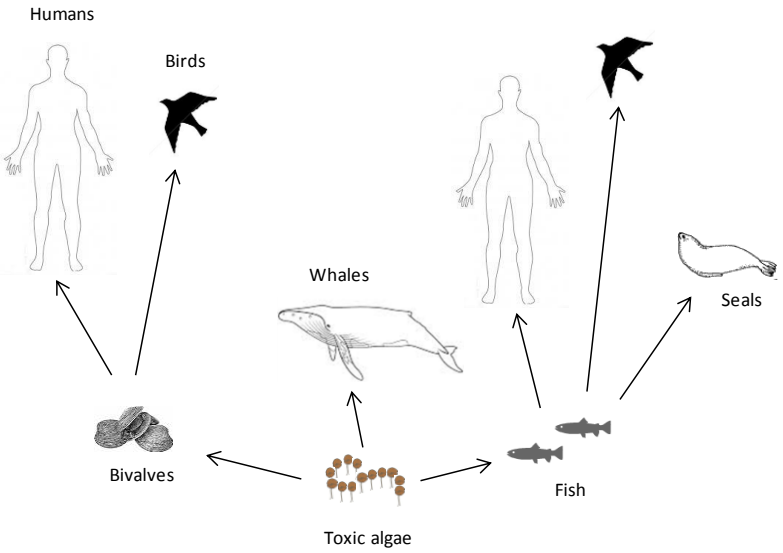


Figure 1.1. Toxic algae in the food chain and their routes of exposure (adapted from Gerssen et al., 2010).

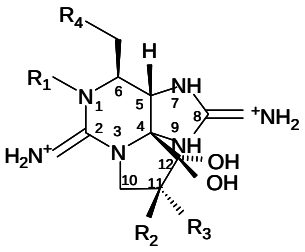
1.2. Paralytic shellfish toxins (PSTs)

1.2.1. Chemical structures

The PSTs form a group of closely related tetrahydropurine compounds, which can be broadly characterized as hydrophilic or hydrophobic, generally divided into sub-groups based on substituent side chains such as carbamate, sulfate, hydroxyl, hydroxybenzoate, or acetate (Llewellyn, 2006). The basic structures of PSTs are composed of a 3,4-propinoperhydropurine tricyclic system (Shimizu, 2000). Since its initial discovery, 57 naturally occurring PSTs have been identified, mainly derived from marine dinoflagellates or resulted of metabolic transformation in bivalves (Wiese et al., 2010) (Table 1.3).

The first isolated toxin of the group PSTs was saxitoxin (STX), obtained from the Alaskan butter clam, *Saxidomus giganteus* in 1957 (Schantz et al., 1957). The structure of STX was characterised by means of x-ray crystallographic and nuclear magnetic resonance spectroscopic studies (Bordner et al., 1975; Schantz et al., 1975). STX is included in the group of guanidinium-containing marine natural products, due to the presence of two guanidino groups which are responsible for its high polarity. Subsequently, gonyautoxins (GTX1-6) and

Table 1.3. The paralytic shellfish toxins produced by marine phytoplankton or resulted from metabolic transformation (adapted from Wiese et al., 2010).



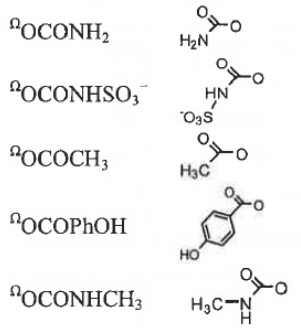
Toxin	R1	R2	R3	ΩR4	R5
STX	H	H	H	$OCONH_2$	OH
NEO	OH	H	H	$OCONH_2$	OH
Mono-sulfated					
GTX1	OH	H	OSO_3^-	$OCONH_2$	OH
GTX2	H	H	OSO_3^-	$OCONH_2$	OH
GTX3	H	OSO_3^-	H	$OCONH_2$	OH
GTX4	OH	OSO_3^-	H	$OCONH_2$	OH
GTX5 (B1)	H	H	H	$OCONHSO_3^-$	OH
GTX6 (B2)	OH	H	H	$OCONHSO_3^-$	OH
Di-sulfated					
C1	H	H	OSO_3^-	$OCONHSO_3^-$	OH
C2	H	OSO_3^-	H	$OCONHSO_3^-$	OH
C3	OH	H	OSO_3^-	$OCONHSO_3^-$	OH
C4	OH	OSO_3^-	H	$OCONHSO_3^-$	OH
Decarbamoylated					
dcSTX	H	H	H	OH	OH
dcNEO	OH	H	H	OH	OH
dcGTX1	OH	H	OSO_3^-	OH	OH
dcGTX2	H	H	OSO_3^-	OH	OH
dcGTX3	H	OSO_3^-	H	OH	OH
dcGTX4	OH	OSO_3^-	H	OH	OH
Deoxy-Decarbomoylated					
doSTX	H	H	H	H	OH
doGTX1	OH	H	OSO_3^-	H	OH
doGTX2	H	H	OSO_3^-	H	OH
Mono-Hydroxy Benzoate Analogs					
GC1	H	H	OSO_3^-	OCOPhOH	OH
GC2	H	OSO_3^-	H	OCOPhOH	OH
GC3	H	H	H	OCOPhOH	OH
*GC4	OH	H	OSO_3^-	OCOPhOH	OH
*GC5	OH	OSO_3^-	H	OCOPhOH	OH
*GC6	OH	H	H	OCOPhOH	OH

Table 1.3. (cont.)

Toxin	R1	R2	R3	ΩR4	R5
Di-Hydroxy Benzoate Analogs					
ǂGC1a	H	H	OSO_3^-	DHB	OH
ǂGC2a	H	OSO_3^-	H	DHB	OH
ǂGC3a	H	H	H	DHB	OH
ǂGC4a	OH	H	OSO_3^-	DHB	OH
ǂGC5a	OH	OSO_3^-	H	DHB	OH
ǂGC6a	OH	H	H	DHB	OH
Sulfated Benzoate Analogs					
ǂGC1b	H	H	OSO_3^-	SB	OH
ǂGC2b	H	OSO_3^-	H	SB	OH
ǂGC3b	H	H	H	SB	OH
ǂGC4b	OH	H	OSO_3^-	SB	OH
ǂGC5b	OH	OSO_3^-	H	SB	OH
ǂGC6b	OH	H	H	SB	OH
Other PST Analogs					
M1	H	OH	H	$OCONHSO_3^-$	OH
M2	H	OH	H	$OCONH_2$	OH
M3	H	OH	OH	$OCONHSO_3^-$	OH
M4	H	OH	OH	$OCONH_2$	OH
*M5					

*Not structurally characterized

ǂR4 group putatively assigned based on major ions obtained via MS



ΩDHB: Di-hydroxil-benzoate

ΩSB: Sulfated-benzoate

neosaxitoxin (neoSTX/NEO) were isolated from toxic bivalves and dinoflagellates and their structures identified (Shimizu, 2000).

Usually, a PST-producing organism synthesises a characteristic suite of toxins made up of several PST analogues. These analogues differ in side group moieties and thus are commonly grouped according to these variable residues (Wiese et al., 2010). The most commonly occurring PSTs are hydrophilic and may be non-sulfated, such as STX and NEO, mono-sulfated, such as the GTX1-6, or di-sulfated (C1-4 toxins) (Llewellyn, 2006) (Table 1.3). Decarbamoyl variants of these analogues also exist, such as decarbamoylsaxitoxins (dcSTX, dcNEO), decarbamoylgonyautoxins (dcGTX1-4), and the 13-deoxy-decarbamoyl derivatives (doSTX, doGTX1-2). Three structural families of STX are named by the identity of the R_4 side chain as either N-sulfocarbamoyl ($OCONHSO_3^-$), decarbamoyl (OH), or carbamoyl toxins ($OCONH_2$).

1.2.2. Properties

The stability of toxins varies greatly depending on their structures and pH of the medium. STX is extremely stable even at high temperature and low pH. However, above pH 8 it degrades rapidly at ambient temperature, which suggests STX may not survive in seawater too long unless it is stabilized by complexation with other dissolved substances. Toxins with the presence of N1-hydroxyl groups, such as NEO, are also more labile to acid and heat than STX. Only heated at low pH (between 2 to 4), the toxins with the N-sulfocarbamoyl moiety may be partially converted to the corresponding carbamate toxins through hydrolysis (Shimizu, 2000). In aqueous solution, STX possesses two pK_a´s of 8.22 and 11.28 which belong to the 7,8,9 and 1,2,3 guanidinium groups, respectively (Rogers and Rapoport, 1980). This polarity nature explains the ready solubility of STX in water and lower alcohols and insolubility in organic solvents. The net charge of the PSTs, which is an important factor to consider in analytical separations, varies with the pH and structures (Shimizu, 2000).

1.2.3. Mechanism of toxicity

The mechanism of action of these compounds is the blockage of sodium channels of nerve membranes, which results in stoppage of the propagation of neural impulses and paralysis of neuromuscular systems (Kao, 1966). This is mediated by the interaction between the positively charged guadinium groups of the PSTs with negatively charged carboxyl groups at site 1 of the voltage-gated sodium channels in a equimolar ratio (Catterall et al., 1980). It is documented that PSTs also bind to calcium and potassium channels (Llewellyn, 2006).

The toxicity of the PSTs is usually expressed as STX or STX equivalents. The toxicity was defined in terms of a mouse unit (MU) to quantify toxic activity, as the mouse is more sensitive to the PSTs when compared to species such as fish, amphibians, reptiles and animals of a low order (Mons et al., 1998). The MU is defined as the dose of toxin which kills one 20 g mouse in 15 min (Sommer and Meyer, 1937). STX presents a LD_{50} (intraperitoneal route) in mice of only 10 μg kg^{-1} body weight and an oral LD_{50} of 263 μg kg^{-1} body weight (Batoréu et al., 2005). The relative toxicity of STX analogues has been studied in mouse bioassays (Genenah and Shimizu, 1981; Koehn et al., 1982; Oshima, 1995a; Sullivan et al., 1983; Vale et al., 2008) and *in vitro* (Vale et al., 2008). Based on an evaluation of the relative potencies of PSTs studied since 1981, and giving greater weight to more recent data, in 2009 the European Food Safety Authority (EFSA) proposed new toxicity equivalence factor (TEF) values of each analogue based on intraperitoneal acute toxicity in mice (EFSA, 2009). Due to side chain variability, each PST has a different binding affinity to voltage-gated sodium channel receptors, which consequently results in different toxicities. Substitutions at N-1 and/or C-11 decrease the toxicity relatively to STX (Genenah and Shimizu, 1981). The toxicity equivalence factor varies from 1.0 for STX, NEO, GTX1 and dcSTX to 0.1 attributed to B1, B2, C2 and C4 (EFSA, 2009).

1.2.4. PST-producing species and their geographic distributions

Paralytic shellfish toxins (PSTs) are synthetised by a limited number of marine toxic

dinoflagellates, namely *Pyrodinium bahamense*, *Alexandrium* spp. and *Gymnodinium catenatum* (Llewellyn, 2006), and by marine bacteria (Ogata et al., 1990; Kodama et al., 1990), and freshwater cyanobacteria (Carmichael, 1997). *Pyrodinium bahamense* is responsible for the occurrence of PSTs in the Indo-West Pacific waters (Usup et al., 2012), *Alexandrium* spp. in temperate waters of both Atlantic and Pacific oceans (Beppu et al., 2008; Martin et al., 2009; MacKenzie et al., 2004), and *G. catenatum* in a large geographic area from California, Mexico, Argentina, Venezuela, Japan, Philippines, Tasmania and Iberian Peninsula (Hallegraeff et al., 2012).

G. catenatum is a unarmored chain-forming dinoflagellate, usually with 8-16 cells, sometimes reaching up to 64 cells in length. The optimal growth of *G. catenatum* has been observed in culture for temperature range of 15-20^{o}C and salinity interval of 23-34 (Blackburn et al., 1989).

Hallegraeff et al. (2012) have proposed two possible mechanisms for *G. catenatum* blooms: (i) the autochthonous case - high abundance of cells derived from an inoculum by local cyst beds or motile cells triggered by organic enrichment related to rainfall events (e.g., Tasmanian estuaries); (ii) the allochthonous case - *G. catenatum* blooms that are inoculated from offshore, and build up during upwelling relaxation (e.g., NW Spanish Rias). Blooms in Portuguese coastal waters have been reported at mid-shelf, related to the relaxation of upwelling events in the western region (Moita et al., 1998, 2003). Slow currents or close circulation contribute to the maintenance of this species near the coast (Pitcher et al., 2010).

Chemical confirmation of toxin production by *G. catenatum* was first achieved by Oshima et al. (1987) using specimens from Tasmania, Australia. Since then, this organism has been reported in several geographic areas as producer of more than 20 STX derivatives (Negri et al., 2007). The toxin profile of PSTs varies among strains and populations and, so far, no single population has been found to contain all STX derivatives (Camino-Ordás et al., 2004; Park et al., 2004; Band-Schmidt et al., 2005). A high proportion of C1+2 and/or C3+4 in the toxin profile seems to be a characteristic of *G. catenatum* worldwide, except for

Singaporean and Malay strains that lack C toxins entirely or have only a low proportion of C1+2 (Holmes et al., 2002; Mohammad-Noor, 2010). Only north-eastern Atlantic strains have a higher B1 and B2 content (>25% molar fraction) although either or both derivatives are absent in some Andalusian strains (Camino-Ordás et al., 2004). Strains from the Gulf of California and the Mexican coast are characterised by a high proportion of NEO (up to 46%), (Band-Schmidt et al., 2006), whereas this derivative generally occurs in lower molar percentages (less than 14%) in Singaporean and Spanish strains (except for one Andalucian strain, Camino-Ordás et al., 2004).

1.2.5. Toxin composition and environmental conditions

Culture-related factors such as growth temperature, chemical compostion of the medium, and chain-length are known to modify both total toxin content and the relative proportions among PSTs (Granéli and Flynn, 2006; Band-Schmidt et al., 2010). Paralytic shellfish toxins are a suite of nitrogen-rich alkaloids, and consequently sufficient nitrogen supply from water is essential for the biosynthesis of these toxins. A number of studies found that nitrogen deficiency could decrease the PST yield and cellular toxin content in toxic species of *Alexandrium* (Anderson et al., 1990; Yu et al., 2001; Leong et al., 2004), while excess nitrogen availability under phosphorus-limitation condition could increase the cellular toxin content (Flynn et al., 1994; John and Flynn, 2000; Murata et al., 2006; Hattenrath et al., 2010). G. *catenatum* showed slower responses of the cellular toxin content to changes in the nutrient regime than *Alexandrium* spp. (Flynn et al., 1996).

1.2.6. Marine organisms sensitive to PSTs

Bivalve molluscs show marked inter-species variation in their capacity to accumulate PSTs, which has a neural basis (Twarog et al., 1972). In general, bivalve species with nerves insensitive to PSTs (e.g., *Mytilus edulis*) readily feed on toxic cells (Bricelj et al., 1990) and thereby accumulate high toxin levels. In contrast, species that attain relatively low toxicities such as the oyster *Crassostrea* spp. are highly sensitive to PSTs and exhibit physiological and

behavioural mechanisms to avoid or reduce exposure to toxic cells (Lassus et al., 1999; Tran et al., 2010). These mechanisms range from feeding rate inhibition to shell clapping and complete shell valve closure (Shumway and Cucci, 1987; Bricelj and Shumway, 1998). Softshell clams (*Mya arenaria*) from areas exposed to toxic blooms are more resistant to PSTs, as demonstrated by whole-nerve assays, and accumulate toxins at greater rates than sensitive clams from unexposed areas. Resistance is caused by natural mutation of a single aminoacid residue, which causes a 1,000-fold decrease in affinity at the STX-binding site in the sodium channel pore of resistant clams (Bricelj et al., 2005). Histological observations of tissues of mussels and oysters exposed to PST producers indicated the presence of hemocytes in the gills, as well as degeneration of muscle tissue (Galimany et al., 2008; Haberkorn et al., 2010). Although PST-producing *Alexandrium tamarense* did not affect the survival and metamorphosis of the scallop *Argopecten irradians concentricus* at its early development stages, scallop activity, including mobility of D-shape larvae, attachment and climbing ability of juveniles, were affected in acute experiments indicating that *A. tamarense* has a detrimental impact on bivalves at early life stages (Yan et al., 2003).

The transport of PSTs through the food chain and the accumulation of toxins in zooplankton have been identified as important mechanisms by which toxins become available to higher trophic levels (Turner et al., 2000; Durbin et al., 2002; Jiang et al., 2007). Through this process, PSTs have also been confirmed or implicated in the deaths of sea birds, whales and monk seals. In the cases of mass mortality events involving birds, piscivorous birds are the most affected (Landberg, 2002). Lefebvre et al. (2004) examined the effects of dissolved saxitoxin exposure during early developmental stages of fish. Although the observed toxicological effects of STX exposure were reversible, a short-term toxin exposure may negatively impact the survival of fish several weeks later.

1.3. Bioaccumulation processes

The bioaccumulation process of a particular toxin in an organism can be seen as a mass balance involving uptake, biotransformation and elimination of the toxic to the

environment. Considering that these processes take place simultaneously and direct measurements are unfeasible, predicting toxic concentrations in an organism requires the use of an indirect approach, such as dynamic modelling. In a one-compartment model the bioaccumulation process can be seen as a balance between two kinetic processes, uptake and depuration, as quantified by first-order rate constants K_1 and K_2, respectively (Figure 1.2) (Connell and Miller, 1984).

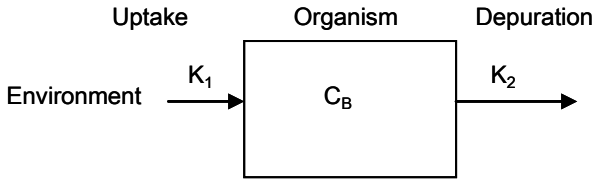


Figure 1.2. Schematic representation of a one-compartment model for bioaccumulation (adapted from Connell and Miller, 1984).

The rate of change of toxin concentration in an organism is given by:

$$\frac{dC_B}{dt} = K_1 C_E - K_2 C_B \qquad (1.1)$$

where C_B is the biotic concentration, C_E the concentration in the environment and t the time.

In a two-compartment model, digestive gland and associated tissues (viscera) form the compartment where toxins first enter in the organism and other tissues receiving toxins from the viscera the second compartment (Silvert and Cembella, 1995; Yu et al., 2005). More complex models involving enzymatic transformation, environmental variables or more than one compartment in mussels have been tested (Blanco et al., 1997, 2003; Li et al.,2005; Yu et al., 2007).

1.3.1. Uptake of PSTs

The ability of bivalves to accumulate toxins may depend largely on clearance rates,

individual species` capabilities for selective ingestion and/or absorption of toxic cells, as well as the density and distribution of the toxic dinoflagellates in the water column (Shumway et al., 1985). Clearance rate representing filtration ability provides a useful index to compare the toxin sensitivity and, thus, the potential for uptake by various species (Shumway and Cucci, 1987; Bricelj et al., 1991). In particular, the mussel *Mytilus edulis* can maintain a relatively constant feeding rate over a wide range of acclimation temperatures (ca. 10 to 20^{o}C), therefore, toxin uptake in this species is likely to be less affected by geographical or seasonal differences in temperature than in species where clearance rate is strongly influenced by seasonal temperature (Bricelj and Shumway, 1998). In addition, clearance rate can be used to compare the response within a species to variations in dinoflagellate cell density (Bricelj et al., 1996). The species-specific differences in the clearance rate found by Contreras et al. (2011) during short-term exposure to PSTs in five bivalve species of New Zealand were reflected in the total content of toxins in the tissues. The scallop *Pecten novazelandiae* and the clam *Paphies donacina* decreased their clearance rates in the presence of *Alexandium tamarense* and also accumulated fewer toxins in their tissues than the most resistant bivalves (*Perna canaliculus* and *Dosinia anus*).

As with organic matter, not all the toxin contained in phytoplankton cells is absorved by the bivalve. The main variables that seem to control the process are the gut transit time (the time the food is maintained in the digestive system) and the digestibility and/or the post-ingestive selection of the toxic cells (Moroño et al., 2001). One important factor affecting the assimilation of toxins is the relative amount of toxins (in relation to the total seston volume) available in the food (Moroño et al., 2001). These authors argued that the toxin assimilation efficiency is regulated mainly by the probability of contact between toxins and cellular walls of the digestive system.

As mentioned before, bivalves exhibit differential behaviour responses when exposed to a bloom in order to avoid or reduce exposure to toxic cells (Wildish et al., 1998; Tran et al, 2010). These responses that influence the toxin uptake may be related to the relative toxicity of compounds produced by the algae, toxin content in the tissues of bivalves (Bricelj

et al., 1996), and the history of toxic algal bloom exposure in any given ecosystem (Shumway and Cucci, 1987).

1.3.2. Biotransformation of PSTs

Cell extracts of PST-producing dinoflagellates are capable of enzymatically modifying PSTs. Oshima et al. (1995b) showed that GTX2+3 can be converted into GTX1+4 by incubation with *Alexandrium tamarense* homogenate. In addition, the introduction of a sulfate moiety on the carbamoyl group, resulting in the formation of C1 and C2 toxins, has been shown following incubation with *G. catenatum* homogenate. In dinoflagellates, biotransformation is likely to occur via PST tailoring enzymes which are a part of the PST biosynthesic pathway (Wiese et al., 2010).

Contaminated bivalves contain a mixture of several PSTs, and may have different toxin profiles from those of the dinoflagellate to which they were exposed. For example, in controlled feeding experiments, N-sulfocarbamoyl toxins produced by dinoflagellates are converted to more toxic decarbamoyl derivatives in the *Spisula solidissima*, whereas toxin conversion in the *Mya arenaria*, are limited primarily to epimerization of N-sulfocarbamoyl derivatives and gonyautoxins, e. g., transformation of toxin C2 to C1 and GTX3 to GTX2 (Bricelj et al., 1996). The conversion of the GTXs and NEO to STX by reductive elimination of O-sulfate and N1-hydroxyl groups, respectively, has been observed when toxins were incubated with the homogenate of the scallop *Placopecten magellanicus*, pointing to the action of reductant, such as cysteine and glutathione (Shimizu and Yoshioka, 1981; Oshima, 1995b). Moreoever, enzymatic conversion of N-sulfocarbamoyl PSTs to the respective decarbamoyl derivatives was confirmed in incubated tissue homogenates of littleneck clams (*Prothotheca staminea*) recognising carbamoylase as the enzyme responsible for the toxin conversion (Sullivan et al., 1983). Incubation *in vitro* of bivalve tissues with toxic dinoflagellates or purified toxin extracts allowed elucidation of the role of enzymatic activities (Fast et al., 2006; Artigas et al., 2007). The purification and characterization of the PST-transforming enzymes carbamoylase I from *Mactra chinensis* and sulfocarbamoylase I

from *Peronidia venulosa* confirmed the conversion of the carbamoyl or N-sulfocarbamoyl moieties of PSTs through enzymatic hydrolysis (Lin et al., 2004; Cho et al., 2008).

Biotransformation of the PSTs by bacteria was first suggested by Kotaki and co-authors who proposed that marine bacteria, such as *Vibrio* and *Pseudomonas* spp., are capable of metabolizing PSTs (Kotaki et al., 1985). The ability of bacteria to degrade PSTs has been further described by Smith et al. (2001), who screened marine bacterial isolates from various bivalve species for their ability to metabolize a range of PSTs suggesting that bacteria may play an important role in the clearance of PSTs from bivalves.

1.3.3. Elimination of PSTs

Feeding experiments with toxic algae are commonly used to estimate depuration rates in laboratory assays (Bricelj et al., 1991; Blanco et al., 2003; Estrada et al., 2007). Depuration studies with natural contaminated bivalves after a toxic bloom are much less documented (Blanco et al., 1997; Botelho et al., 2010a). The relevance of assessing depuration kinetics in bivalves previously exposed to toxins comes from the importance to predict the time required to reduce the total PST concentration to a level that is safe for human consumption. Depuration rates of PSTs vary greatly among bivalve species. For example, depuration of PSTs in the mussel *Mytilus edulis* is relatively fast (Bricelj and Shumway, 1998), whereas removal of toxins from the butter clam is comparatively slow due to the strong binding of the siphon tissue with the toxin STX (Beitler and Liston, 1990). Once incorporated by a bivalve, PSTs will be transported differentially to various tissues, from which they are eliminated at varying rates (Yu et al., 2005). In general, PSTs accumulate at highest levels in the viscera of bivalves, but the ranking of toxicity among tissues often shifts during depuration (Bricelj et al., 1990). Several studies have also shown that depuration occurs most rapidly in the viscera (e.g., Waiwood et al., 1995).

In most cases, depuration patterns could be adequately fitted by a single-compartment, negative exponential model. However, this simple approach often markedly underestimated the time required to reach the PSTs regulatory level, especially in

species such as *Saxidomus giganteus* and *Spisula solidissima* (Bricelj and Shumway, 1998). A better fit to the data, and more accurate prediction of the time required to reach safety levels for human consumption, was provided in some cases by a biphasic detoxification model, consisting of an initial more rapid detoxification phase and subsequent slower (exponential) phase of toxin elimination (Bricelj and Cembella, 1995; Silvert and Cembella, 1995). It has been suggested that the initial detoxification phase represents gut evacuation of unassimilated toxin, whereas the second phase represents the release of toxins assimilated and incorporated in tissues (Bricelj and Shumway, 1998).

Various studies reported the accumulation and depuration profiles of individual PSTs (C1, C2, GTX1, GTX2, GTX3, GTX4, dcGTX2, dcGTX3, dcSTX, STX and NEO) after exposure to *Alexandrium* species or *G. catenatum* (Sekiguchi et al., 2001; Choi et al., 2003; Samsur et al., 2006, 2007). However, the elucidation of how individual PSTs behave during depuration was mainly focused on the toxins C1, C2, GTX1, GTX2, GTX3, GTX4, STX and NEO, which are characteristic of bivalves exposed to *Alexandrium* spp. (Ichimi et al., 2001; Blanco et al., 2003). Botelho et al. (2010a) studied the depuration kinetics of B1, dcSTX and dcGTX2+3, which are characteristic of the toxin profile in bivalves exposed to *G. catenatum*, under depuration laboratory conditions and over a declining bloom of the dinoflagellate in the field.

1.3.4. Responses at organism level to accumulated toxins

In general, when organisms are exposed to toxic substances, detoxifying enzymes will be induced to metabolize the toxic chemicals into more polar forms to facilitate excretion. During these metabolic processes reactive oxygen species may be formed as by-products causing widespread damage. To counterbalance this, many organisms have evolved antioxidant defence systems to prevent the formation of these free radicals (De Zwart et al., 1999). The changes in the antioxidant defence systems are often used to monitor oxidative stress in biological systems. Choi et al. (2006) examined the oxidative stress-related responses in clams (*Ruditapes philippinarum*) exposed to PSTs produced by *Alexandrium*

tamarense, predominantly C2 toxins. It appears that not all the antioxidant parameters showed responses to the increasing PST concentrations. In particular, clams showed considerable variations in their antioxidative responses. In a study with giant lions-paw scallops (*Nodipecten subnodosus*) exposed to *G. catenatum*, the digestive gland showed higher CAT and glutathione peroxidase (GPX) activities than other tissues (Estrada et al., 2007).

The sub-cellular distribution of domoic acid (ASP toxin) in the digestive gland cells suggests that it is weakly bound or free in the cytosol (Mauriz and Blanco, 2010). The sub-cellular partitioning of okadaic acid (DSP toxin) in the digestive gland of *Mytilus galloprovincialis* indicates its association with high-density lipoproteins (Rossignoli and Blanco, 2010). This approach was not used to provide better knowledge in the distribution and linkage of PSTs in bivalve tissues.

1.4. Effects of ingested PSTs on humans

Human can develop PSP through ingestion of contaminated bivalves, gastropods, or crustaceans containing high concentrations of PSTs (Gessner and Middaugh, 1995). Symptoms in humans associated with mild to moderate levels of intoxication consist of tingling and numbness of the perioral area and extremities, loss of motor control, drowsiness, incoherence, nausea, vomiting and diarrhoea. In the cases of severe intoxication, muscular paralysis and pronounced respiratory difficulties also occur (Van Dolah, 2000). PSP may be fatal through respiratory paralysis occurring within 2-24 h after ingestion (Gessner and Middaugh, 1980). There is no specific antidote for PSP, thus the clinical management of intoxicated victims is entirely supportive (Kao, 1993). This syndrome has been identified a serious risk for shellfish consumers for centuries. The first description of PSP dates from 1793 in the Pacific Northwest of North America (Halstead and Schantz, 1984). The location of these cases is related to the global distribution of the various PST-producing species. Figure 1.3 shows the cumulative global increase in the recorded distribution of the causative species and the confirmed appearance of PSTs in bivalves

(Anderson, 2009). While numerous fatal cases of PSP have been reported globally, the successful implementation of monitoring programs in many countries has helped to reduce human illnesses and fatalities (Etheridge, 2000).

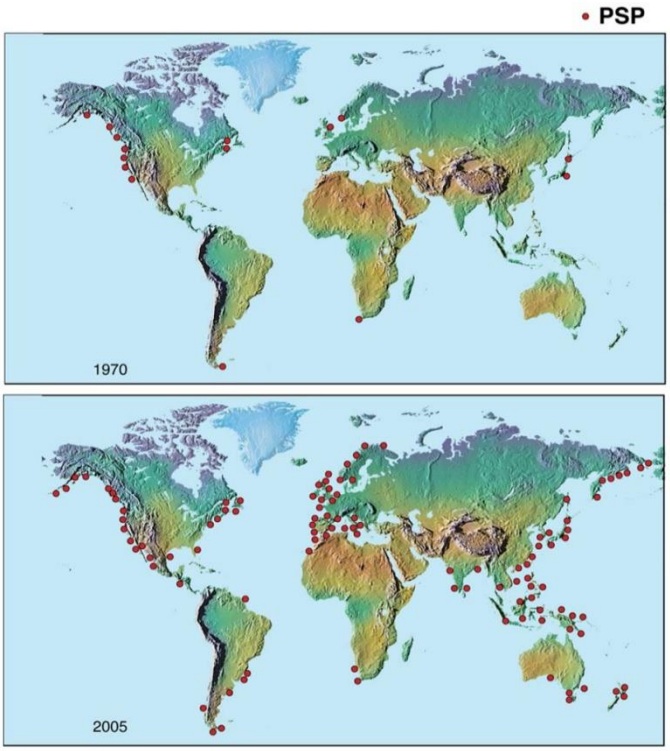


Figure 1.3. The global expansion in the distribution of PSTs in bivalves and PSP episodes - 1970 versus 2005. (Credit: U.S. National Office for Harmful Algal Blooms, Woods Hole Oceanographic Institution, Woods Hole, MA; adapted from Anderson, 2009)

1.5. Monitoring of toxin-producing species and bivalve toxicity

1.5.1 Design of monitoring programmes

Since the 1970s to 1980s, several countries run national programmes to survey PST-producing species and marine toxin concentrations in bivalves to alert the consumers about toxicity episodes (Mons et al., 1998; Batoréu et al., 2005). Currently, more than 50 countries are combining monitoring of toxin-producing species and commercial bivalve toxicity (Anderson, 2009). Estimation of uptake and elimination rates of toxin in bivalves

was the first step to select the best species to be used in surveillance programmes. Species that rapidly achieve elevated toxin levels when exposed to toxic algae, such as *Mytilus* spp., are suitable as sentinel organisms providing early warning of a bivalve toxicity episode. This species has long been used as the primary indicator for monitoring levels of PSTs in several geographic areas. This ability was confirmed in coastal waters of Maine, USA, since PST levels in *Mytilus edulis* occurred several days earlier than those in *Mya arenaria*, thus providing an adequate protection of harvesting of this species. It is also known that *M. edulis* generally becomes 2 to 4 times more toxic than neighbouring *Mya arenaria* (Bricelj and Shumway, 1998).

Monitoring bivalve toxicity provides a valuable, time- and space- integrated historical record of the occurrence and intensity of toxic blooms. Continuous time series of PST toxicity for the same bivalve species sampled at the same location are useful to learn about possible seasonal and multi-annual trends of toxicity episodes.

1.5.2. Closure of legal harvest of bivalves

Legal controls of marine toxins in bivalves are prescribed by the European food safety legislation (Anon., 2004a; Anon., 2004b). This legislation requires all European Union (EU) member states to have in place an official control monitoring system that ensures there are checks for the presence of marine toxins in bivalve production areas. Production area is here interpreted as a natural production area or aquaculture area. There is also a requirement for the monitoring of toxin-producing phytoplankton in those areas. Every time a sample presents toxicity values exceeding the EU regulatory limit (RL), the closure of legal harvest of bivalves is mandatory. The sector regains the activity when two consecutive samples (sampled at least 48 hours apart) show values below RL (Anon., 2004b). In the EU and USA (Anon., 2004b), as well as in most countries (FAO, 2004), the RL for PSTs is 800 µg STX di-HCl equivalents per kg of shellfish meat. This value has been lowered to 300 and 400 µg STX di-HCl equiv. kg^{-1} in Mexico and the Philippines, respectively (FAO, 2004). Exceptionally, harvest of *Acanthocardia tuberculata* (Mediterranean cockle) is authorised by

the European Commission to Spain in areas where the PST level in edible parts of those molluscs is between 800 and 3000 µg per kg tissue, if heat treatment is carried out (EC, 1996).

1.6. Economic impact of PSTs

The toxicity episodes by PSTs have a array of economic impacts, including the costs of conducting monitoring programmes for bivalves and other affected resources, short- and long-termed closure of harvestable bivalves, reductions in seafood sales, mortalities of wild and farmed fish, impacts on tourism and tourism-based related businesses, and medical treatment of exposed populations (Anderson et al., 2009). The annual cost of monitoring PSTs in the Bay of Fundy and British Columbia, Canada, was valued at US $102 K and $82 K, respectively, representing about 4 to 5% of the value of shellfish harvested in 1988 (Cembella and Todd, 1993). As example of single PST episodes, the total economic loss to the oyster industry on the Pacific U.S. coast in 1980 was estimated at US $ 0.6 million, and an 8-month closure on mussel harvesting in the Philippines in 1983, resulted in an estimated loss of $US 2.2 million (Bricelj and Shumway, 1998).

1.7. The Portuguese situation

The first association of *G. catenatum* blooms with PSP episodes in Portugal was reported in 1986 (Franca and Almeida, 1989). A detailed neurological characterization of PSP symptoms was performed on hospitalised patients due to a severe event in 1994 after consumption of mussels harvested in the NW coast (Carvalho et al., 1998). The Portuguese monitoring programme for marine toxins was started in 1994 by the Portuguese Institute of Marine Research (IPIMAR) and subsequent Governmental Institutes (Sampayo et al., 1997; Vale et al., 2008). PSTs were one of the first toxin groups under surveillance, accomplished by the monitoring of their phytoplankton producers (Sampayo et al., 1997; Vale et al., 2008). Currently, the programme covers 38 production areas located in coastal lagoons, estuaries and open coastal zones including most of the commercial bivalve species harvested in these

areas (*Mytilus* spp., *Cerastoderma edule*, *Ruditapes decussatus*, *Venerupis pullastra*, *Scrobicularia plana*, *Ensis* spp., *Solen* spp., *Venus verrucosa*, *Ostrea* spp., *Crassostrea* spp., S*pisula solida*, *Donax trunculus*, *Ensis siliqua*, *Callista chione* and *Chamelea gallina*) (IPMA database, 2014).

A review of marine toxin monitoring in bivalves from Portugal between 1986 and 2006 indicated that PSTs occurred intermittently in autumn (Vale et al., 2008). In recent years, high concentrations of PSTs in bivalves have been sporadically registered (IPMA database, 2014).

1.8. Motivation and objectives

The first works on marine toxins were focused on the assessment of bivalve toxicity in order to protect human health. Whole bivalve toxicity was first assessed through bioassays. As chemical methodologies were improved, bioassays were progressively substituted by analytical procedures providing the possibility to quantify individual toxins, and thus to characterise the toxin profiles both in algae and bivalves. Paralytic shellfish poisoning is a key issue due to severity of symptoms in humans. In addition, toxin-producing species may produce a variety of paralytic shellfish toxins, some of them poorly documented. Individual quantification of those toxins, in particular the less documented compounds, or those that are recurrently found in bivalves from the Iberian waters, will help to elucidate tissue partitioning of toxins, kinetics, and transformation mechanisms of the assimilated toxins.

This thesis focused on the accumulation of PSTs in commercial bivalve species harvested in Portuguese estuarine and coastal waters, including kinetics of assimilated toxins, sub-cellular partitioning, and differences in profiles of PSTs between bivalves exposed to *G. catenatum* blooms and bivalves with low toxicity values. Moreover, an overview of the occurrence of PSTs episodes in Portugal was carried out, as well as the linkage between nutrient composition and toxins in bivalves.

The scientific objectives of this study include:

- To search differences between uptake and depuration kinetics of the major toxins produced by *G. catenatum* cells (dcGTX2+3, dcSTX, B1, C1+2) in the clam *Ruditapes decussatus*; the selection of this species is related to the high production in Portugal;

- To test whether nutrient concentration and composition influence the abundance of toxic dinoflagellates and consequently the bivalve toxicity; this hypothesis was tested in a eutrophic lagoon and adjacent coastal waters with recurrent toxicity episodes;

- To investigate the sub-cellular partitioning of the major toxins produced by *G. catenatum* cells (dcGTX2+3, dcSTX, B1, C1+2) in the cockle *Cerastoderma edule*, under post-bloom natural conditions; this species is highly abundant in estuarine and coastal lagoons;

- To examine the inter-annual and seasonal variation of bivalve toxicity episodes by PSTs in the Portuguese waters; the commercial bivalve species *Spisula solida*, *Donax trunculus*, *Mytilus* spp., *Cerastoderma edule* were considered.

- To compare profiles of PSTs between bivalves exposed to *G. catenatum* blooms and bivalves of low toxicity; differences between these two circumstances were examined in *Spisula solida*, *Donax trunculus*, *Mytilus* spp., and *Cerastoderma edule* from Portuguese waters.

1.9. Dissertation outline and content

After this introductory chapter, Chapter 2 presents a synthesis of the experimental and analytical methodologies used to reach the objectives. Chapters 3 to 7 include the following scientific articles published or submitted at peer-reviewed journals developed during the PhD study:

- Botelho, M.J., Vale, C., Grilo, R.V., Ferreira, J.G., 2012. Uptake and release of paralytic shellfish toxins by the clam *Ruditapes decussatus* exposed to *Gymnodinium catenatum* and subsequent depuration. Marine Environmental Research 77, 23-29.

- Pereira, P., Botelho, M.J., Cabrita, M.T., Vale, C., Moita, M.T., Gonçalves, C., 2012. Winter-summer nutrient composition linkage to algae-produced toxins in shellfish at a eutrophic coastal lagoon (Óbidos lagoon, Portugal). Estuarine Coastal and Shelf Science 112, 61-72.

- Botelho, M.J., Raimundo, J., Vale, C., Ferreira, J.G., 2014. Partitioning of paralytic shellfish toxins in sub-cellular fractions of the digestive gland of the cockle *Cerastoderma edule*: Changes under post-bloom natural conditions. Ecotoxicology and Environmental Safety 104, 365-372.

- Botelho, M.J., Vale, C., Ferreira, J.G. Identification of seasonal and multi-annual trends of bivalve toxicity by PSTs in Portuguese estuarine and coastal waters. Estuarine, Coastal and Shelf Science, in submission.

- Botelho, M.J., Vale, C., Ferreira, J.G. Profiles of paralytic shellfish toxins in bivalves of low and elevated toxicities following exposure to *Gymnodinium catenatum* blooms in Portuguese estuarine and coastal waters. Chemosphere, in submission.

The order of the chapters reflects the chronological order of the aforementioned publications. Chapter 3 deals with the uptake and depuration kinetics of paralytic shellfish toxins in the clam *Ruditapes decussatus* exposed to toxic algae. Chapter 4 examines the possible effect of the nutrient pool in the water column on the maintenance of high abundance of toxic cells, and consequently increasing the duration of bivalve toxicity episodes. After these studies published in 2012, the partitioning of those toxins in sub-cellular fractions of the digestive gland of the cockle *Cerastorderme edule* was investigated, as well as alterations due to toxin biotransformation under post-bloom

conditions (Chapter 5). Historical data on paralytic shellfish toxin concentrations and toxicity of commercial species harvest in Portugal was used to examine multi-annual and seasonal variations. Bivalve species and geographical areas more vulnerable to toxicity episodes were identified (Chapter 6). Chapter 7 shows the different toxin profiles of commercial bivalves, emphasizing the effect of biotransformation of the assimilated toxins under post-bloom conditions. Chapter 8 presents a review of the various topics dealt with in Chapters 3 to 7, and provides an integrative discussion to key questions linked to the thesis objectives. Supplementary areas of research related to this thesis were identified. Chapter 8 contains a broad discussion on the management application of this work.

2

2. Methodologies

2.1. Algal cultures

2.1.1. *Gymnodinium catenatum*

The culture of the dinoflagellate *Gymnodinium catenatum* (strain C37-07, Figure 2.1) was obtained from the IPMA algae collection and was originally isolated in 2007 from the adjacent coastal area of Aveiro, Portugal. This strain was maintained in natural seawater enriched with GSe medium (salinity 28, Blackburn et al., 2001) at 18°C under a 16 h light: 8 h dark photocycle with a light intensity of 15 µE m^{-2} s^{-1}. The strain was then mass cultured in 10 L culture flasks under the same conditions. Cells of the toxic microalgae were counted in Palmer-Maloney chambers under an Zeiss IM 35 inverted microscope (Hallegraeff et al., 1995).

Figure 2.1. Light microscopy photo of *Gymnodinium catenatum*; marine toxic algae collection (IPMA, ex-IPIMAR); scale bar=30 µm

2.1.2. *Isochrysis galbana*

The non-toxic microalga *Isochrysis galbana* was grown in Wallerstein and Miquel medium (Bandarra et al., 2003) at 18°C in 75 L plastic bags under constant illumination at salinity 25. Cells of *I. galbana* were counted in an automatic particle counter (Coulter EPICS

XL).

2.2. Collection and processing of samples

2.2.1. Algal culture

Aliquots of 400 mL *G. catenatum* culture were harvested in the late exponential growth phase. Algal mass was obtained by filtering the culture under light vacuum pressure (100 mmHg) through GF/C glass filters (1.2 µm), and freezing in 0.1 M acetic acid at -80^{o}C until toxin analyses.

2.2.2. Seawater

Surface water samples were collected for phytoplankton identification, cell counting and toxin analysis. The samples for phytoplankton identification and cell counting were preserved in Lugol's solution immediately after collection. The samples for toxin analysis passed through a GF/C glass filters (1.2 µm), under light vacuum pressure (100 mmHg), and the material retained in the filters was frozen in 0.1 M acetic acid at -80^{o}C until analysis.

Water samples for measure of phytoplankton biomass as chlorophyll *a* (Chl *a*) concentration, and for nutrient determination were collected at surface (0.5 m), middle (10 m) and bottom (20 m) at each sampling site. For Chl *a* quantification, samples were filtered through GF/F glass filters and frozen at -20^{o}C until analysis. Samples were filtered through MSI Acetate Plus filters for determination of nutrients.

2.2.3. Particulate organic matter

Particulate organic matter released by bivalves (faeces and pseudofaeces) during a laboratory feeding experiment was collected from the 5 L plastic tanks. Seawater from three tanks was transferred to an inverted conic recipient and particles were left to settle for 12 hours. Material was then collected by filtration through 0.45 µm polycarbonate membranes, and kept frozen in 0.1 M acetic acid at -80^{o}C until toxin analyses.

2.2.4. Bivalves

Whole soft tissues. Bivalves from three pools of 10 specimens each were dissected and composite samples (n=10) of whole soft tissues were weighed and stored at -80^{o}C until toxin analyses.

Digestive glands. Bivalves were dissected and composite samples of digestive glands (three pools of 10 specimens) were weighed and frozen at -80^{o}C until toxin analysis.

2.2.5. Sub-cellular fractionation of digestive glands

For sub-cellular toxin analyses, bivalve digestive gland samples were homogenised in a buffer solution and subjected to differential fractionation (Figure 2.2) according to the procedure adopted from Campbell et al. (2005) and Raimundo et al. (2008).

Treatment of samples. The digestive gland composite samples were placed in an ice bath and homogenised in a buffer solution (5 mL of buffer g^{-1} of digestive gland), consisting of 0.1 M Tris-HCl and ammonium formate 0.15 M to a final pH of 7.8 (Mauriz and Blanco, 2010; Raimundo et al., 2008). The homogenisation was completed in 3 min at 11000 rpm in a Polytron Kinematica AG homogenizer (Rossignoli and Blanco, 2010). A 3 g aliquot of the homogenate tissue was taken as initial sample for toxin extraction and the remaining homogenate was used for differential fractionation.

Differential fractionation. The aliquots of each homogenate pool of digestive glands were transferred to centrifuge tubes. The homogenate was first fractioned by centrifugation at 700 x g for 15 min at 4^{o}C to separate the nuclei, granules and plasmatic membranes, designated as "nuclei+debris" fraction (P1); the supernatant was further centrifuged at 9 000 x g for 20 min at 4^{o}C to separate the mitochondria fraction (P2); the lysosomes (P3), and microsomes (P4) fractions were obtained by centrifuging the supernatant at 30 000 x g for 25 min, and at 100 000 x g for 40 min at 4^{o}C, respectively. The remained soluble part, mainly constituted by cytosolic components, was designated by cytosolic fraction.

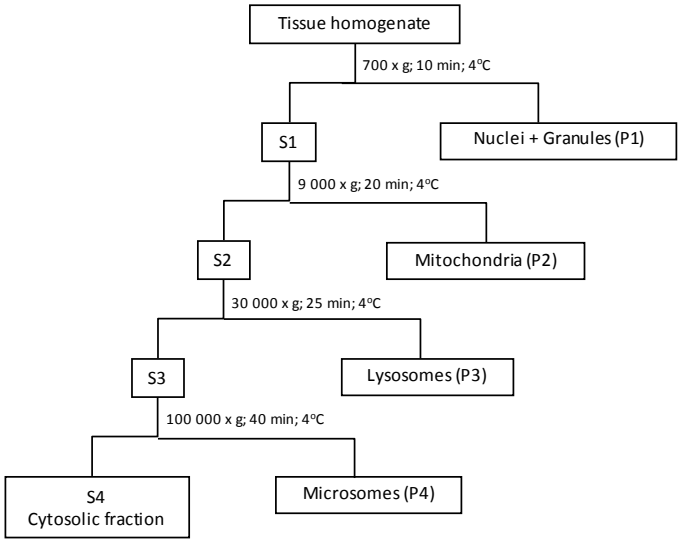


Figure 2.2. Schematic procedure of the sub-cellular fractionation by sequential centrifugation; S - supernatant fraction; P - pellet fraction.

2.3. Phytoplakton and nutrients

2.3.1. Phytoplankton

Phytoplankton identification and cell counts were performed following the sedimentation method of Uthermöl (1958). In water samples with high sediment particles content overlapping of cells and sediment particles are likely to occur. To minimize the underestimation of small phytoplankton species 5-25 mL of water were used in counting chambers. Cell identification and counting were carried out under an inverted microscope Zeiss IM 35.

Phytoplankton biomass was measured as Chl *a* concentration determined by fluorescence (Perkin Elmer Fluorometer) using the modified method of Lorenzen (1966).

2.3.2. Water quality and nutrients

Temperature, salinity, dissolved oxygen and pH were measured *in situ* in surface (0.2-0.5 m), middle (10 m) and bottom (20 m) waters using a YSI (650 m) multi-parametric probe.

The determinations of nitrate+nitrite (NO_3^-+NO_2^-), ammonium (NH_4^+), phosphate

(PO_4^{3-}) and silicate $Si(OH)_4$ were carried out using a TRAACS 2000 (Bran+Luebbe) autoanalyser (Grasshoff et al., 1983). In addition, total dissolved nitrogen and total dissolved phosphorus were measured according to Koroleff´s method (1983) modified by the ISO 11905-1 (1997). Dissolved organic nitrogen and dissolved organic phosphorus were estimated by subtracting the mass of dissolved inorganic nitrogen (NO_3^-+NO_2^-+ NH_4^+) and of PO_4^{3-} from the mass of total dissolved nitrogen and total dissolved phosphorus, respectively.

2.4. Paralytic shellfish toxins

2.4.1. Chemical methodologies for PSTs

During the last decades, chemical methodologies for determination of PSTs have been under development and validation, attempting to replace the mouse bioassay as the official method (Anon., 2005a). A instrumental chemical method of liquid chromatography with fluorescence detection (LC-FLD) involving pre-chromatographic oxidation was adopted by the Association of Official Analytical Chemists (AOAC 2005.06) as an official method for determination of PSTs in bivalves (Anon., 2005b). After validation through a collaborative study for STX, NEO, GTX2+3, GTX1+4, dcSTX, B1, C1+2 and C3+4 in bivalves (mussels, clams, oysters, and scallops) the method was integrated in European Directives to act as a legal alternative to the mouse bioassay (Lawrence et al., 2005; Anon., 2006). Recently, a refinement and extension of the AOAC method to other bivalve species, offers possibilities to achieve reliable analytical alternatives, as well as including the additional toxins dcNEO and dcGTX2+3 (Turner et al., 2009; 2010). As the method was applied in routine analysis difficulties have been reported for profiles derived from various toxin-producer phytoplankton species (Ben-Gigirey et al., 2007).

The AOAC method, also called ‘‘the Lawrence method’’ was primarily developed for bivalve samples contaminated by *Alexandrium* spp. blooms, where N-sulfocarbamoyl and decarbamoyl toxins made a smaller contribution to the PSTs profile (Lawrence et al., 1991). A different situation occurs with bivalves contaminated by *Gymnodinium catenatum* in Portuguese and Galician waters. The non-hydroxylated compounds (B1, C1+2, dcSTX,

dcGTX2+3) emerge as major components of the toxin profiles (Franca et al., 1989; Camino-Ordás et al., 2004). Due to these specificities in toxin profiles in bivalves exposed to *G. catenatum* an adaptation of this method was developed. The modification was centered on the inversion of the sequence of oxidation reactions of the AOAC method, firstly quantifying the non-hydroxylated toxins in the peroxide oxidation (Botelho et al., 2010). Under these conditions one can assume that if non-N-hydroxylated compounds are absent, then N-hydroxylated toxins will also be absent. Consequently, no further action is necessary, having the merit of being less-time consuming.

The determination of PSTs in samples of bivalve tissues, toxic algae, and particulate organic matter presented in this thesis were based on AOAC 2005.06 (Anon., 2005b).

2.4.2. Toxin extraction, cleanup and oxidation

Bivalve samples

The extraction, cleanup and oxidation procedures for PSTs determination in bivalve samples were performed according to the following steps:

Acid extraction. Three mL of 1% acetic acid were added to approximately 5 g bivalve homogenate in a centrifuge tube, mixed by vortexing, and the mixture boiled for 5 min in a water bath. The tubes were then placed in a beaker of cold water for 5 min to cool down and centrifuged at 3600 x g for 10 min at room temperature. The supernatant was saved, and the pellet was extracted again with 3 mL of 1% acetic acid. The two supernatants were mixed, and the final volume was brought up to 10 mL with water.

Cleanup. An octadecyl bonded phase silica C18 SPE (Solid Phase Extraction) cartridge (500 mg/3 mL) (Supelclean, Supelco, USA) was preconditioned with 6 mL of methanol followed by 6 mL of water. Then 1 mL of extract (0.5 g bivalve equivalent) was loaded into the cartridge, washed with 2 mL of water and the washing was combined with the effluent. The pH of the extract was adjusted to 6.5 with 0.2 M NaOH and diluted to exactly 4 mL. Aliquots of this extract were used for oxidation of PSTs with peroxide and periodate respectively prior to LC analyses.

Sequential fractionation. A second column purification, SPE ion exchange cartridge with carboxylic acidsiliane (COOH) bonded to silica gel (500 mg/3 mL) (Bakerbond, J.T. Baker, USA) was used only for extracts found to contain N-hydroxylated PSTs after C18 cleanup. This procedure was necessary to separate NEO from B2, and GTX1+4 from C3+4 toxins since they produced the same oxidation product. The cartridges were conditioned by washing with 10 mL of 0.01 M aqueous ammonium acetate. A 2 mL aliquot of C18 clean extract was placed on the column and the eluate collected into a 15 mL graduated conical test tube. The column was eluted with 4 mL of ultrapure water and the eluates combined and adjusted to 6 mL of final volume. Fraction 1 was considered to contain the C toxins. The same COOH cartridge was again eluted with 4 mL of 0.05 M NaCl solution, the eluate collected and the volume adjusted to 4 mL with ultrapure water (fraction 2). This fraction contains toxins GTX1+4, GTX2+3, B1, B2 and dcGTX2+3. Further elution of the column with 5 mL of 0.3 M NaCl solution followed by adjustment to 5 mL with ultrapure water, produces fraction 3, which contains toxins STX, NEO, dcSTX and dcNEO.

Peroxide oxidation. Aqueous hydrogen peroxide (25 µL, 10% v/v) was added to 250 µL of 1 M NaOH in a 1.5 mL glass vial and vortex mixed. Then 100 µL PST standard solution or sample extract after C18 or ion exchange cleanup was added, and allowed to react for 2 min at room temperature. Glacial acetic acid (20 µL) was added and vortex mixed before analysis by LC.

Periodate oxidation. The periodate reagent was prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.03 M ammonium formate and 0.3 M disodium hydrogen phosphate and the pH was adjusted to 8.2 with 0.2 M NaOH (Gago-Martínez et al., 2001). A matrix modifier was made with an extract prepared from PST-free oysters as described below for C18 cleanup. Aliquots (100 µL) of PSTs standard solution, or sample extract after C18 or ion exchange COOH, were added to 100 µL of matrix modifier solution in a 1.5 mL glass vial. Periodate oxidant (500 µL) was added and the mixture thoroughly mixed and allowed to react during 1 min. Then glacial acetic acid (5 µL) was added and the mixture was allowed to stand for a further 10 min before analysis by LC.

The same procedure of periodate and peroxide oxidations was followed substituting the oxidant reagent by water in order to detect natural fluorescent compounds.

Toxic algae samples

Toxins of *G. catenatum* cells retained on filters were extracted according to the method described in Artigas et al. (2007) with the following modifications: the extraction was done by freeze/thaw cycle followed by ultrasonication in an ice bath for 30 seconds at 60% amplitude and 20 W (Vibra Cell, Sonics & Materials Inc.). Cell debris after ultrasonification was examined under an inverted microscope revealing full disruption of the surveyed samples. The crude extract was centrifuged at 2500 x g for 10 min, and then cleanup was performed using an SPE C18 cartridge (Sep-Pak Light, Waters). The 130 mg C18 cartridge was conditioned with 1.5 mL methanol and 1.5 mL ultra-pure water. Subsequently an aliquot of 250 µl of supernatant was loaded, then washed with 500 µL of ultra-pure water. The pH of the extract was adjusted to 6.5 with 0.2 M NaOH, and diluted to exactly 1 mL.

Particulate organic matter

Toxins in particulate organic matter released by bivalves were extracted using the same procedure as for dinoflagellate cells, except for the cleanup step, which was skipped due to the low toxin content. After centrifugation the extract was filtered through disposable 0.2 µm regenerated cellulose syringe filters.

2.4.3. Liquid chromatography analysis

The LC analysis was carried out by a Hewlett-Packard/ Agilent system (Palo Alto, CA, USA) consisted of a 1050 Series quaternary pump, 1100 Series in-line degasser, autosampler, column oven, and 1200 Series fluorescence detector. The Agilent Chemstation software (revision A.10) performed data acquisition and peak integration. Separation was performed using a reversed-phase column Supelcosil C18, 15 x 4.6 mm id, 5 mm (Supelco, Bellefonte, PA, USA) equipped with a guard column Supelguard Supelcosil C18, 2 x 4.0 mm id, 5 mm

(Supelco, Bellefonte, PA, USA). The column was kept in a column oven at 30^{o}C. Detection wavelengths were set at 340 nm for excitation and 395 nm for emission. The mobile phase gradient used to elute the PST 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 next 4 min and 100% mobile phase A (0.1 M ammonium formate, pH=6) was used for 5 min before next injection. The flow rate was 1 mL min^{-1}. The injection volumes were 50 mL and 100 mL, for the oxidation products of peroxide and periodate reaction, respectively.

2.4.4. Identification and quantification of PSTs

All PSTs were identified and quantified by comparison of peak areas of sample oxidation products and of working standards. The compounds GTX2+3, GTX1+4, C1+2 and C3+4 were quantified jointly due to the fact that the oxidation process creates the same oxidation product. Due to the lack of commercial standards, the identification of C3+4 and B2 was done taking into account their retention time described in the LC method (Ben-Gigirey et al., 2007; Lawrence and Niedzwiadek, 2001).

Since the non-N-hydroxylated toxins form highly fluorescent derivatives with hydrogen peroxide oxidation but not N-hydroxylated toxins, chromatograms of peroxide oxidised samples are used for the detection and quantification of non-N-hydroxylated toxins (dcGTX2+3, C1+2, dcSTX, B1, GTX2+3 and STX) (Figure 2.3). Periodate oxidation of the C18 extract was used to quantify the dcNEO toxin, avoiding dilution effect during the SPE-COOH fractionation. This toxin may also be determined from the C18-fraction 3-periodate oxidation extract as mentioned in Turner et al. (2009). To separate N-hydroxylated toxins, namely C3+4 from GTX1+4, and B2 from NEO, an SPE-COOH ion exchange column was used. The LC measurements after periodate oxidation of each fraction allowed to detect and/or quantify potential N-hydroxylated toxins (C3+4 in fraction 1; GTX1+4 and B2 in fraction 2; NEO in fraction 3). The peaks used for quantification of GTX1+4 and NEO were those recommended by Lawrence et al. (2005). Figure 2.4 presents chromatograms illustrating

toxin separation in a mussel sample with a toxic profile characteristic of *Gymnodinium catenatum* after peroxide and periodate oxidation of C18 extract, and periodate oxidation of fractions 1 to 3.

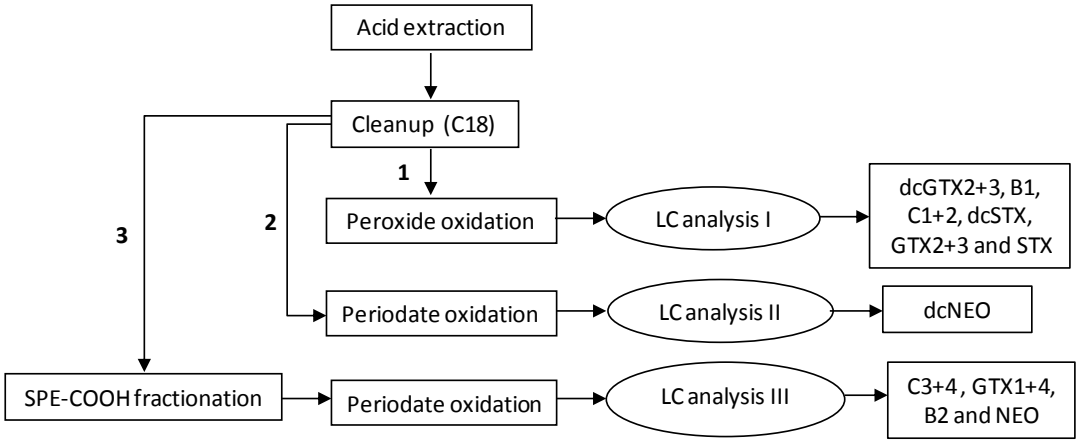


Figure 2.3. Flow diagram with the sequence of oxidation reactions and corresponding toxin identification after C18 cleanup or SPE-COOH fractionation (1=peroxide oxidation of C18 extract; 2=periodate oxidation of C18 extract; 3=periodate oxidation of fractions 1 to 3)

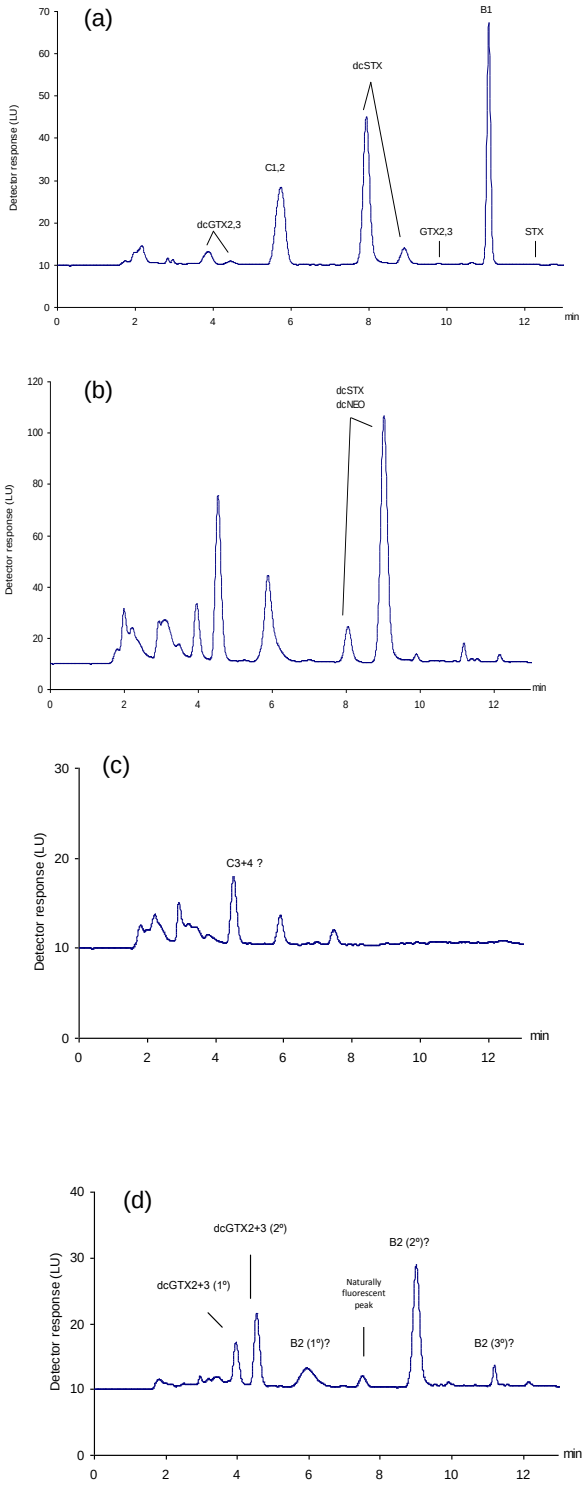


Figure 2.4. Chromatograms obtained for a mussel sample with a toxic profile characteristic of *Gymnodinium catenatum.* (a) quantification of dcGTX2+3, C1+2, dcSTX, GTX2+3, B1 and STX (peroxide-C18); (b) quantification of dcNEO (periodate-C18); (c) detection of C3+4 (periodate-SPE-COOH-F1); (d) quantification of GTX1+4 and detection of B2 (periodate-SPE-COOH-F2).

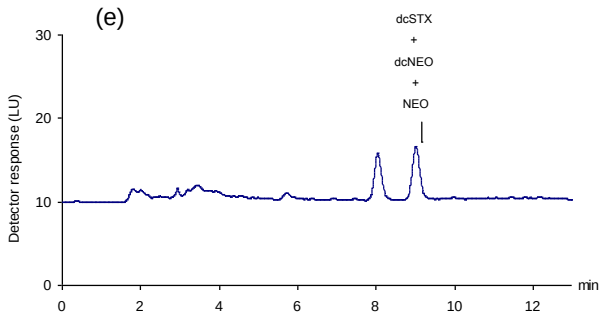


Figure 2.4. (cont.) (e) quantification of NEO (periodate-SPE-COOH-F3).

The abundant dcSTX in extracts from shellfish samples contaminated by *Gymnodinum catenatum* frequently leads to the interference in the dcNEO quantification due to similar retention time after periodate oxidation (Botelho et al., 2010). To resolve the overlapped areas, dcNEO was quantified by subtracting the dcSTX contribution. This contribution was estimated from the calibration curve oxidized with periodate, using the dcSTX concentration obtained previously under perioxide oxidation. A similar calculation was applied to quantify (i) GTX1+4 in the extract from fraction 2 followed by periodate oxidation, subtracting the contribution of dcGTX2+3, and (ii) NEO in the extract from fraction 3 followed by periodate oxidation, subtracting the contribution of dcNEO and dcSTX. The procedure to quantify N-hydroxylated toxins in presence of dcSTX has been adopted in Turner et al. (2009).

2.4.5. Estimation of B2 concentration

Despite the unavailability of commercial certified reference material for the toxin B2, its concentration was estimated through the hydrolysis conversion into NEO toxin. The analytical procedure was based on the Marine Biotoxins Report of the Community Reference Laboratory for the determination of PSP toxins in shellfish including B2 (GTX6) after hydrolysis (Anon., 2007). Aliquots of fraction 2 (SPE-COOH fractionation) containing the toxin B2 were used for periodate oxidation, prior to LC-FLD analyses. The LC measurements after periodate oxidation of this fraction allowed the detection of B2. 125 μL of 1 M chloridric acid solution were added to 500 μL of fraction 2 in a glass tube. The tube

was closed and heated for 20 minutes at 90°C in a water bath, and then allowed to cool down at room temperature. The reaction was then neutralized adding small volumes of 1 M sodium hydroxide solution. The solution was mixed after each addition until a volume of 125 µL was obtained. After neutralization, the pH of the hydrolyzed extract was adjusted to 8.2. Hydrolysed extracts were submitted to periodate oxidation and B2 toxin was indirectly quantified through conversion into NEO. It was assumed that the molarity of B2 toxin hydrolyzed was equal to the molarity of the NEO toxin present in fraction 2 after hydrolysis.

2.4.6. Certified reference materials

In order to check the precision and accuracy of the measurements, certified reference materials were used in this work. Certified calibration solutions for PSTs were purchased from the Certified Reference Materials Program of the Institute for Marine Biosciences, National Research Council, Halifax, Canada (CRM-STX-e, CRM-NEO-b, CRM-GTX2&3-b, CRM-GTX1&4-b, CRM-dcSTX, CRM-dcGTX2&3, CRM-GTX5-b (B1), CRM-C1&2, CRM dcNEO-b and NEO-b. Working standard solutions were prepared by appropriate dilution of the corresponding stock solution and stored at 4°C during 1 month. Two standard toxin mixtures were prepared: GTX1+4 and NEO (Mix I); GTX2+3, dcGTX2+3, STX, dcSTX, B1 and C1+2 (Mix II). The dcNEO was used individually in working standard solutions.

For quantification of PSTs in bivalve tissues, matrix-matched calibration curves with cleaned-up bivalve tissue extract were used as described in Botelho et al. (2010). Calibration curves prepared in solvent were used for toxin quantification in *G. catenatum* and particles extracts.

2.4.7. Performance and quality control

Limits of detection (LOD) were experimentally determined using a signal:noise ratio of 3:1. Oxidation of standard toxin mixtures and subsequent quantification in triplicate were used to assess the variability of those amounts. The non-N-hydroxylated toxins exhibited lower LODs than N-hydroxylated toxins, reflecting higher sensitivity of the methodology for

those compounds. LODs (nmol L^{-1}) were 3.9 (C1+2), 4.0 (B1), 4.0 (STX), 4.9 (dcSTX), 8.2 (dcGTX2+3), 8.5 (GTX2+3), 25 (dcNEO), 30 (GTX1+4) and 31 (NEO).

Recoveries and losses of the analytical procedure were assessed in mussel and clam tissues at two concentration levels, following the methodology described in Quevauviller and Morabito (2000). PST-free clam and mussel matrices were used to assess recoveries. The matrices were spiked in triplicate with the addition of a toxin mixture including C1+2, B1, GTX2+3, STX, dcSTX and dcGTX2+3 to produce two concentration levels. The spiked level I contained toxin concentrations ranging from 20 ng g^{-1} (dcSTX) to 142 ng g^{-1} (C1+2), while spiked level II from 82 ng g^{-1} (dcSTX) to 567 ng g^{-1} (C1+2). Intervals of the mean recoveries for the quantified PSTs were: 71-74% (C1+2), 84-133% (B1), 87-110% (GTX2+3), 85-93% (STX), 77-116 (dcSTX) and 52%-107% (dcGTX2+3). For the toxin NEO, a interval of mean recoveries of 87-97% was obtained in matrices spiked in triplicate with 227 ng g^{-1} (level II). Repeatability values in terms of relative standard deviation were from 0.3 to 21%. Instrumental detection limits (nmol L^{-1}) were 3.9 (C1+2), 4.0 (B1), 4.0 (STX), 4.9 (dcSTX), 8.2 (dcGTX2+3), 8.5 (GTX2+3) and 31 (NEO).

3

3. Uptake and release of paralytic shellfish toxins by the clam *Ruditapes decussatus* exposed to *Gymnodinium catenatum* and subsequent depuration

Botelho, M.J., Vale, C., Grilo, R.V., Ferreira, J.G., 2012. Uptake and release of paralytic shellfish toxins by the clam *Ruditapes decussatus* exposed to *Gymnodinium catenatum* and subsequent depuration. Marine Environmental Research 77, 23-29. (doi:10.1016/j.marenvres.2012.01.002).

Abstract

A laboratory experiment was performed with the clam *Ruditapes decussatus,* fed with the toxic dinoflagellate *Gymnodinium catenatum* and the non-toxic algae *Isochrysis galbana* (14 days) and subsequently only with *I. galbana* (15 days). Individual paralytic shellfish toxins were determined by LC-FLD in *G. catenatum* cells, whole clam tissues, and particulate organic matter (POM) produced by clams. The toxins dcSTX and dcGTX2+3 in the algae were less abundant than C1+2 and B1, but were predominant in clams during both the exposure and depuration phases. The toxin dcNEO was only detected in clams during a short period, indicating conversion from other compounds. The toxin composition of the POM indicated the export of dcSTX as faeces or pseudo-faeces along the entire experiment (2.5-14 nmol mg^{-1}), B1 was present in a short period of the exposure and C1+2 and dcGTX2+3 absent. A mass balance calculation indicated that approximately 95% of C1+2 and 85% of B1 supplied to the clams were converted into other toxins or lost in solution. Conversely, the net gain of 512, 61 and 31 nmol for dcSTX, dcGTX2+3 and dcNEO, respectively, suggests the conversion from other assimilated compounds by clams during exposure and depuration phases.

Keywords: Paralytic shellfish toxins; *Ruditapes decussatus*; Particulate organic matter; Uptake; Depuration; Mass balance

3.1. Introduction

Paralytic shellfish toxins (PSTs) are a group of neurotoxic alkaloids produced in the marine environment by dinoflagellates like *Alexandrium* spp., *Pyrodinium bahamense* and *Gymnodinium catenatum* (Llewellyn, 2006). The documented PSTs are grouped into three structural families in decreasing order of toxicity (Oshima, 1995a; Anon., 2009): carbamate (saxitoxin-STX, neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), decarbamoyl (dcGTX1 to dcGTX4, dcSTX and dcNEO), and N-sulfocarbamoyl (B1, B2, C1 to C4). Most of the *Alexandrium* species have a carbamate-dominated toxin profile, while decarbamoyl and N-sulfocarbamoyl

toxins are dominant components in *G. catenatum* (Cembella et al., 1987; Chou et al., 2004; Ordás et al., 2004; Band-Schmidt et al., 2005; Costa et al., 2010). It is well documented that PSTs are efficiently accumulated by filter-feeders during blooms of toxic phytoplankton species, being causative agents of paralytic shellfish poisoning in humans (Sommer and Meyers, 1937; Gessner and Middaugh, 1980).

Various studies have proved that shellfish exposed to dinoflagellates exhibit different PSTs profiles from the toxin producers (Oshima et al., 1990; Bricelj et al., 1990; Cembella et al., 1993; Samsur et al., 2006). Metabolic interconversion of assimilated PSTs achieved by enzymatic and chemical reactions in shellfish tissues may contribute to those differences (Shimizu and Yoshioka, 1981; Kotaki et al., 1985; Oshima, 1995b; Bricelj and Shumway, 1998). Incubation in vitro of toxic dinoflagellates or purified toxins extracts allowed elucidating the role of enzymatic activities (Sullivan et al., 1983; Fast et al., 2006; Artigas et al., 2007). Different uptake and depuration kinetics of individual PSTs may also contribute to the registered modifications on toxin profiles between the toxic algae and the exposed shellfish (Blanco et al., 2003; Yu et al., 2007; Botelho et al., 2010a).

Despite the low concentration of individual toxins in the dissolved fraction their quantification provides additional information on the elimination of individual PSTs (Bricelj and Shumway, 1998; Sekiguchi et al., 2001). Toxins in particulate organic matter rejected by shellfish are at higher concentrations, but may be difficult to interpret if encompassing particles from different pathways, namely faeces, pseudofaeces and wasted food (Samsur et al., 2006; Estrada et al., 2007; Samsur et al., 2007).

This work reports the levels of the toxins C1+2, B1, dcSTX, dcGTX2+3 and dcNEO in whole tissues of the clam *Ruditapes decussatus* and in particulate organic matter rejected by the clams during a 29-day feeding experiment, including exposure to *Gymnodinium catenatum* and subsequent depuration. Toxin composition of toxic algae, clams and particulate fraction in conjunction with mass balance calculations for individual toxins, allowed identifying the toxins exported as faeces or pseudo-faeces, as well as the compounds biotransformed during exposure and depuration phases.

3.2. Material and Methods

3.2.1. Algal culture

The culture of the dinoflagellate *Gymnodinium catenatum* (strain C37-07, IPIMAR collection) was maintained in natural seawater enriched with GSe medium (salinity 28, Blackburn et al., 2001) at 18^{o}C under a 16 h light: 8 h dark photocycle with a light intensity of 15 µmol photons m^{-2} s^{-1}. The strain was then mass cultured in 10-L culture flasks under the same conditions to supply to the clam feeding experiment. Cells of *G. catenatum* were harvested in the late exponential growth phase. The non-toxic microalgae *Isochrysis galbana* was grown in Wallerstein and Miquel medium (Bandarra et al., 2003) at 18^{o}C in 75-L plastic bags under constant illumination at salinity 25. Cells of the toxic microalgae were counted in Palmer-Maloney chambers under an Zeiss IM 35 inverted microscope and of *I. galbana* in an automatic particle counter (Coulter EPICS XL).

3.2.2. Clams

A total of 450 clams (*Ruditapes decussatus*) were obtained from growth banks at Ria Formosa, a coastal lagoon located in southern Portugal with an annual production of 5000 T. Clams were collected in November 2008, after several years of undetected *G. catenatum* blooms in the lagoon or adjacent coastal zone. Animals were acclimatized during 15 days to the laboratory conditions in a 50-L aerated polyethylene tank with filtered seawater and fed daily with the non-toxic microalga *I. galbana* culture (2x10^{9} cells). Whole tissue wet weight and shell length of the clams ranged within 1.3-1.8 g and 2.8-3.9 cm, respectively.

3.2.3. Feeding experiment

Clams were divided into 42 groups of 10 individuals each and placed into 5 L plastic tanks filled with filtered natural seawater stored in a reservoir. The seawater in the tanks was continuously aerated and renewed daily. Water temperature remained at 12±1^{o}C. The feeding experiment consisted of two phases schematically illustrated in figure 3.1. During phase I (14 days) each 10 clam group was fed twice a day with 1x10^{4} cells of *G. catenatum* culture and

$5x10^7$ cells of *I. galbana* culture. During phase II (15 days) clams were fed twice a day only with $5x10^7$ cells of *I. galbana* culture. No control animals were considered in the experiment, since *I. galbana* does not produce toxins and clam responses to toxin exposure was not studied. Throughout the 29 days of the experiment no clam deaths occurred and changes in the whole tissue weight were negligible.

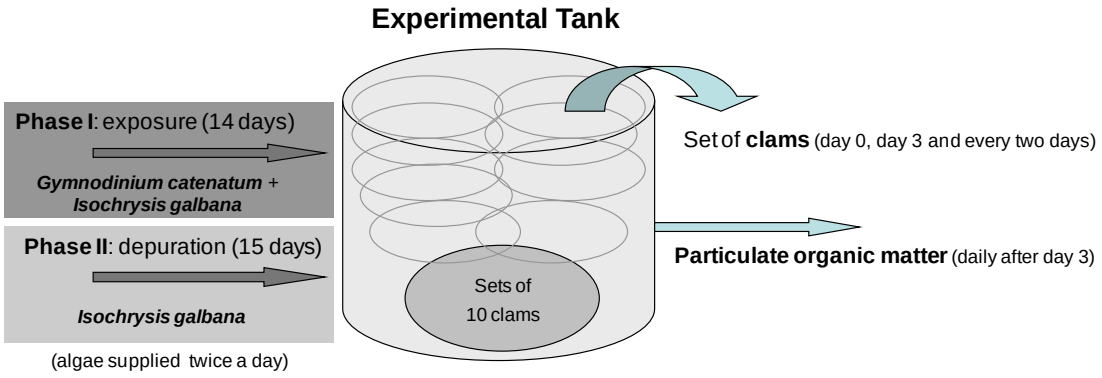


Figure 3.1. Schematic representation of the laboratory feeding experiment.

3.2.4. Samples

Toxic algae. Aliquots of 400 mL *G. catenatum* culture were harvested at days 3, 9 and 14 of the phase I for toxin analysis. Algal mass was obtained by filtering the culture under light vacuum pressure (100 mmHg) through GF/C glass filters (1.2 µm), and freezing in 0.1 M acetic acid at -80°C until analysis. ***Clams***. Specimens from three pools of 10 clams each were sacrificed in days 0, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29, dissected and composite samples (n=10) of whole soft tissues were prepared and stored at -80°C until analysis. ***Particulate organic matter.*** Particulate organic matter (POM) produced by clams was collected from the experimental tanks daily after day 3. Seawater of three tanks was transferred to an inverted conic recipient and particles were left settling for 12 hours. Material was then collected by filtration through 0.45 µm polycarbonate membranes, and kept frozen in 0.1 M acetic acid at -80°C until analysis.

3.2.5. Reagents

All chemicals and solvents used were LC or analytical grade. Sodium hydroxide and hydrogen peroxide were from Merck (Germany). Ultra-pure water was obtained by a *Milli-Q* system Millipore (France). Acetonitrile, acetic acid, methanol and ammonium formate were purchased from Sigma-Aldrich (Germany).

3.2.6. Toxin extraction and oxidation

Toxic algae. Toxins of *G. catenatum* cells retained on filters were extracted according to the method described in Artigas et al. (2007), with the following modifications. The extraction was done by freeze/thaw cycle followed by ultrasonication in an ice bath for 30 seconds at 60% amplitude and 20 W (*Vibra Cell*, Sonics & Materials Inc.). Cell debris after ultrasonification was examined under an inverted microscope revealing full disruption of the surveyed samples. The crude extract was centrifuged at 2500 x g for 10 min, and then cleanup using a Solid Phase Extraction (SPE) C18 cartridge (*Sep-Pak Light*, Waters, USA). The 130 mg C18 cartridge was conditioned with 1.5 mL methanol and 1.5 mL ultra-pure water. Subsequently an aliquot of 250 µl of supernatant was loaded, then washed with 500 µL of ultra-pure water. The pH of the extract was adjusted to 6.5 with 0.2 mol L^{-1} NaOH, and diluted to exactly 1 mL.

POM. Toxins in the collected POM were extracted using the same procedure as for dinoflagellate cells, except for the cleanup step, which was skipped due to the low toxin content. After centrifugation the extract was filtered through disposable 0.2 µm regenerated cellulose syringe filters.

Clams. Approximately 5 g of clam homogenate were double-extracted with 1% acetic acid solution (first extraction with heating), following AOAC 2005.06 (Anon., 2005b). The extract passed through a SPE C18 cartridge (500 mg/3 mL, *Supelclean*, Supelco, USA).

Aliquots of *G. catenatum*, clams and particles extracts were used for peroxide and periodate oxidation of PSTs prior to liquid chromatography with fluorescence detection (LC-FLD) analyses. The procedure used in the oxidation of PSTs was based on the AOAC method (Anon., 2005) with a procedural modification due to dominance of N-sulfocarbamoyl and decarbamoyl

compounds in the *G. catenatum* toxic profile (Botelho et al., 2010b). Similar procedures of peroxide and periodate oxidations were followed, substituting the oxidant reagent by water in order to detect naturally fluorescent compounds.

3.2.7. LC-FLD analysis

The LC system consisted of a Hewlett-Packard/Agilent (Germany) Model 1050 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. The PST oxidation products were separated using a reversed-phase *Supelcosil* LC-18, 150 x 4.6 mm, 5 µm column (Supelco, USA) equipped with a guard column *Supelguard Supelcosil* C18, 20 x 4.0 mm id, 5 µm (Supelco, USA). The column was kept in an oven at 30^{o}C. The mobile phase gradient used 2 mobile phases: A (0.1 M ammonium formate, pH=6) and B (0.1 M ammonium formate in 5 % acetonitrile, pH=6). The elution gradient consisted of 0-5% B in the first 5 min, 5-70% B in the next 4 min and back to 0% B in the next 5 min. Flow rate was 1 mL/min and the injection volumes were 50 µL and 100 µL, for the oxidation products of peroxide and periodate reaction, respectively. The excitation and emission wavelengths for fluorimetric detection were set at 340 nm and 395 nm respectively.

3.2.8. Quality control

The certified reference materials, CRM-STX-e, CRM-NEO-b, CRM-GTX2&3-b, CRM-GTX1&4-b, CRM-dcSTX, CRM-dcGTX2&3, CRM-GTX5-b (B1), CRM-C1&2 and CRM-dcNEO-b were obtained from the Institute for Marine Biosciences, National Research Council Canada (IMB, NRCC, Halifax, NS, Canada). For PSTs quantification in shellfish tissues, matrix matched calibration curves with cleaned-up clam tissue extract were used as described in Botelho et al. (2010a). Calibration curves prepared in solvent were used for toxin quantification in *G. catenatum* and particles extracts. Evaluation of linear ranges for PSTs and instrumental limits of detection (LOD), are described in Botelho et al. (2010b). Instrumental LODs were 3.9 nmol L^{-1} (C1+2); 4.0 nmol L^{-1} (B1); 4.0 nmol L^{-1} (STX); 4.9 nmol L^{-1} (dcSTX); 8.2

nmol L^{-1} (dcGTX2+3); 8.5 nmol L^{-1} (GTX2+3); 25 nmol L^{-1} (dcNEO); 30 nmol L^{-1} (GTX1+4) and 31 nmol L^{-1} (NEO). Recoveries of the analytical procedure were assessed through clam tissues spiked in triplicate, with the addition of a toxin mixture at two concentration levels. Intervals of the mean recoveries for the quantified PSTs in clams were: 71-74% (C1+2), 97-98% (B1), 77-114% (dcSTX), 85-107% (dcGTX2+3) and 55-56% (dcNEO). Repeatability values in terms of relative standard deviation were from 1 to 18%. Moreover, recoveries were assessed in POM spiked with B1 and dcSTX in triplicate at two concentration levels. Only the toxins quantified in POM were considered. The following intervals were obtained: 101-107% (B1) and 76-97% (dcSTX). Repeatability values were from 12 to 18%.

3.2.9. Mass balance calculation

The mass balance for individual toxins in clams was calculated for a basis of 100 clams by the following equation:

$$T_a + T_g = T_c + T_p + T_l + T_r \qquad (3.1)$$

Where:

T_a: mass of individual toxin in the toxic algae supplied to the clams

T_g: mass of individual toxin in clams gained by conversion of other assimilated compounds

T_c: mass of individual toxin accumulated in whole clam tissues

T_p: mass of individual toxin in particulate organic matter of the experimental tanks

T_l: mass of individual toxin in clams lost by conversion

T_r: mass of individual toxin released by clams to solution

The mass balance was calculated assuming that clams have ingested the whole algal mass supplied, and consequently the particulate organic matter collected in the experiments tanks consisted of material produced by clams. This assumption is consistent with the lack of *G. catenatum* intact cells in seawater from the tanks that was examined microscopically on a daily basis. In equation 3.1 T_g, T_l and T_r are unknown terms, their sum being substituted by T_{ng} that

may be considered as a net gain quantity. By resolving the equation 3.1 T_{ng} was calculated by:

$$T_{ng} = T_c + T_p - T_a \qquad (3.2)$$

3.2.10. Statistical analysis

Data statistical analysis were performed using the STATISTICA 6.0 Statistical Software System. The fitting of time-course variation of toxin concentrations to the experimental points was assessed by SigmaPlot 8.0 (Systat Software Inc.).

3.3. Results

3.3.1. Toxin profile of *Gymnodinium catenatum*

Figure 3.2 shows the median, the percentile 25% and 75%, minimum and maximum of concentrations of the toxins C1+2, B1, dcSTX and dcGTX2+3 quantified in the culture of *Gymnodinium catenatum*. Samples were taken in replicates (n=4) every 5 days during the 14 days of the exposure phase of the experiment. The N-sulfocarbamoyl analogues B1 and C1+2 were the major toxins produced by this culture, concentrations exceeding one order of magnitude the values found for the decarbamoyl analogues dcSTX and dcGTX2+3. Variation of the concentrations for the three sampling periods was comparable to values of the four replicates at each sampling period for C1+2 (33 and 31%), B1 (41 and 31%) and dcSTX (31 and 31%). A larger discrepancy was observed for dcGTX2+3 (42 and 19%). Modifications on cell size, chain length, and toxin leakage may contribute to the observed variability (Granéli and Flynn, 2006; Band-Schmidt et al., 2010).

3.3.2. Toxin concentrations in *Ruditapes decussatus*

Mean concentrations (± one standard deviation) of the toxins C1+2, B1, dcSTX, and dcGTX2+3 in whole soft tissues of *R. decussatus* collected at days 0, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29 are presented in figure 3.3. In general, toxin concentrations in clams

increased during the feeding with *G. catenatum*, although the accumulation pattern differed among the compounds. The first concentration augment was registered for dcSTX in day 3, enhancements of B1 and dcGTX2+3 were found in day 5, while a sharp increase of C1+2 was only observed 11 days after exposure to the toxic algae. The maximum levels of C1+2 and dcGTX2+3 were registered by the end of the exposure period, while of B1 and dcSTX were found three days after, during the subsequent depuration period. The maximum concentrations pointed to a higher accumulation of decarbamoyl toxins (dcSTX and dcGTX2+3) than N-sulfocarbamoyl (B1 and C1+2). In addition to the above mentioned toxins, dcNEO was also found in clams (not shown). However, values were only above detection limit in days 11, 13 and 15 not exceeding 0.30 nmol g^{-1}. Results showed an opposite PSTs composition between clams and *G. catenatum* (Figures 3.2 and 3.3).

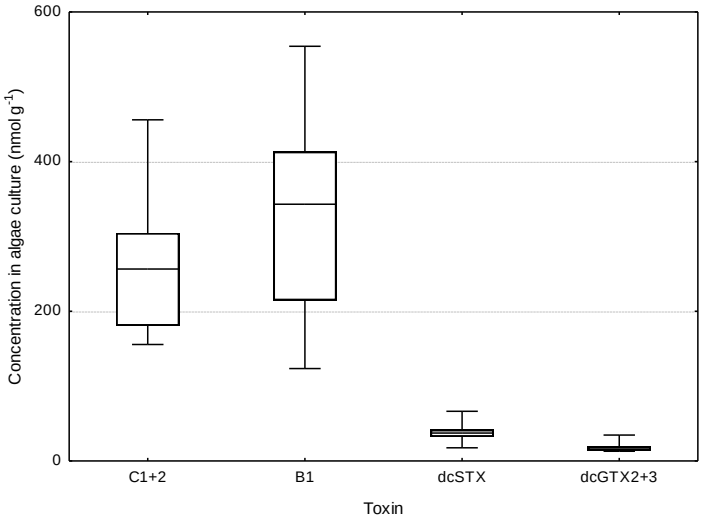


Figure 3.2. Median, percentile 25% and 75%, minimum and maximum of C1+2, B1, dcSTX, dcGTX2+3 concentrations (nmol g^{-1}) in the culture of *Gymnodinium catenatum* at days 2, 7 and 12 of the clam exposure experiment; four replicates at each sampling date.

Clams fed by a non-toxic diet exhibited a substantial decrease of C1+2 concentrations (18% of the peak value) eight days after depuration. Conversely, values of B1 and dcSTX remained at 68% and 50%, respectively, of the maximum concentrations after 14 days. An intermediate situation was found for dcGTX2+3 that exhibited a reduction of 37% at the end of the experiment. Furthermore a higher dispersion of dcGTX2+3 concentrations was registered

between replicates in days 9, 17, 19 and 21.

3.3.3. Best-fitting curves

The best fitting curves of the experimental points were searched for each quantified toxin. Table 3.1 shows the two equation types for exposure and depuration conditions, power equations ($y=ax^b$) and exponential decay equations ($y=ae^{-bx}$), respectively. Correlation coefficients and levels of significance are also given.

The calculated *b* values of the power equations were 2.45±1.07 (C1+2), 1.55±0.202 (B1), 1.18±0.122 (dcSTX) and 1.33±0.298 (dcGTX2+3). The fitting curve for C1+2 presented a level of significance above 0.05 (Table 3.1). The equations governing the time-dependent toxin concentrations under depuration conditions mirror a first-order kinetic, where *a* is the toxin concentration in the clam at the initial depuration conditions and *b* is the depuration rate. The levels of significance for C1+2, dcSTX and dcGTX2+3 curves were below 0.05, corroborating the validity of the first-order decay approach. The calculated depuration rates (*b*) were 0.17±0.024 day^{-1} for C1+2, 0.049±0.012 day^{-1} for dcSTX and 0.065±0.0085 day^{-1} for dcGTX2+3. The fitting curve for B1 presented a level of significance above 0.05 (Table 3.1), indicating a poorer first-order decay approach. Figure 3.3 shows computed and measured toxin concentrations in clams during the two phases of the experiment.

3.3.4. Toxin composition of particulate organic matter

The mass of particulate organic matter (POM) produced daily by 100 clams, from 10 experimental tanks, during phase I increased from 0.50 to 2.5 mg (Figure 3.4). The amount was more irregular under depuration conditions, varying between 0.83 and 5.5 mg. Concentrations of the toxins C1+2, dcGTX2+3 and dcNEO in POM were always below the detection limit. Conversely, the toxin dcSTX was quantified, concentrations increasing from 2.5 to 5.9 nmol mg^{-1} as clams were fed with the toxic algae, and ranging between 3.8 and 14 nmol mg^{-1}, under a non-toxic diet (Figure 3.4). Furthermore, three periods of dcSTX enrichment were observed in the produced POM. An intermediate situation was found for B1, concentration being

undetectable, except during 4 days of the phase I of the experiment that reached a maximum of 2.5 nmol mg^{-1}.

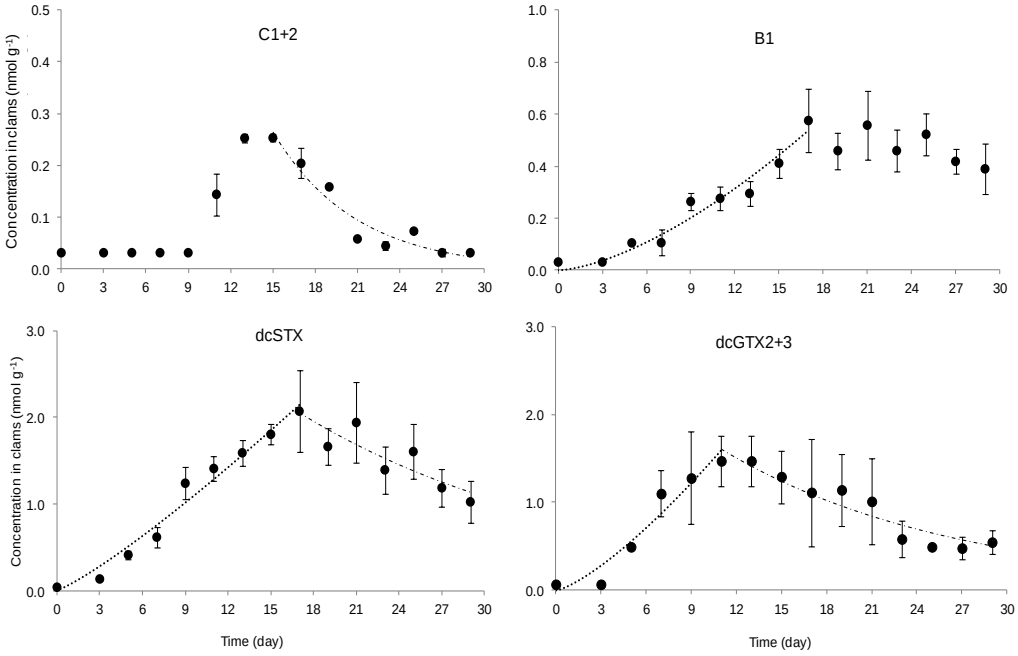


Figure 3.3. Concentrations of the toxins C1+2, B1, dcSTX and dcGTX2+3 (nmol g^{-1}) in the clam *Ruditates decussatus* exposed 14 days to *Gymnodinium catenatum* and 15 days under depuration conditions; mean concentrations (*n*=3; ±SD) and best fitting curves for exposure and depuration periods.

Table 3.1. Best fitting curves for C1+2, B1, dcSTX and dcGTX2+3 in *Ruditapes decussatus* during phase I (exposure) and phase II (depuration); calculated parameters (standard error); correlation coefficients (r) and levels of significance (*p*).

Phase I (exposure)			
Equation type: $y=ax^b$			
Toxin	*a*	*b*	r
C1+2 ‡	0.0004 (0.001)	2.45 (1.07)	0.812**
B1	0.007 (0.004)	1.55 (0.202)	0.976*
dcSTX	0.076 (0.024)	1.18 (0.122)	0.986*
dcGTX2+3	0.065 (0.043)	1.33 (0.298)	0.967*

‡ Valid to x>9

Table 3.1. (cont.)

Phase II (depuration)			
Equation type: y= a exp $^{-bx}$			
Toxin	a	b	r
C1+2	0.26 (0.021)	0.17 (0.024)	0.965*
B1	0.56 (0.038)	0.025 (0.010)	0.733**
dcSTX	2.06 (0.146)	0.049 (0.012)	0.886*
dcGTX2+3	1.61 (0.097)	0.065 (0.008)	0.948*

*p< 0.05 and **p>0.05

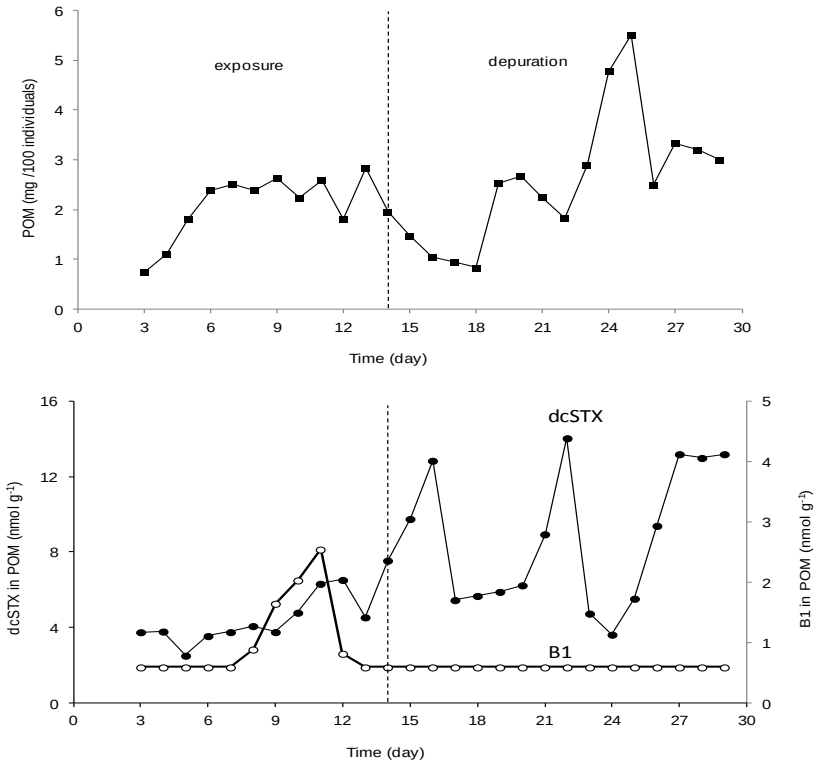


Figure 3.4. Variation of the amount (mg) of particulate organic matter (POM) produced by 100 individuals of *Ruditapes decussatus* exposure to *Gymnodinium catenatum* and under depuration conditions; concentrations (nmol g^{-1}) of the toxins B1 and dcSTX in the POM.

3.3.5. Mass balance calculation

Table 3.2 gives the amounts of toxins supplied to clams, accumulated in whole clam tissues and in POM produced by clams during the exposure phase (I), depuration phase (II) and the entire experiment. The quantities of C1+2, B1, dcSTX, dcGTX2+3 and dcNEO, expressed in

nmol, were computed on a basis of 100 clams, corresponding to 10 experimental tanks. In the mass balance calculation, the value of detection limit for each compound was accounted when concentrations were undetected.

Table 3.2 presents also the net gain values (T_{ng}) of each toxin calculated according to the Eq. 3.2. Negative values of T_{ng}, like for C1+2 and B1 in the phase I, mean that amounts supplied to the clams were not accounted in the whole clam tissues or POM. Approximately 95 and 85% of the mass of C1+2 (T_a=392 nmol) and B1 (T_a=470 nmol), respectively, added to the experiment tanks were converted into other toxins by clams or lost in solution (T_{ng}=-372 nmol and T_{ng}=-401 nmol, respectively). Conversely, positive values of dcSTX, dcGTX2+3 and dcNEO, imply a net gain most likely resulted from the conversion of other assimilated compounds by clams. The net gain (T_{ng}) of dcSTX (250 nmol) and dcGTX2+3 (108 nmol) were approximately 5 times the amounts added to the tanks (50 and 21 nmol, respectively). The net gain of dcNEO (22 nmol) has no correspondence in the algae supplied. Under depuration (phase II), T_{ng} for C1+2, B1 and dcGTX2+3 were one order of magnitude lower (-15, 38 and -47 nmol, respectively) than during the phase I. Only 9 nmol of dcNEO were accounted. Contrarily, comparable T_{ng} were obtained for dcSTX in phases I (250 nmol), and phase II (262 nmol). The calculation of the T_{ng} values for the entire experiment, points to a substantial loss of C1+2 and B1, amounting -750 nmol and a gain of 604 nmol of the sum of dcSTX, dcGTX2+3 and dcNEO.

3.4. Discussion

3.4.1. Conversion of toxins assimilated by clams into decarbamoyl toxins

The concentrations of the toxins C1+2, B1, dcSTX, dcGTX2+3 and dcNEO as well as their relative proportion differed considerably in whole tissues of the clam *Ruditapes decussatus* and *Gymnodinium catenatum* culture The dcSTX and dcGTX2+3 were predominant in clams, although being detected at minor concentrations in the algae. Conversely, C1+2 and B1 were more abundant in the algae and minor in clams. In particular, C1+2, which is dominant in the supplied toxic algae, was detected later than the other toxins in the clams during the exposure

Table 3.2. Mass (nmol) of toxins accumulated in *Ruditapes decussatus* (T_c), supplied to the experiment (T_a) and present in particulate organic matter (T_p) after phase I, phase II, and phases I+II. Values of T_{ng} were computed according to Eq. 3.2. Amounts were calculated for a basis of 100 clams.

	Toxin	**T_c**	**T_a**	**T_p**	***T_{ng}***
			nmol		
Phase I	C1+2	20	392	---	*-372*
	B1	41	470	28	*-401*
	dcSTX	180	50	120	*250*
	dcGTX2+3	129	21	---	*108*
	dcNEO	22	---	---	22
Phase II	C1+2	-15	---	---	*-15*
	B1	17	---	21	*38*
	dcSTX	-26	---	288	*262*
	dcGTX2+3	-47	---	---	*-47*
	dcNEO	9	---	---	*9*
	Toxin	**T_c**	**T_a**	**T_p**	***T_{ng}***
			nmol		
	C1+2	5	392	---	*-387*
	B1	58	470	49	*-363*
	dcSTX	154	50	408	*512*
	dcGTX2+3	82	21	---	*61*
	dcNEO	31	---	---	*31*

period (Figure 3.3). This delay on the accumulation of C1+2 pointed to different behaviour during the uptake, namely specific metabolic transformations. The undetected concentrations of dcNEO in the algae in conjunction with the presence in clams in days 11, 13 and 15 indicate the conversion of other assimilated toxins into dcNEO.

The modifications observed on PSTs profile between clams and the supplied algae cells were less drastic than those reported in other experiments with the clam T*apes japonica* fed by *Alexandrium catenella* (Samsur et al., 2006) and *Gymnodinium catenatum (*Samsur et al., 2007). In these feeding experiments, decarbamoyl derivatives were found in clams (*Tapes japonica*) being undetectable in *A. catenella* or *G. catenatum* cells that were used as food. The appearance of those compounds in clams was attributed to enzymatic hydrolysis of N-sulfocarbamoyl compounds supplied only once at the beginning of the experiment (Sullivan, 1983; Oshima, 1995b). The same explanation may be invoked to the results obtained in the current work. However, hydrolysis may have been more effective since *Gymnodinium catenatum* cells were supplied daily during the exposure phase. Ingestion by clams during 14 days may have resulted in an ongoing biotransformation process, explaining the progressive difference of toxin profiles between *R. decussatus* and the ingested *G. catenatum* cells. The predominance of decarbamoyl toxins during the depuration phase of the experiment, indicates that biotransformation of other assimilated toxins into dcSTX and dcGTX2+3 pursued beyond the ingestion of the toxic algae. It is recurrently observed in national monitoring programmes that toxicity in shellfish remains beyond the presence of high density of toxic algae cells. The results of the current work show that dcSTX may be responsible for the prolonged toxicity in clams exposed to a *G. catenatum* bloom. Two factors contribute to this extension of toxicity. Firstly, the slower depuration rate of dcSTX in shellfish (Botelho et al., 2010a), and the high toxicity equivalent factor in comparison to the other toxins produced by *G. catenatum* (Oshima, 1995a; Anon., 2009). For example, the toxicity equivalent factor of dcSTX is 10 times higher than of B1 and C1+2.

The mass balance calculated for the entire experiment (Table 3.2) evidenced the net gain of decarbamoyl compounds and the conversion of C1+2 and B1 in comparable quantities (750

and 604 nmol, respectively). It appears that dcSTX and dcGTX2+3 resulted mainly from the biotransformation of C1+2 and B1, although other toxins assimilated by clams should not be negligible. This hypothesis is in line with several works pointing to the transformation of saxitoxin derivatives in shellfish contaminated by *G. catenatum* into dcSTX (Oshima et al., 1990; Lin et al., 2004; Vale, 2008). The slower depuration rates of dcSTX and dcGTX2+3 in comparison to C1+2 observed in the current experiment indicate a tendency to higher retention time of those compounds in clams, either resulted from assimilation or conversion from other toxins.

3.4.2. Elimination of dcSTX through clam faeces/pseudo-faeces

Toxin composition of particulate organic matter in rearing tanks is of difficult interpretation because often it reflects the mixture of wasted food, faeces and pseudo-faeces produced by shellfish (Samsur et al., 2006, 2007; Estrada et al., 2007). The contribution of wasted food to the POM collected in the current experiment is most likely negligible. Firstly, because the dominant toxic component of the *G. catenatum* culture added to the experimental tanks (C1+2) were below the detection limit in the POM. Moreover, daily microscopic examination of seawater from the tanks, showed no *G. catenatum* intact cells. This indicates that clams have ingested the algal mass supplied daily, and particulate organic matter collected during the period that clams were exposed to the toxic algae (phase I) is, thus, admittedly constituted by a mixture of clam faeces and pseudo-faeces.

A relevant aspect of this work is that dcSTX was the only toxin quantified in the POM produced by clams during the exposure and depuration phases of the 28-day experiment. The toxin B1 was only detected during a short period of the exposure phase. The toxin profile of the particles produced by clams is different from that in the whole clam tissues. Estrada et al. (2007) proposed that PSTs in faeces are an indicator of the toxin content in the digestive system. The validity of this relationship cannot be tested in the current work because clam tissues were analyzed as a whole, and toxin concentration in the clam gut content may have been diluted with the low values in other tissues, as shown for other shellfish (Cembella et al., 1994; Shumway et al., 1994).

The toxin composition of faeces or pseudo-faeces produced during the exposure phase should reflect the ingested toxic algae composition and metabolic transformations of the assimilated toxins. However, only dcSTX, and punctually B1, were found in the POM. The absence of C1+2 and dcGTX2+3 suggests their biotransformation as ingested by clams. A similar explanation could be invoked regarding B1, since it was only quantified in the 8th day of exposure to the toxic algae. Although It should not be excluded the possibility of toxins being released in solution due to the hydrophilic nature of PSTs (Shimizu, 2000), the contribution of the soluble fraction to the mass balance should be low as suggested by Sekiguchi et al. (2001).

Under depuration, dcSTX concentrations in the POM were irregular with pronounced maximum (Figure 3.4). These concentration peaks cannot reflect the composition of ingested food since the diet was only composed by non-toxic algae. Presumably, metabolic processes have a key role on the composition of the eliminated particles. Furthermore, dcSTX was the major excreted toxin by *R. decussatus* after fed by *G. catenatum*, which indicates its provenance from reactions associated with the metabolic transformation. Results of the two phases of the experiments pointed to the conversion of assimilated toxins, namely B1 and C1+2, into dcSTX and dcGTX2+3. This hypothesis is in accordance with previous works with the clam *Mactra chinensis* (Lin et al., 2004) and *Perdonidia venulosa* (Cho et al., 2008). The gain of decarbomoyl toxins evidenced in the mass balance approach is in line with conversion of toxins by clams. The toxin composition of the POM showed the export of dcSTX as faeces or pseudo-faeces. The elimination of C1+2 and dcGTX2+3 as rejected particles were negligible, being likely transformed into other compounds or loss in solution.

3.5. Conclusion

Toxin composition of particulate organic matter rejected by the clam *Ruditapes decussatus* under depuration conditions after being exposed to *Gymnodinium catenatum* indicates the release of dcSTX, pointing to interconversion of other assimilated toxins during the exposure.

Acknowledgments

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4

4. Winter-summer nutrient composition linkage to algae-produced toxins in shellfish at a eutrophic coastal lagoon (Óbidos lagoon, Portugal)

Highlights

- Nutrient molar ratios contrast between winter and summer in a eutrophic coastal lagoon.
- Winter-summer variations lead to the predominance of dinoflagellate species in summer.
- Toxic G*ymnodinium catenatum*, *Dinophysis* cf. *acuminata* and *D. acuta* were observed in the lagoon in summer.
- Those species are on the basis of mussels toxicity as provided by PSTs and DSTs.

Pereira, P., Botelho, M.J., Cabrita, M.T., Vale, C., Moita, M.T., Gonçalves, C., 2012. Winter-summer nutrient composition linkage to algae-produced toxins in shellfish at a eutrophic coastal lagoon (Óbidos lagoon, Portugal). Estuarine, Coastal and Shelf Science. 112, 61-72. (doi:10.1016/j.ecss.2011.07.016).

Abstract

The current work examines the linkage of pronounced winter-summer fluctuations on the nutrient composition with phytoplankton assemblages and mussel toxicity produced by the presence of toxic dinoflagellates. The work was performed at the Óbidos lagoon, a coastal eutrophic ecosystem that is permanently connected to an area characterized by frequent upwelling episodes. The lagoon and adjoining coastal area exhibit recurrent incidents of diarrhetic and paralytic shellfish poisoning.

Data used in this work include: (i) inorganic and organic nutrients at five sites of the lower, middle and upper Óbidos lagoon, and inorganic nutrients at two sites of the adjacent coastal area; biannual campaigns were performed in winter and summer between 2006 and 2010; (ii) phytoplankton assemblages in three sites of the lagoon (located at lower and upper areas) in winter and summer of 2009; (iii) algae-derived toxicity of wild mussels from the lower lagoon and coastal area, on a 1-2 week time scale, over 2006 and 2009. Nutrient molar ratios in Óbidos lagoon contrast between winter and summer. The lower medians DIN:P (31 and 0.8) and Si:P (11 and 3.3) in summer reflect the excess of phosphate. Excess was mainly attributed to phosphorus regeneration in sediments of the upper lagoon with accentuated symptoms of eutrophication. Dissolved organic nitrogen and dissolved organic phosphorus were also higher in summer, in particular in this area. No significant winter-summer differences were recorded for nutrient ratios in the adjacent coastal area. Phytoplankton assemblages pointed to a winter-summer contrast characterized by a shift of non-siliceous-based phytoplankton to diatoms. Toxic dinoflagellate species (*Gymnodinium catenatum, Dinophysis cf. acuminata and D. acuta*), presumably imported from the adjacent coast following upwelling episodes in summer, were observed in the lower lagoon. In summer of the two surveyed years toxins produced by dinoflagellates were registered in mussels from the lower lagoon and coastal area. However, mussel toxicity in the lagoon exceeded values of the coastal area, which points to high cell density of toxic dinoflagellates resulted from favorable nutrient conditions. This work suggests that connectivity between eutrophic lagoons and upwelling systems stimulates the increase of toxic algae and consequently enhancing shellfish toxicity.

Keywords: Eutrophication; Nutrient composition; Toxic phytoplankton; Marine toxins

4.1. Introduction

The increasing number of coastal ecosystems with eutrophication symptoms led environmental managers to identify eutrophication as a major worldwide problem (Cloern, 2001; Hauxwell and Valiela, 2004; Lillebø et al., 2007). Many coastal lagoons display symptoms of eutrophication, particularly those with deficient connection to sea that concentrate the nutrient loads supplied from the watershed (Pérez-Ruzafa et al., 2005). The nutrient retention combined with low turbidity favour high primary production leads in extreme cases to intermittent periods of low oxygenation. Anoxic conditions near the water-sediment interface promote the release of nutrients from the sediments to the overlying water (Pereira et al., 2009a, 2010; Lillebø et al., 2002), followed by the enhancement of algal biomass. Besides light intensity, temperature, salinity and nutrient levels, the ratio between nutrients, in both inorganic and organic forms, influence phytoplankton abundance and diversity (Newton and Mudge, 2005; Glibert et al., 2007; Lopes et al., 2007). Although the Redfield molar ratios for phytoplankton growth (Si:DIN:P=16:16:1) are merely used to define the resource availability (del Amo et al., 1997), the shift away from these proportions may alter the species dominance and composition. Eventually, the overall result is the loss of diversity (Tilman et al., 1982). Since human activities in the last century have selectively enhanced nitrogen and phosphorus loads relatively to silicon into coastal systems, a tendency of a shift from diatom dominated communities towards flagellates have been reported (Cloern, 2001).

The linkage between harmful algal blooms (HABs) and eutrophication has been examined in various coastal regions, particularly the USA in order to identify mitigation measures (Anderson et al., 2008; Bricker et al., 2008; Heisler et al., 2008). The increasing number and frequency of toxic algal blooms have been regarded with concern, since algae-produced toxins accumulated in shellfish are a threat to human health. Blooms of diatoms (*Pseudo-nitzchia* spp.) and dinoflagellates (*Alexandrium fundyense, Alexandrium catenella* and *Gymnodinium catenatum*) have been registered in coastal systems under

eutrophic conditions (Trainer et al., 2003; Poulton et al., 2005; Thessen and Stoecker, 2008; Band-Schmidt et al., 2010). Cause-effect relationships between nutrient ratios and toxic algal blooms have been difficult to achieve due to several confounding factors. A "roundtable discussion" searched the linkage between water quality, eutrophication and HABs (Heisler et al., 2008). Among the short list of the consensus statements, nutrient contamination, nutrient composition, and exogenous nutrient sources were highlighted as promoters of HABs. Moreover, episodes of toxic phytoplankton species possibly linked to those conditions were referred (Heisler et al., 2008).

The current work examines whether pronounced winter-summer changes of nutrient composition, like observed in several coastal lagoons and estuaries, facilitate the development of toxic algal blooms. This hypothesis was tested in the Óbidos coastal lagoon (Portugal) where along the past decades recurrent episodes of toxic algal blooms and shellfish toxicity have been reported in the summer/autumn (Vale et al., 2008; Botelho et al., 2010a). This lagoon has two specificities that may be linked to that historic record. Firstly, oceanographic conditions in the coastal area of the region favour the occurrence of toxic blooms of *G. catenatum* and *Dinophysis* spp., particularly in summer and early autumn (Moita et al., 2001, 2003, 2006). Moreover, toxic dinoflagellate cells entering the lagoon are exposed to low concentrations of dissolved inorganic nitrogen and silicate with respect to phosphate (Pereira et al., 2009a), which may facilitate their development (Anderson et al., 2002).

4.2. Materials and Methods

4.2.1. The Óbidos lagoon study area

The Óbidos lagoon is a shallow coastal ecosystem (mean depth 2 m), located on the west coast of Portugal with a wet area of 7 km^2 and permanently connected to the sea through a narrow inlet (Figure 4.1). The tidal amplitude ranges fortnightly up to 2 m (Oliveira et al., 2006). The lagoon comprises areas of different morphological and sedimentary characteristics: sand banks and narrow channels in the lower part; a large and shallow middle area consisting in a mixture of sand and mud; muddy bottom sediments in the upper inner branch (Barrosa). This

part is 1 m deep and water circulation is mostly driven by tides and by a small tributary (Cal River) that drains agricultural fields. Urban effluents from a nearby town (Caldas da Rainha, 50 000 inhabitants) also have been discharged to this branch by the Cal River. Consequently, Barrosa presents the highest nutrient availability of the lagoon, being classified as eutrophic (Pereira et al., 2009a). High nutrient concentrations cause abundant macroalgae (*Ulva* spp. and *Enteromorpha* spp.) and a broad daily variation of dissolved oxygen concentration during the summer months (Pereira et al., 2009b, 2010). In this branch, dissolved oxygen decreases to low saturation values during the summer nights indicating hypoxic conditions (Pereira et al., 2009b, 2010). Ecotoxicological studies with *Ulva* spp. and *Carcinus maenas* pointed to biochemical alterations in autochthonous populations from Barrosa branch, but not in specimens from lower/middle lagoon (Pereira et al., 2009c). The other branch in the upper lagoon has no significant freshwater inputs. The middle lagoon was previously characterised by a better water quality regarding nutrient availability (Pereira et al., 2009a).

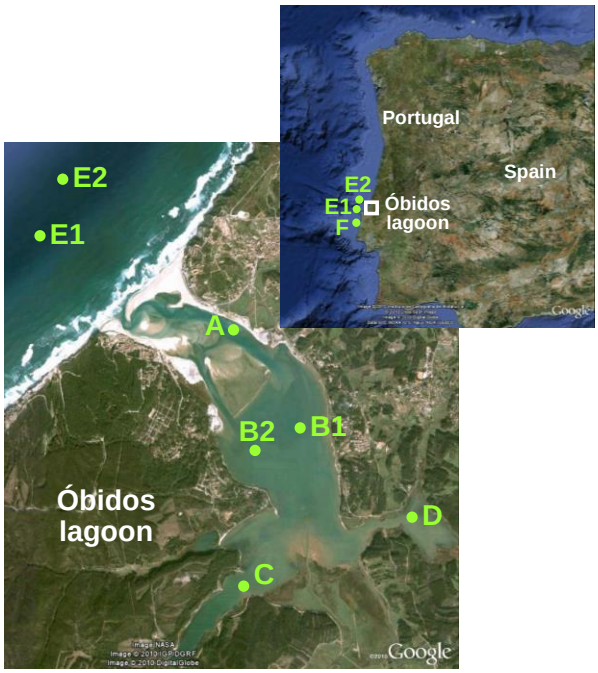

Figure 4.1. Location of the sampling sites at Óbidos lagoon and coastal area: A - lower lagoon; B1 and B2 - middle lagoon; C and D - upper lagoon (Bom-Sucesso branch and Barrosa branch, respectively); E1 and E2 - coastal area adjacent to the Óbidos lagoon; F - southern coastal area.

The Óbidos lagoon and adjacent coastal area are included in the national surveillance programme of toxic phytoplankton species and shellfish toxicity performed by IPIMAR. An overview of the past 20 years indicates recurrent episodes of shellfish toxicity in this lagoon caused by toxins produced by the dinoflagellates *G. catenatum* (paralytic shellfish toxins, PSTs), as well as *D. acuminata* and *D. acuta* (diarrheic shellfish toxins, DSTs) (Vale et al., 2008; Botelho et al., 2010a). Interdiction of shellfish harvesting during weeks are recurrent between spring to early autumn. The first poisoning event was reported in the 1940s (Correia, 1946; Pinto and Silva, 1956).

4.2.2. Sampling

Nine water sampling campaigns were carried out at Óbidos lagoon, biannually, between 2006 and 2010: five in winter and four in summer. The selection of the five sampling sites took into consideration results from previous studies on environmental quality (Pereira et al., 2009a, 2009b). The sampling sites were located in the following zones: A - lower lagoon; B1 and B2 - middle lagoon; C and D – upper lagoon (Figure 4.1). Water was sampled at high tide and low tide in 2006 and 2009, and only at low tide in 2007, 2008 and 2010. At each campaign, temperature, salinity, dissolved oxygen (DO) and pH were measured *in situ* at 0.2 m depth using an YSI, 650 meter. At the same time, 2 L of water was sampled for nutrients and chlorophyll *a* determinations. Water samples for phytoplankton identification and cell counts were collected in the sites A, C and D under summer conditions (early October) and winter 2009. Surface waters were sampled and preserved with Lugol's solution immediately after collection.

Between 2006 and 2009, seven campaigns were performed in two sites (E1 and E2) of the coastal area adjacent to the Óbidos lagoon in the same seasonal periods (Figure 4.1). Temperature, salinity and DO were measured *in situ* at surface (0.5 m), middle (10 m) and bottom (20 m) waters using an YSI, 650 meter. Water was also collected at these depths for nutrient and chlorophyll *a* determinations.

Thirty specimens of the mussel *Mytilus* galloprovincialis (length=5.8±0.5 cm) were sampled on a weekly basis in the lower part of the Óbidos lagoon (site A) and fortnightly in the coastal site F, located 50 km south of the lagoon (Figure 4.1). Samples were obtained from the national surveillance programme of toxins in shellfish performed by IPIMAR.

4.2.3. Chemical analysis and phytoplankton

4.2.3.1. Nutrients

Water samples were filtered through MSI Acetate Plus filters for determinations of nitrate+nitrite (NO_3^-+NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate $Si(OH)_4$ and analyses carried out using an autoanalyser TRAACS 2000 (Bran+Luebbe) (Grasshoff, 1983). In addition, total dissolved nitrogen and total dissolved phosphorus were measured according to Koroleff's method (1983) modified by the ISO 11905-1:1997. The dissolved organic nitrogen (DON) and dissolved organic phosphorous (DOP) were estimated by subtracting the amount of dissolved inorganic nitrogen (NO_3^-+NO_2^-+NH_4^+) from the amount of total dissolved nitrogen, and the amount of PO_4^{3-} from the amount of total dissolved phosphate, respectively.

4.2.3.2. Phytoplankton

Phytoplankton biomass was measured as chlorophyll *a* (Chl *a*) concentration. Water samples were filtered through Whatman GF/F filters which were immediately frozen at -20°C. Chl *a* was determined in a Perkin Elmer Fluorometer, using the modified method of Lorenzen (1966). Phytoplankton identification and cell counts were performed following the sedimentation method of Uthermöl (1958). In water samples with high sediment particles content, overlapping of cells and sediment particles are likely to occur, due to their similar sizes. To minimize the underestimation of small phytoplankton species, 5 to 25 mL of water were used in counting chambers. The phytoplankton quantitative and qualitative analyses were carried out under an inverted microscope Zeiss IM 35.

4.2.3.3. Toxins

After collection, mussels were dissected and composite samples (n=30) of soft whole tissues were prepared for each date and sampling site. The accumulated toxins were extracted

and concentrations of individual toxins determined using liquid chromatographic methodologies, with mass (DSTs) and fluorimetric (PSTs) detection. The analytical procedures are described in Vale and Sampayo (2002) for DSTs, and in Lawrence et al. (1991) and Botelho et al. (2010b) for PSTs. Concentrations of individual PSTs were converted into total toxicity levels according to tabled factors (Quilliam et al., 2007).

4.2.4. Data analyses

Prior to statistical analyses, data was tested for normality and equality of variances. Since ANOVA assumptions were not established, Kruskal-Wallis analysis was performed to compare sampling sites using Statistica 6.1. Differences between medians were considered significant when $p<0.05$. Principal Component Analysis (PCA) was applied to matrixes of 11 environmental variables (temperature, salinity, DO, NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, DON, DOP, $Si(OH)_4$ and Chl *a*) and objects (sites, seasons). A logarithmic transformation log (X+1) of the data was applied in order to approximate the distributions to normality and reduce the proportion of variances explained by the different variables. The variables were then auto-scaled (standardized) so as to be treated with equal importance. The PCA was performed on the correlation matrixes. The software used for these analyses was NTSYS-PC (Numerical Taxonomy and Multivariate System Analysis) Version 2.0 software package.

4.3. Results

4.3.1. Physicochemical characteristics

Water temperature ranged within broader intervals inside the lagoon in summer due to elevated values registered in the upper branches (Table 4.1). In the winter campaigns, salinity generally decreased from site A to D, reflecting the freshwater discharge into the Barrosa branch. Otherwise, the high salinity registered in site D in summer (e.g., 2006 and 2009 campaigns), indicates that evaporation was not compensated by the negligible freshwater inputs. Dissolved oxygen measured in sites A, B1 and B2 suggests that lower and middle lagoon were well oxygenated. In site D (upper lagoon) it was registered low DO (e.g., 53%) in early

morning, and 158% saturation in the afternoon. Such a broad daily fluctuation was not registered in winter. Values of pH showed no considerable winter-summer variations. Measurements in the sites E1 and E2 (adjacent coastal area) indicate narrow intervals of temperature (14-18^{o}C), salinity (35-36) and dissolved oxygen (85-115%) between the two seasons.

Table 4.1. Values of temperature (T), salinity, O_2 and pH registered at five sites in the Óbidos lagoon: A – lower lagoon, B1 and B2 - middle lagoon, Bom-Sucesso branch – C and Barrosa branch - D. Minimum and maximum intervals are presented for 2006 and 2009, and single values for 2007, 2008 and 2010.

Year	Season	Site	T (^{o}C)	Salinity	DO (%)	pH
2006	Summer (27-Jul)	A	19-20	36	104-111	8.2
		B1	21	36	101-108	8.1-8.2
		B2	-	-	-	-
		C	24-25	36-37	109-121	8.2-8.3
		D	24-25	37	74-96	8.2
	Winter (31-Jan)	A	9-12	33-35	108-119	-
		B1	11	34-35	103-108	-
		B2	-	-	-	-
		C	9-11	32-33	102-107	-
		D	8-11	28-31	103-124	-
2007	Summer (18-Jul)	A	20	36	107	-
		B1	23	36	125	-
		B2	21	36	107	-
		C	23	35	108	-
		D	24	33	83	-
	Winter (12-Dec)	A	12	36	98	8.0
		B1	13	18	101	8.2
		B2	13	35	120	8.1
		C	13	35	110	8.2
		D	15	33	99	8.0

Table 4.1. (cont.)

Year	Season	Site	T (°C)	Salinity	DO (%)	pH
	Summer	A	18	36	101	8.1
	(16-Jul)	B1	23	36	124	8.3
		B2	23	36	121	8.2
		C	26	36	156	8.6
2008		D	25	34	165	8.3
	Winter	A	9	34	63	7.9
	(15-Dec)	B1	10	34	73	7.6
		B2	10	35	73	7.8
		C	10	33	61	7.8
		D	11	25	81	7.8
		A	18-19	36	109-116	8.1-8.2
	Summer	B1	19	36	83-125	8.0-8.4
	(27-Aug)	B2	19	36	119	8.4
	(15-Out)	C	21	36	89-131	8.0-8.3
		D	18-21	34-36	53-158	8.1-8.4
2009	Winter	A	13-14	35-36	63-120	8.1
	(17-Dec)	B1	11-14	35-36	73-119	8.1
	(18-Dec)	B2	14	36	73	-
		C	12	34-35	61-116	8.1-8.2
		D	11-12	27-34	81-112	8.1
	Summer	A	19-20	36	104-111	8.2
	(29-Jul)	B1	21	36	101-108	8.1-8.2
		B2	-	-	-	-
2010		C	24-25	36-37	109-121	8.2-8.3
		D	24-25	37	74-96	8.2

4.3.2. Nutrients and chlorophyll *a*

Table 4.2 gives the nutrient and chlorophyll *a* concentrations in winter and summer conditions between 2006 and 2010, in the five surveyed sites of the lagoon. The most noticeable result is the statistical difference ($p<0.05$) of silicate, phosphate and chlorophyll *a* concentrations between site D and sites A, B1 and B2. The spatial variation of nutrients inside the lagoon was more pronounced in winter. Furthermore, nutrients in sites A, B1, B2 and C were higher in winter than in summer, except for phosphate. For example, nitrate+nitrite in summer varied between 0.24 and 14 µM, while in winter from 4.8 to 74 µM. Lower consumption by producers in winter and the external input of inorganic nitrogen and silicates due to tributary discharges and drainage, may both contribute to high levels. The same seasonal trend was also registered in site D for the inorganic forms of nitrogen and silicates. Conversely, higher levels of phosphate were found in summer. Considering all data obtained in site D, levels of phosphate in winter ranged from 1.5 to 4.5 µM, but in summer were between 2.5 and 8.5 µM. In general, dissolved organic forms of nitrogen and phosphorus in summer exceeded the values observed in winter.

Nutrient concentrations in sites E1 and E2 (adjacent coastal area) showed no consistent differences between summer and winter (Table 4.3) and were lower than in the lagoon, particularly in winter. Chlorophyll *a* enhanced in summer of 2007 and 2009

4.3.3. Principal Component Analysis (PCA)

Because nutrient concentrations varied considerably between the three sets of the sampling sites (D; A, B1, B2, C; and E1, E2), PCAs were performed separately for each set (Figure 4.2). The separation of site D from the other sampling sites in PCA was also based on previous works highlighting the specificity of that area (e.g., Pereira et al., 2009a). In the PCA of site D, nitrate+nitrite, ammonium and silicate were projected in the opposite side to salinity, temperature and phosphate along the axis PC1 (51.7%). PC2 explained 15.1% of variance and

separated chlorophyll *a* from dissolved organic forms of nitrogen (DON) and phosphorus (DOP). Chlorophyll *a* was projected close to phosphate.

The PCA applied to the sites A, B1, B2 and C (PC1+PC2=43.5%+19.4%) showed differences relatively to PCA of site D for phosphate and chlorophyll *a*. Phosphate was poorly represented in the plan PC1-PC2, being not associated with temperature and salinity, like in site D. Chlorophyll *a* was projected far from inorganic nitrogen and silicates and close to DON and DOP. Although being projected along the PC2 axis with low variance, that association was not registered in the site D. Both PCAs separate summer from winter samples, reinforcing the accentuated seasonal differences on the nutrient availability, as well as organic forms of nitrogen and phosphorus in the lagoon.

The PCA applied to E1 and E2 (adjacent coastal area) showed no nutrients decoupling or winter-summer separation. Nitrate+nitrite, phosphate and silicate opposite to dissolved oxygen, temperature and salinity along PC1 (29.4%), while ammonium opposite to chlorophyll *a* along PC2 (18.8%). Chlorophyll *a* was close to inorganic forms of nitrogen and phosphate.

4.3.4. Nutrient molar ratios

The molar ratios of dissolved inorganic nitrogen DIN (NH_4^++NO_3^-+NO_2^-) to phosphate (P) and silicate (Si), DIN:P, Si:DIN and Si:P were calculated. Winter and summer results were considered separately in three data sets: site D (Barrosa branch); sites A, B1, B2 and C (lower and middle lagoon); and sites E1 and E2 (adjacent coastal area). Figure 4.3 shows the median of three ratios, the percentile 25% and 75%, as well as maximum and minimum values. The molar ratios were always significantly higher ($p<0.05$) in winter than in summer for the lagoon data sets. The DIN:P ratio varied 25-fold between winter and summer, while for Si:DIN and Si:P ratios ranged only 5- and 3-fold, respectively. Winter and summer ratios in the coastal area were not significantly different ($p>0.05$).

Table 4.2. Values of NH_4^+, NO_3^-+NO_2^-, dissolved organic nitrogen (DON), PO_4^{3-}, dissolved organic phosphorous (DOP), $Si(OH)_4$ and chlorophyll *a* (Chl *a*) registered at five sites in the Óbidos lagoon: A – lower lagoon, B1 and B2 - middle lagoon, C – Bom-Sucesso branch and D – Barrosa branch. Minimum and maximum intervals are presented for 2006 and 2009, and single values for 2007, 2008 and 2010.

Year	Season	Site	NH_4^+ (μM)	NO_3^-+NO_2^- (μM)	DON (μM)	PO_4^{3-} (μM)	DOP (μM)	$Si(OH)_4$ (μM)	Chl *a* (μg L^{-1})
2006	Summer (27-Jul)	A	0.56-0.67	0.29-0.40	11-18	0.07-0.78	0.17	1.6-2.9	0.36-0.58
		B1	0.47-0.92	0.25	14-17	0.76-1.6	0.10-0.26	2.4-3.7	0.57-0.72
		B2	-	-	-	-	-	-	-
		C	0.92-1.6	0.25-0.40	9.0-17	2.0-3.9	0.10	3.0-3.1	0.50-1.1
		D	1.2	0.31-0.75	20-45	3.4-8.5	0.10-1.8	3.1-3.2	1.0
	Winter (31-Jan)	A	13-20	17-33	4.9-5.5	0.74-1.2	0.40-0.44	7.8-14	0.67-0.76
		B1	20-53	26-74	2.7-4.7	1.3-2.9	0.21-0.81	16-25	2.6
		B2	-	-	-	-	-	-	-
		C	25-46	34-62	5.8-10	1.6-2.5	0.39-0.75	13-22	0.82-1.1
		D	20-81	28-124	1.8-4.8	2.2-4.5	0.59-0.67	22-29	8.5-12
2007	Summer (18-Jul)	A	1.5	1.2	13	0.62	0.44	4.2	1.7
		B1	1.0	0.83	23	0.68	0.30	3.9	1.4
		B2	1.0	1.0	29	0.97	0.69	3.1	1.7
		C	1.0	1.2	39	2.1	0.38	4.6	3.7
		D	0.90	1.3	71	6.5	2.4	12	10
	Winter (12-Dec)	A	6.8	7.1	4.5	0.70	1.0	6.1	0.23
		B1	13	4.8	7.9	1.2	3.3	14	0.24
		B2	12	7.5	5.5	1.0	1.7	9.2	0.31
		C	16	6.4	2.9	1.2	0.91	11	0.94
		D	53	9.2	6.9	3.3	2.5	31	0.77
2008	Summer (16-Jul)	A	1.3	0.80	19	1.4	3.4	6.9	0.10
		B1	0.83	0.80	20	2.3	3.5	5.6	0.60
		B2	0.58	0.60	22	1.5	1.5	7.5	0.60
		C	0.58	0.70	-	3.3	1.3	3.6	0.90
		D	14	1.2	33	7.1	-	8.7	1.7
	Winter (15-Dec)	A	20	20	4.5	0.82	0.01	11	0.40
		B1	24	19	13	0.74	0.13	15	0.30
		B2	12	18	8.3	0.70	0.01	9.4	0.10
		C	27	25	20	1.0	0.01	16	0.20
		D	55	38	54	2.4	2.0	26	0.30

Table 4.2. (cont.)

Year	Season	Site	NH_4^+ (μM)	NO_3^-+NO_2^- (μM)	DON (μM)	PO_4^{3-} (μM)	DOP (μM)	$Si(OH)_4$ (μM)	Chl a (μg L^{-1})
2009	**Summer**	A	1.4-2.3	0.50-1.4	7.9	0.19-1.2	0.87	2.6-6.7	0.82-1.0
	(27-Aug)	B1	1.6-3.9	0.34-0.68	16	0.52-2.7	0.71	3.7-9.4	0.84-1.9
	(15-Out)	B2	1.9	1.7	12	1.3	0.44	5.3	1.8
		C	1.0-2.0	0.38-1.1	20	2.4-2.7	0.89	6.8-8.8	3.5-4.2
		D	1.5-11	0.24-2.5	35	2.5-4.1	1.6	11-32	3.6-13
	Winter	A	3.6-4.6	7.2-9.3	13	0.52-0.93	0.10	5.8-6.4	0.52-0.93
	(17-Dec)	B1	11-22	9.9-44	18	0.82-0.90	0.10	8.1-9.7	0.71-0.90
	(18-Dec)	B2	2.7	44	23	0.72	0.23	5.5	0.12
		C	10-17	14-24	22	1.0-1.4	0.22	14-18	1.0-1.4
		D	16-38	18-63	11	1.5-2.5	0.10	16-36	1.5-2.4
2010	**Summer**	A	5.3	9.6	-	0.66	-	2.4	0.90
	(29-Jul)	B1	7.3	14	-	1.4	-	6.7	1.1
		B2	1.9	1.0	-	1.1	-	5.0	0.95
		C	1.4	0.67	-	2.3	-	6.8	2.2
		D	4.6	1.3	-	7.0	-	23	2.8

Table 4.3. Values of NH_4^+, NO_3^-+NO_2^-, PO_4^{3-}, $Si(OH)_4$ and chlorophyll *a* (Chl *a*) registered at 2 sites located at the adjacent coast to the Óbidos lagoon: E1 and E2. Minimum and maximum intervals are presented for winter and summer of 2006, 2007, 2008 and 2009.

Year	Season	Site	NH_4^+ (μM)	NO_3^-+NO_2^- (μM)	PO_4^{3-} (μM)	$Si(OH)_4$ (μM)	Chl a (μg L^{-1})
2006	**Summer**	E1	-	-	-	-	-
	(27-Jul)	E2	1.2-1.6	0.47-1.7	0.05-0.16	0.75-1.8	0.35-1.9
	Winter	E1	-	-	-	-	-
	(31-Jan)	E2	5.3-8.3	5.1-5.8	0.29-0.34	3.4-5.6	0.16-0.20
2007	**Summer**	E1	1.9-3.3	2.0-12	0.18-0.48	5.1-5.8	1.1-6.2
	(02-Aug)	E2	2.6-2.9	1.3-1.8	0.17-0.25	3.8-4.3	1.6-5.5
2008	**Summer**	E1	0.92	8.1-11	0.36-0.50	3.4-3.5	0.30-1.3
	(16-Jul)	E2	0.73-0.91	8.3-24	0.50-1.1	2.9-3.8	0.1-0.4
	Winter	E1	3.6-4.9	3.9-5.0	0.51-0.55	3.6-4.9	1.0-2.0
	(29-Jan)	E2	3.4-3.7	4.1-4.2	0.44-0.55	3.4-3.7	1.7-2.3
2009	**Summer**	E1	1.5-1.6	3.0-3.5	0.44-1.3	1.9-4.4	1.5-2.7
	(31-Aug)	E2	1.0-1.3	1.3-5.0	0.47-0.98	1.1-3.7	1.2-2.3
	Winter	E1	0.33-0.45	6.3-6.9	0.67-0.84	2.2-2.9	0.67-0.84
	(19-Mar)	E2	0.37-0.92	7.7-7.9	0.46-0.74	2.4-3.0	0.46-0.74

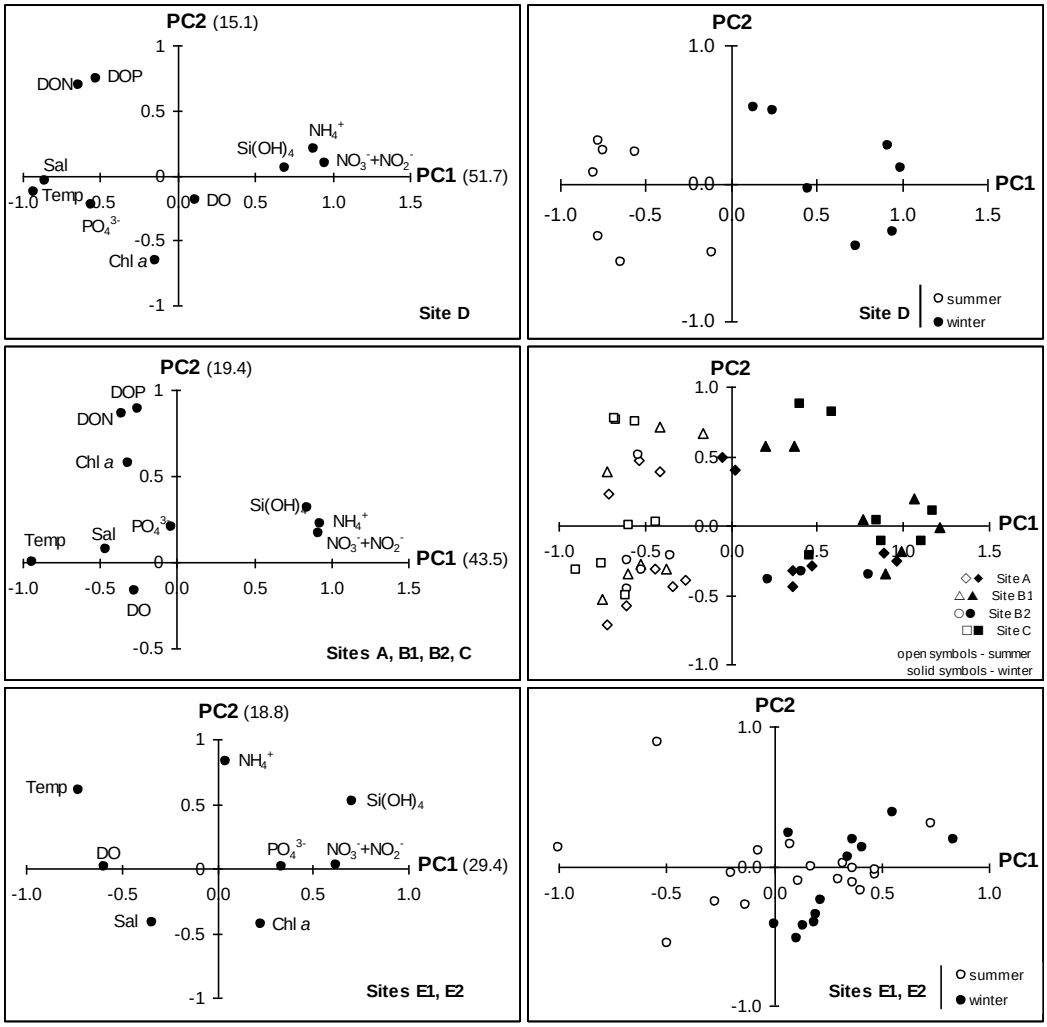

Figure 4.2. Projection of environmental parameters, and sampling sites in summer (open symbols) and winter (black symbols) campaigns obtained from the principal component analyses (PCA) performed on three data sets: site D - Barrosa branch (PCA1); sites A, B1, B2 and C all together – lagoon, except Barrosa branch (PCA2); and sites E1, E2 - coastal area adjacent to the Óbidos lagoon (PCA3). Percentage of total variance is indicated in brackets close to principal components axes.

The DIN:P ratio in the lagoon exceeded the Redfield ratio (16) in winter, reaching the median of 24 in site D and 31 in sites A, B1, B2 and C. Conversely, in summer DIN:P medians were 0.8 (site D) and 2.5 (sites A, B1, B2 and C). The situation differed in the adjacent coastal area: DIN:P ratio showed no significant ($p>0.05$) difference between winter and summer, and the medians in the two periods (13 and 18, respectively) were closer to 16. The median of Si:DIN ratio in the lagoon was close to 1 (Redfield ratio) in winter, and doubled in summer. In the coastal sites adjacent to the lagoon the median was close to 1 either in winter or summer. Most of the Si:P ratios in the lagoon were below the Redfield ratio (16) due to a decrease of Si relatively to P. This divergence to the Redfield ratio was more pronounced in summer.

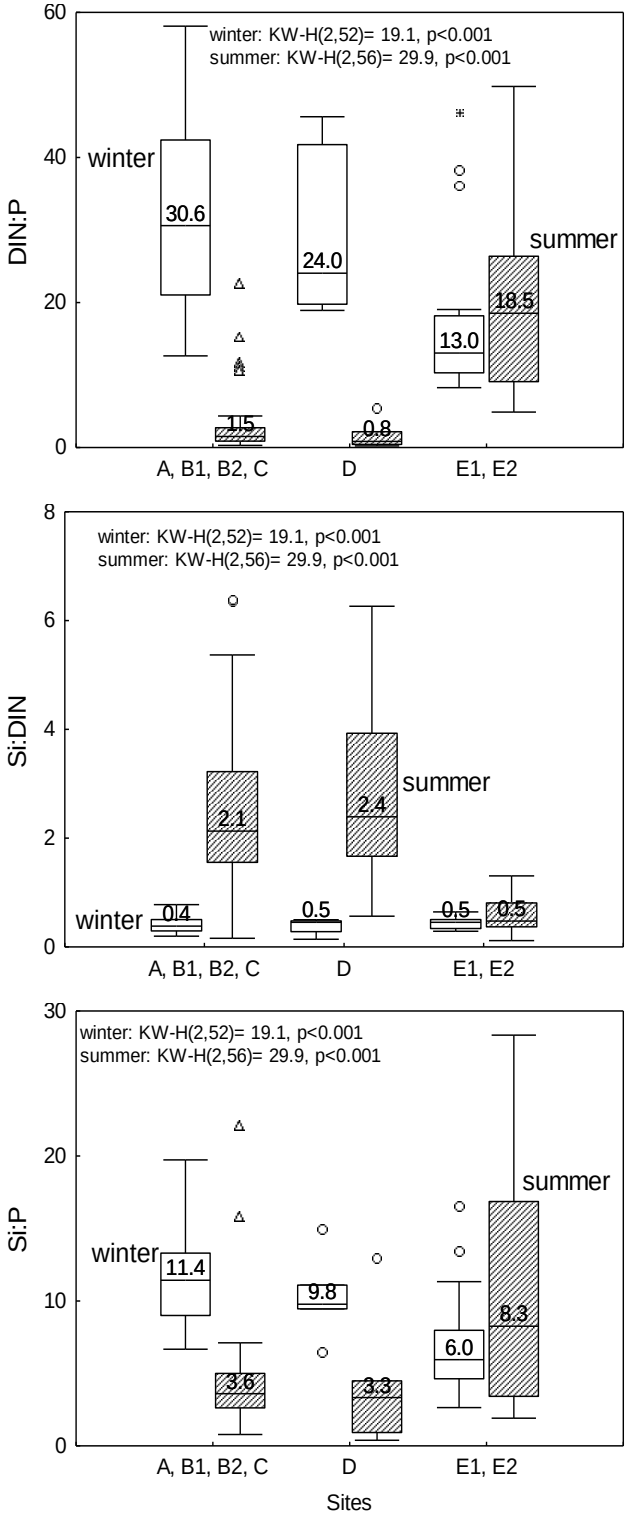

Figure 4.3. Median, percentile 25% and 75%, maximum and minimum of molar ratios DIN:P, DIN:Si and SI:P in summer (open box) and in winter (shaded box). Three data sets were considered for the surveyed period 2006-2010. Sites A, B1, B2 and C all together – lagoon, except Barrosa branch; site D - Barrosa branch; and sites E1, E2 - coastal area adjacent to the Óbidos lagoon. Outliers (○) and extreme (▵) values are identified. Results of Kruskal-Wallis test are presented.

4.3.5. Phytoplankton assemblages

Figure 4.4 shows the contribution of phytoplankton main groups to the total biomass, considering the sites A, C and D in summer and winter of 2009. Phytoplankton assemblages were dominated by Cryptophyceae, followed by Bacillariophyceae and Dinophyceae. Community in site D was clearly different from sites A and C. Contribution of Cryptophyceae was higher in site D (78-87%) than in sites A (49-50%) and C (53-59%). Bacillariophyceae represented only 7-8% of the total biomass in site D, while reached 22-42% and 24-28% in sites A and C, respectively. Dinophyceae had a minor contribution in all sites. Winter-summer differences between main groups were only registered in site A. In winter, the autotrophic ciliate *Mesodinium rubrum* was very abundant at sites C and D ($2x10^4$ cel L^{-1} and $6x10^4$ cel L^{-1}, respectively), being sustained by cryptophytes.

Table 4.4 lists the phytoplankton species of Bacillariophyceae and Dinophyceae identified in winter and summer campaigns of 2009. It is noticeable a lower number of Bacillariophyceae species in summer relatively to winter in the three sampling sites: 68 to 36% (A) 60 to 43% (B) and 50 to 38% (C). In winter, diatoms consisted mainly of marine species like *Chaetoceros* spp., *Pseudo-nitzschia* spp., *Rhizosolenia* spp. and *Leptocylindrus* spp.. An opposite trend was observed for Dinophyceae, since the number of species was lower in winter than in summer: 56 to 71% (site A) and 33 to 56% (site D). Estuarine species, such as *Licmophora* spp. and *Alexandrium* spp. were present in site D. The toxic dinoflagellate species are highlighted in bold in Table 4.4, namely *Dinophysis cf. acuminata*, *Dinophysis acuta, Dinophysis caudata, Dinophysis tripos and Gymnodinium catenatum*. These species were only found in summer at sites A and D, and registered in both seasons at site C. Concentrations of these species varied from 30 to 230 cells L^{-1} in all surveyed samples, and *Dinophysis cf. acuminata* reached 10^4 cells L^{-1} in site D in summer.

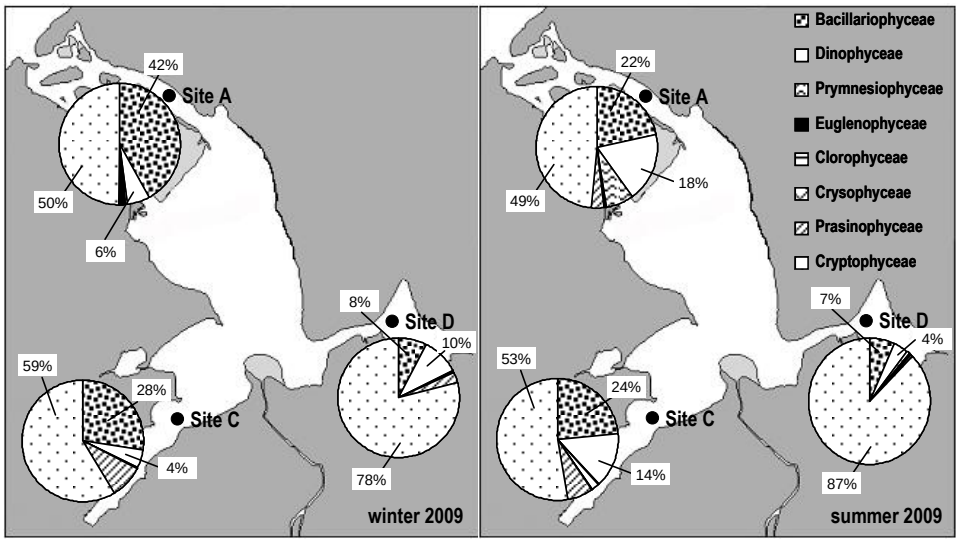

Figure 4.4. Phytoplankton main groups contribution (%) to total biomass, at sites A (lower lagoon), C (Bom-Sucesso branch) and D (Barrosa branch), during summer and winter campaigns of 2009.

4.3.6. Toxins in mussels

Figure 4.5 presents the annual variation of toxicities estimated from concentrations of paralytic shellfish toxins (PSTs) and diarrhetic shellfish toxins (DSTs) in *Mytilus galloprovinciallis*. During 2006 and 2009 surveillances, toxicities were compared in site A (lower lagoon) and site F (coastal area). Records during 2006 showed single (site F) and multiple (site A) toxicity episodes of PSTs and DSTs in mussels between June and September. Moreover, enhanced toxicities from the two toxin groups occurred in the same period of time. For several weeks toxicity exceeded largely the regulatory limit of PSTs (80 µg STX equivalents per 100 g tissue) and DSTs (16 µg OA equivalents per 100 g tissue) established for human consumption (Anon., 2004b). During 2009, toxicity associated with DSTs was virtually absent. Toxicity values due to PSTs in mussels exceeded slightly the regulatory limit in site A (lower lagoon), but not is the site F (adjacent coastal area).

Table 4.4. Phytoplankton species observed in winter and summer campaigns of 2009 in the Óbidos lagoon: lower lagoon (site A), Bom-Sucesso branch (site C) and Barrosa branch (site D). Species identified both in winter and summer 2009, at each site are shown within a shaded box. Potentially toxic species are highlighted in bold.

Phytoplankton main groups	Site A		Site C		Site D	
	winter	summer	winter	summer	winter	summer
	Chaetoceros spp. *Cylindrotheca closterium* *Nitzschia longissima* *Pleurosigma* spp./*Gyrosigma* spp. *Pseudo-nitzschia* spp. *Rhizosolenia* spp.	*Chaetoceros* spp. *Cylindrotheca closterium* *Nitzschia longissima* *Pleurosigma* spp./*Gyrosigma* spp. *Pseudo-nitzschia* spp. *Rhizosolenia* spp.	*Chaetoceros* spp. *Cylindrotheca closterium* *Pseudo-nitzschia* spp. *Nitzschia* spp.	*Chaetoceros* spp. *Cylindrotheca closterium* *Pseudo-nitzschia* spp. *Nitzschia* spp.	*Licmophora* spp. *Melosira* spp. *Nitzschia* spp. *Pleurosigma* spp./*Gyrosigma* spp. *Navicula* spp.	*Licmophora* spp. *Melosira* spp. *Nitzschia* spp. *Pleurosigma* spp./*Gyrosigma* spp. *Navicula* spp.
Bacillariophyceae	*Navicula* spp. *Bacteriastrum* spp. *Cerataulina* spp. *Cocconeis* spp. *Coscinodiscus* spp. *Dactyliosolen* spp. *Fragillaria* spp. *Guinardia* spp. *Nitzschia* spp. *Paralia sulcata* *Proboscia alata* *Skeletonema costatum* *Surirella* spp. *Synedra* spp. *Thalassionema* spp. *Thalassiosira* spp.	*Leptocylindrus minimus* *Asterionella* spp. *Climacosphaenia* spp. *Leptocylindrus danicus* Oxytoxum spp./*Torodinium* spp.	*Cerataulina* spp. *Detonula pumila* *Guinardia* spp. *Melosira* spp. *Navicula* spp. *Skeletonema costatum*	*Oxytoxum* spp./*Torodinium* spp. *Pleurosigma* spp./*Gyrosigma* spp. *Rhizosolenia* spp.	*Chaetoceros* spp. *Cylindrotheca closterium* *Fragillaria* spp. *Guinardia* spp. *Skeletonema costatum*	*Nitzschia longissima* *Rhizosolenia* spp. *Thalassiosira* spp.
% species new to each season	68%	36%	60%	43%	50%	38%
	Glenodinium spp. *Gymnodinium* spp./*Gyrodinium* spp. <50mm *Protoperidinium bipes* *Scrippsiella* spp.	*Glenodinium* spp. *Gymnodinium* spp./*Gyrodinium* spp. <50µm *Protoperidinium bipes* *Scrippsiella* spp.	***Dinophysis* cf. *acuminata*** *Gymnodinium* spp./*Gyrodinium* spp. <50mm *Gyrodinium falcatum* *Prorocentrum* spp. *Protoperidinium* spp. *Scrippsiella* spp.	***Dinophysis* cf. *acuminata*** *Gymnodinium* spp./*Gyrodinium* spp. <50µm *Gyrodinium falcatum* *Prorocentrum* spp. *Protoperidinium* spp. *Scrippsiella* spp.	*Glenodinium* spp. *Gymnodinium* spp./*Gyrodinium* spp. <50mm *Prorocentrum* spp. *Scrippsiella* spp.	*Glenodinium* spp. *Gymnodinium* spp./*Gyrodinium* spp. <50µm *Prorocentrum* spp. *Scrippsiella* spp
Dinophyceae	*Gyrodinium falcatum* *Gyrodinium fusiforme* *Oxytoxum* spp./*Torodinium* spp. *Prorocentrum* spp. *Pyrocystis* spp.	*Ceratium furca* *Ceratium fusus* *Ceratium pentagonum* ***Dinophysis caudata*** ***Dinophysis tripos*** *Gonyaulax* spp. ***Gymnodinium catenatum*** *Protoperidinium* spp. *Protoperidinium divergens* *Protoperidinium quinquecorne*	***Dinophysis acuta*** *Fragilidium* spp. *Gyrodinium fusiforme* *Protoperidinium bipes* *Protoperidinium diabolum*	*Glenodinium* spp. ***Gymnodinium catenatum*** *Oxytoxum* spp./*Torodinium* spp. *Protoperidinium quinquecorne*	*Gyrodinium falcatum* *Oxytoxum* spp./*Torodinium* spp.	*Alexandrium* spp. ***Dinophysis* cf. *acuminata*** *Fragilidium* spp. *Protoperidinium* spp. *Protoperidinium quinquecorne*
% species new to each season	56%	71%	45%	40%	33%	56%

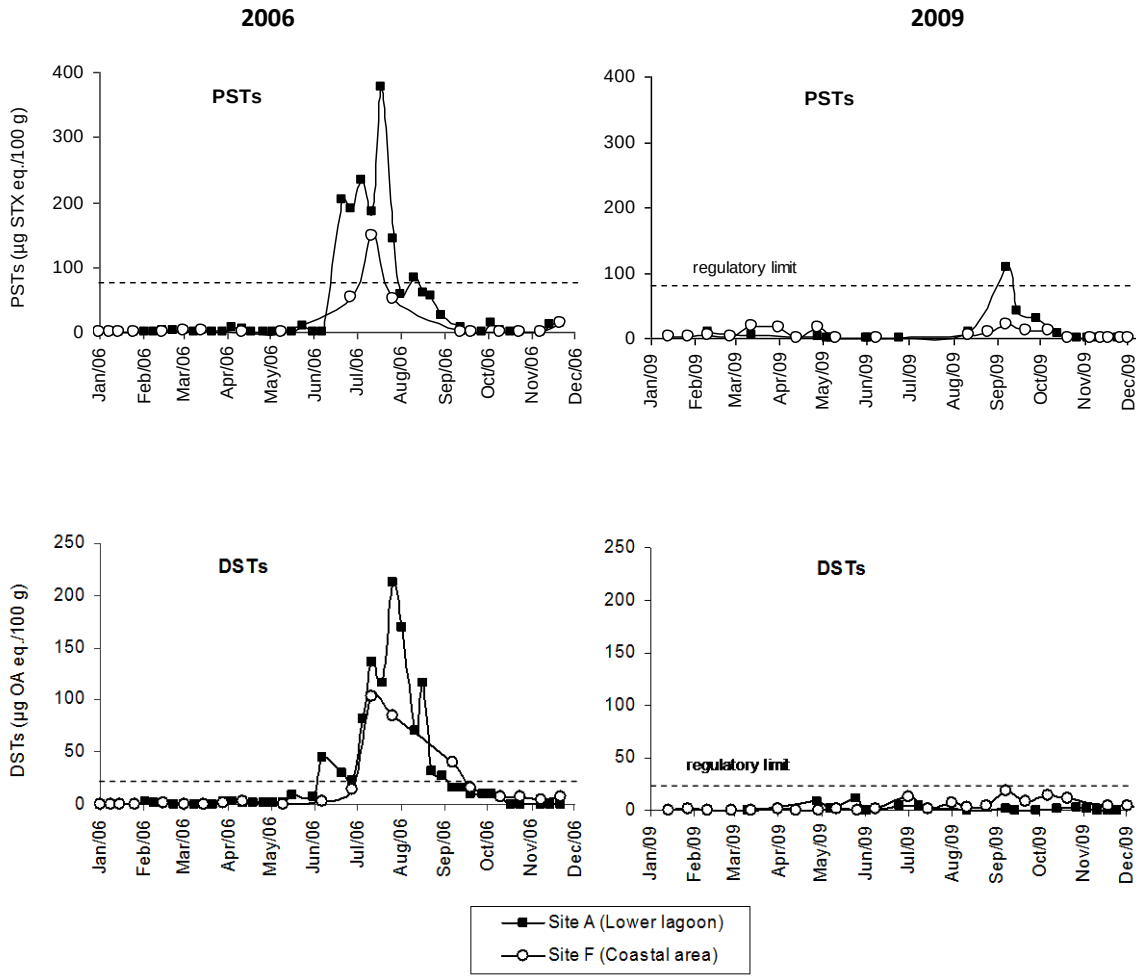

Figure 4.5. Annual variation of toxicity derived from accumulated paralytic shellfish toxins (PSTs, µg STX eq./100g) and diarrheic shellfish toxins (DSTs, µg OA eq./100g) in *Mytilus galloprovincialis* collected at site A (lower lagoon) and site F (coastal area south of the Óbidos lagoon). Data of 2006 and 2009 are presented.

4.4. Discussion

4.4.1. Winter-summer decoupling of phosphorus from nitrogen and silicon

Nutrient availability points to a clear-cut contrast between the Barrosa upper branch and the rest of the Óbidos lagoon. The contrast on water quality has been shown in previous works (Pereira et al., 2009a, 2010). Concentrations of chlorophyll *a*, dissolved inorganic nitrogen, phosphate and silicate are within the range of values reported for Mondego estuary (Lillebø et al., 2005) and Ria de Aveiro (Lopes et al., 2007), two estuaries in the same region that receive nutrients from diffuse and local sources. Values were also comparable to Ria Formosa, a coastal lagoon with a high production of the clam *Ruditapes decussatus* (Falcão and Vale, 2003). Chlorophyll *a* in the Óbidos lagoon was lower

than in highly eutrophic systems as the Barnegat Bay-Little Egg Harbour estuary (Kennish et al., 2007). Despite the high abundance of macroalgae and phytoplankton biomass in the upper branch (Table 4.2), the elevated nutrient concentrations indicate that consumption is not balanced by inputs. The PCA for site D emphasizes that freshwater discharged in winter constitutes the external supply of ammonium, nitrate+nitrite and silicate into the upper branch. Conversely, phosphorus levels mirror its regeneration and release from the sediment in warm periods, when dissolved oxygen in the overlying water lowers during the night (Pereira et al., 2010). These results are in accordance to various works asserting the relevance of the internal sources in ecosystems suffering from eutrophic symptoms (Lillebø et al., 2007). Moreover, DON and DOP appeared at higher concentrations in summer, possibly reflecting the increase of organic matter degradation under high temperature. The decomposition of the abundant *Ulva* spp. and *Enteromorpha* spp. in Barrosa branch probably contribute to the enhanced levels of those organic forms.

Phosphate supplied to the upper branch (site D) drove mainly from endogenous sources that are more pronounced in summer, while dissolved inorganic nitrogen and silicate were mainly provided from winter freshwater inputs. This contributes to the winter-summer contrast between phosphorus and nitrogen/silicon availability. Classic works have documented the phosphorus fractionation in sediments (Slomp et al., 1998), but the abrupt escape of phosphorus from the bottom is admittedly from the reduction of Fe(III) to soluble Fe(II) as dissolved oxygen at the water-sediment interface is consumed (Sundby, 2006). The low oxygen conditions occurring recurrently during the night in Barrosa induce the reduction of Fe(III) and subsequent release of phosphorus from Fe-oxides (Pereira et al., 2010). The low flushing time of water in the lagoon favours the dispersion of the released phosphorus and produced DON and DOP from the inner branch (site D) to the rest of the lagoon (total area of 7 km^2). The association of chlorophyll *a* with organic forms of N and P, as suggested by the PCA applied to lower and middle lagoon (Figure 4.2), raise the possibility of organic nutrients being a promoting factor to the lagoon phytoplankton production, as well as influencing changes on species composition as observed in other works (Taft et al., 1977; Bronk, 2007). Indeed, PCA pointed to chlorophyll *a* opposed to inorganic nitrogen and silicon and the relation to phosphate is difficult to discern (Figure 4.2). Shallowness and low turbidity suggest that light is unlike to be a limiting factor to primary production. High abundance of

filter-feeder organisms in the middle and lower parts of the lagoon implies high consumption of chlorophyll *a*, which is in accordance to its low level (Carvalho et al., 2006).

The different sources of phosphorus relatively to nitrogen and silicon have a marked impact on the winter-summer fluctuation of nutrient ratios (Figure 4.4). This pattern has been commonly reported for several coastal ecosystems (e.g., Lopes et al., 2007). Among the three analysed ratios, the fluctuation of DIN:P ratio in the Óbidos lagoon was more pronounced since nitrogen and phosphorus sources occurred in different seasons, promoting a clear-cut decoupling of these elements. The extremely low DIN:P ratios in summer support the hypothesis of phosphate being in excess in the entire lagoon. Furthermore, Si:P ratios being far below the Redfield ratio (16) suggest also an excess of phosphate relatively to silicate. A remarkable shift to 16 was observed in summer (medians of 3.3 and 3.6).

4.4.2. Impact of nutrient ratios on phytoplankton assemblages

It is well recognized that phytoplankton assemblages are influenced by composition, besides the total quantity, of the nutrient pool (Anderson et al., 2002; Heisler et al., 2008). Species composition in the Óbidos lagoon suggests a predominance of non-siliceous-based phytoplankton relative to diatoms in summer, despite the dominance of Cryptophyceae and presence of the authotrophic ciliate *Mesodinium rubrum*. This predominance agrees with low median ratios of Si:P (3.3 and 3.6) and Si:DIN (0.4 and 0.5) relative to Redfield ratios (16 and 1, respectively). Several works have associated the low ratios between these elements with minor contribution of diatoms to phytoplankton assemblages (Rocha et al., 2002; Domingues et al., 2005). Nevertheless, at the Óbidos lagoon the decrease of diatoms in summer does not seem to be solely related with Si decrease, since the concentration range (1.6-32 µM) is higher than the half-saturation constants reported in the literature (K_m=1-5 µM) for marine and coastal diatoms (Fisher et al., 1988). It should not be excluded the possibility of organic forms of nitrogen and phosphorus influence also changes on species composition (Taft et al., 1977; Bronk, 2007). A cause-effect relationship between dissolved organic nitrogen levels and the occurrence of brown-tide blooms was reported (Glibert et al., 2007).

The lower lagoon presented higher abundance of marine diatom species (*Cylindroteca closterium*, *Chaetoceros* spp., *Pseudo-nitzschia* spp., *Rhizosolenia* spp. and *Leptocylindrus* spp.) than

upper lagoon, indicating the influx of cells from the coastal area. The proximity of Óbidos lagoon to an area of frequent upwelling (Santos, 2001; Moita et al., 2001, 2003) favours the subsequent tidal transport of diatoms-populated offshore waters to the lagoon. Phytoplankton blooms were not registered in winter and summer surveys in the Óbidos lagoon. However, the toxic dinoflagellates *G. catenatum*, *D*. *acuminata* and *D. acuta*, whose blooms are responsible for the shellfish toxicity episodes in the Portuguese coast (Vale et al., 2008), were found in the lagoon. This species occurred chiefly in summer. The presence of low cell density of these toxic species, particularly in the lower lagoon, may indicate the advection of dinoflagellate low-populated off-shore water into the lagoon. Therefore, the observed phytoplankton assemblages were insufficient to conclude whether nutrient ratios had promoted cell density. Nevertheless, a previous work have reported higher density of *G. catenatum* in the middle ($5x10^3$ cell L^{-1}) and lower part of lagoon ($10x10^3$ cell L^{-1}) (Botelho et al., 2010a) than the current study ($0.1x10^3$ cell L^{-1}). Amorim and Dale (1998) referred the presence of *G. catenatum* cysts in sediments of Óbidos lagoon. However, the lack of direct relationship between cyst distribution and bloom intensity in the Iberian coast, suggests the import from the sea as a major factor (Amorim, 2001; Bravo et al., 2010).

The dominance of Cryptophyceae in the lagoon can also be responsible for the high concentrations of *Mesodinium rubrum* (and related high levels of chlorophyll *a*) observed at the inner branches. This ciliate, although containing a permanent cryptophyte symbiont, requires the ingestion of cryptophyte preys for its sustained growth (Hansen and Fenchel, 2006). High concentrations of *M. rubrum* seem recurrent in the lagoon during winter as observed in the 70s and 80s by Silva and Peixoto (1987). Moreover, recent studies have also shown that cultures of the mixotrophic *Dinophysis,* previously impossible to maintain, were finally possible when *M. rubrum* was added as prey (Park et al., 2006). Therefore, suitable conditions for *Dinophysis* growth seem to be attained at the upper branches of the lagoon since the directly related trophic levels Cryptophytes-*Mesodinium rubrum*-*Dinophysis* were well established in natural conditions.

4.4.3. Enhancement of mussel toxicities in the lagoon

The upwelling system is recognized as being susceptible to promote harmful algal blooms in Iberian Peninsula and other regions of the world (Pitcher et al., 2010). In the Portuguese coast,

blooms of *G. catenatum, D. acuminata* and *D. acuta* have been reported during relaxation conditions of coastal upwelling (Moita et al., 2001, 2003). The DST and PST peaks registered in wild mussels from Óbidos lagoon and coastal area mirror the availability of high levels of these toxic species in the water column. The occurrence of peaks in summer is in accordance with previous data of shellfish toxicity in the Portuguese coast (Vale et al., 2008) and with studies on toxic phytoplankton dynamic (Moita et al., 2001, 2003, 2006).

The difference of DST- and PST- derived toxicities between mussels from the lagoon and the adjacent coast was registered between June and August/September of 2006. The most plausible explanation for the dissimilarity between the two areas is the increasing availability of algae cells in the lower lagoon. As dinoflagellate-populated off-shore waters were advected to the lagoon in summer 2006, phytoplankton cells became exposed to a proportional enrichment of phosphorus relative to silicon and nitrogen, as well as of organic forms of nitrogen and phosphorus. These conditions may have favoured the increase of cell density and, consequently, the mussel toxicity. The explanation agrees with studies examining the linkages between composition of nutrient pool and shellfish toxicity (e.g., Hattenrath et al., 2010). Laboratory works showed that nitrogen and phosphorus availabilities and its molar ratio may enhance PST production (Touzet et al., 2007) and change toxin profile (Poulton et al., 2005) in algae. The modifications on algae toxin composition should not be excluded in the Óbidos lagoon, since phytoplankton transported from coastal water were exposed to a distinct nutrient pool.

In 2009, the divergence on the toxicity between the lower lagoon and coastal area was only found in September for PSTs. The low toxicity signal in mussels from the coastal area suggests a lower abundance of *G. catenatum* in 2009 than in 2006. Differences of oceanographic conditions probably explain the inter-annual variation. Consequently, smaller quantities of cells should have been advected from the coastal area into the lagoon in 2009, although shellfish toxicity in the lagoon exceeded values in the coastal area. In 2009, the concentrations of phosphate and dissolved organic phosphorus in the lower lagoon were lower than in 2006. Nevertheless, it appears sufficient to promote the increase of algae cells and consequent mussel toxicity.

Water residence time has been considered an influencing factor of toxicity when embayment acts as a retention zone for toxic phytoplankton species (Pitcher et al., 2010). The flushing time in the lower lagoon (1-2 days) (Malhadas et al., 2009) cannot explain the toxicity differences between the

lagoon and the adjacent coastal area.

4.5. Conclusions

Nutrients composition, phytoplankton assemblages and toxicity in shellfish of Óbidos lagoon, a narrow-mouth coastal lagoon nearby an upwelling system, point to the connectivity between enhanced toxicity derived from toxic algae and the excess of phosphorus or high nitrogen and phosphorus organic forms occurring in summer. Changes on the proportionality between P, N and Si in eutrophic lagoons or estuaries, which import toxic phytoplankton species from adjoining upwelling systems, may enhance shellfish toxicity. Mitigation of the enhanced shellfish toxicity could imply sediment dredging to reduce internal nutrient regeneration and decrease of flushing time in confined areas.

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5

5. Partitioning of paralytic shellfish toxins in sub-cellular fractions of the digestive gland of the cockle *Cerastoderma edule*: changes under post-bloom natural conditions

Highlights

- Sub-cellular partitioning of PSTs in cockle digestive glands.
- Affinity of PSTs to insoluble cellular fractions.
- Changing of organelle toxin profiles under post-bloom conditions.
- Inter-conversion of toxins in mitochondrial and lysosomal fractions.

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Abstract

Concentrations of paralytic shellfish toxins (C1+2, B1, dcGTX2+3, dcSTX, GTX2+3 and STX) were determined by LC-FLD in composite samples of digestive glands of the cockle *Cerastoderma edule* and in each sub-cellular particulate fractions obtained after differential centrifugation (nuclei+debris, mitochondria, lysosomes and microsomes). The specimens were sampled during the exposure to a bloom of *Gymnodinium catenatum* (day 0) and in the subsequent 8, 12, 14, 19, 21 and 25 days under natural depuration conditions. Toxin profiles of digestive glands were dominated by C1+2 followed by B1 and dcGTX2+3, although the proportion between C1+2 and B1 contents decreased with the time, indicating a slower elimination of B1. All toxins, except GTX2+3 and STX, were quantified in the four sub-cellular fractions. The content of the quantified toxins decreased most markedly in nuclei+debris and microsomal fractions, during the first eight and 12 days, respectively. Conversely, different patterns were observed among toxins in mitochondrial and lysosomal fractions. The less accentuated decreases of dcGTX2+3 and dcSTX contents in the mitochondrial fraction may have resulted from the conversion of other toxins, like C1+2 and B1, associated with enzymatic activities in that fraction. The largest discrepancy was registered in lysosomal fraction for B1, since its content increased after eight days of post-bloom conditions. Input of B1 may come from the conversion of other toxins, like the abundant B2 and C1+2. These transformations are associated to the major role of lysosomes in the intra-cellular digestive process of materials acquired through vesicular transport.

Keywords: Paralytic shellfish toxins; Sub-cellular partitioning; *Cerastoderma edule*; Digestive gland; Lysosomes; Mitochondria

5. 1. Introduction

Paralytic shellfish toxins (PSTs) are a group of neurotoxic alkaloids produced in the marine environment by dinoflagellates like *Alexandrium* spp., *Pyrodinium bahamense* and *Gymnodinium catenatum* (Llewellyn, 2006). During blooms of those phytoplankton species, bivalves uptake the toxins, which are causative agents of paralytic shellfish poisoning in humans (Gessner and Middaugh, 1980; Sommer and Meyers, 1937). More than 50 PSTs have been reported (Weise et al., 2010),

primarily comprising three main structural families according to the functional groups: carbamate (saxitoxin-STX, neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), decarbamoyl (dcGTX1 to dcGTX4, dcSTX and dcNEO) and N-sulforcarbamoyl (B1, B2, C1 to C4). The N-sulfocarbamoyl and decarbamoyl are the dominant toxins for the species *Gymnodinium catenatum* (Band-Schimdt et al., 2005; Botelho et al., 2012; Ordás et al., 2004) and carbamate toxins for *Alexandrium* species (Cembella et al., 1987; Chou et al., 2004).

In general, the proportions among PSTs in bivalves differ from the profile of the dinoflagellate that produces the toxins. These modifications are explained by differential uptake and depuration kinetics of individual toxins (Botelho et al., 2010a; 2012; Yu et al., 2007), in conjunction with metabolic interconversions of the assimilated toxins through enzymatic and chemical reactions in bivalve tissues (Kotaki et al., 1985; Oshima, 1995b; Shimizu and Yoshioka, 1981; Sullivan et al., 1983). The digestive gland of bivalves is the initial repository of ingested food, including xenobiotics and marine toxins. This organ contains by far the greatest proportion of the total toxin body burden in bivalves exposed to toxic algal blooms (Bricelj and Shumway, 1998), and therefore weighs heavily on toxicity of the edible parts. Enzymatic and other oxidation/reduction reactions are involved in the elimination or biotransformation of toxins in the digestive gland (Fast et al., 2006). Other workers have highlighted the major association of domoic acid with the cytosol of the digestive gland of the scallop *Pecten maximus* (Mauriz and Blanco, 2010) and of the octopus *Octopus vulgaris* (Lage et al., 2010). However, the partitioning of toxins among cell organelles has been poorly documented, which limits our understanding of the mechanisms behind the release of toxins at sub-cellular level.

The combined worldwide annual catch of clams, cockles, and arkshells ranges between 0.5-1 million tonnes (FAO, 2012). Within that group, the cockle *Cerastoderma edule* is cultivated or collected in Europe and North Africa for human consumption with an overall production of between 10 and $100x10^3$ tonnes per year (FAO, 2013). This species is both hand and mechanically dredged, with the largest catch being registered in the Netherlands and United Kingdom (FAO, 2013). A part of the harvest is sold fresh and the rest is canned. Because this species is of considerable interest from a fishery point of view, the patterns of marine toxin accumulation take on particular relevance.

The objective of this work is to study the partitioning of C1+2, B1, dcGTX2+3, dcSTX, GTX2+3 and STX among the sub-cellular operational fractions nuclei+debris, mitochondria, lysosomes and microsomes in digestive glands of the cockle *Cerastoderma edule* exposed to a bloom of

Gymnodinium catenatum and under post-bloom natural conditions. We test the hypothesis that sub-cellular partitioning of each of those paralytic shellfish toxins changes during depuration after bloom cessation. To the best of our knowledge these are the first results of sub-cellular partitioning of PSTs in bivalves.

5.2. Materials and Methods

5.2.1. Samples

Cockles (*Cerastoderma edule*) from a natural bank in the lagoon of Aveiro (Nilin et al., 2012) were naturally exposed, in October 2011, to a bloom of *Gymnodinium catenatum* that reached a maximum of $6x10^3$ cells L^{-1} (day 0), decreasing in the subsequent period to $6x10^2$ cells L^{-1} (day 8), $5x10^2$ cells L^{-1} (day 14) and <20 cells L^{-1} (day 19) (IPMA, monitoring programme of toxic phytoplankton). Thirty specimens (shell length=3.0±0.2 cm) were collected at the bloom peak (day 0) and days 8, 12, 14, 19, 21 and 25 under post-bloom natural depuration. The paralytic shellfish toxins C1+2, B1, dcGTX2+3, dcSTX, GTX2+3 and STX were quantified in the digestive gland and its sub-cellular particulate fractions. Additionally, B2 was indirectly quantified in selected samples of digestive gland.

5.2.2. Reagents

All chemicals and solvents used were LC or analytical grade. Sodium hydroxide, periodic acid, hydrogen peroxide and hydrochloridric acid (Merck), and acetonitrile, acetic acid, methanol, trizma hydrochloride, ammonium formate, ammonium acetate, disodium hydrogen phosphate, dithiothreitol and mercaptoethanol (Sigma-Aldrich), were used. Ultra-pure water was obtained from a Millipore Milli-Q system.

5.2.3. Treatment of samples

Cockles were carefully dissected and composite samples of digestive glands (three pools of 10 specimens) were weighed and frozen at -80°C. The composite samples were placed in an ice bath and homogenised in a buffer solution (5 mL of buffer g^{-1} of digestive gland), consisting of 0.1 M Tris-HCl and ammonium formate 0.15 M to a final pH of 7.8 (Mauriz and Blanco, 2010; Raimundo et al., 2008).

The homogenisation was completed in 3 min at 11000 rpm in a Polytron Kinematica AG homogenizer (Rossignoli and Blanco, 2010). To test the effect of protease inhibitor on PST determinations, two aliquots of a digestive gland composite sample were used. The first aliquot was homogenised with the buffer solution. The second one was complemented with a mixture of thiol group protectors, dithiothreitol and mercaptoethanol (1 mM) to inhibit the activity associated with thiol groups (Mauriz and Blanco, 2010). Losses of toxins exceeded 38%, which leads to the rejection of protease inhibitor addition. Under these conditions interferences probably occur in PSTs detection after oxidation reaction. A 3 g aliquot of the homogenate tissue was taken as initial sample for toxin extraction and the remaining homogenate was used for sub-cellular fractionation.

5.2.4. Sub-cellular fractionation

For sub-cellular analyses, the aliquots of each homogenate pool of digestive glands were transferred to centrifuge tubes and subjected to differential fractionation. The procedure was adapted from Campbell et al. (2005) and Raimundo et al. (2008): the homogenate was first fractioned by centrifugation at 700 x g for 15 min at 4^{o}C to separate the nuclei, granules and plasmatic membranes, designated as "nuclei+debris" fraction (P1); the supernatant was further centrifuged at 9 000 x g for 20 min at 4^{o}C to separate the mitochondria fraction (P2); the lysosomes (P3), and microsomes (P4) fractions were obtained by centrifuging the supernatant at 30 000 x g for 25 min, and at 100 000 x g for 40 min at 4^{o}C, respectively. The four particulate fractions obtained by centrifugation were extracted for toxin analyses. The remained soluble part, mainly constituted by cytosolic components, is hereafter designated by cytosolic fraction.

5.2.5. Toxin extraction and oxidation

The extraction, cleanup and oxidation procedures for PSTs determination were based on the AOAC 2005.06 (Anon., 2005b), involving the following steps: (*i*) the 3 g aliquot of digestive gland homogenate, and the P1, P2, P3 and P4 pellet fractions were double-extracted with 1% acetic acid solution (first extraction with heating); (*ii*) the digestive gland and pellets extracts passed through a solid phase extraction (SPE) C18 cartridge (500 mg/3 mL, Supelclean, Supelco, USA) and the pH of the extracts was adjusted to 6.5; (*iii*) an aliquot of C18-cleaned extract were placed in an SPE-COOH

cartridge (500 mg/3 mL, Bakerbond, J.T. Baker, USA) and sequentially eluted to obtain three individual fractions (1 to 3); (iv) hydrogen peroxide (10% v/v) was added to 1 M sodium hydroxide solution and vortex mixed, then the cleaned sample or the PST standard solution was added, and the mixture thoroughly mixed and allowed to react for 2 min at room temperature; glacial acetic acid was added and vortex mixed; (*v*) a sample extract after C18 or SPE-COOH fractionation, or a PST standard solution, was added to a matrix modifier solution prepared with PST-free oysters, and then is added the periodate oxidant; the mixture was thoroughly mixed and allowed to react during 1 min, then glacial acetic acid was added and the mixture was allowed to stand for further 10 min. Aliquots of C18 extract were used for oxidation of PSTs with peroxide and periodate oxidants, while aliquots of SPE-COOH fractions were used for periodate oxidation, prior to liquid chromatography with fluorescence detection (LC-FLD) analyses. Similar procedure of peroxide and periodate oxidations were followed, substituting the oxidant reagent by water in order to detect naturally fluorescent compounds.

The procedure used in the oxidation of PSTs was based on the AOAC method (Anon., 2005b) with a procedural modification (Botelho et al., 2010b). This modification was centred on the inversion of reactions sequence of AOAC method, the peroxide oxidation being performed before the periodate oxidation. The option for the inversion comes from non-N-hydroxylated toxins (B1, C1+2, dcSTX, dcGTX2+3) being the most abundant ones in shellfish samples contaminated by *Gymnodinium catenatum* from Iberian waters. In the AOAC method, these compounds are also quantified in the peroxide oxidation, due to a better detection of the oxidation products, but after a periodate oxidation.

5.2.6. Estimation of B2 concentration

Despite the unavailability of commercial certified reference material for the toxin B2, its concentration was estimated in selected samples of digestive gland from the days 0, 8, 12, 14, 19, 21 and 25, through the hydrolysis conversion into neosaxitoxin (NEO). The analytical procedure was based on the Marine Biotoxins Report of the Community Reference Laboratory for the determination of PSP toxins in shellfish including B2 (GTX6) after hydrolysis (Anon., 2007). Aliquots of fraction 2 (SPE-COOH fractionation) containing the toxin B2 were used for periodate oxidation, prior to LC-FLD analyses (Anon., 2005b). The LC measurements after periodate oxidation of this fraction allowed the

detection of B2. 125 μL of 1 M hydrochloridric acid solution were added to 500 μL of fraction 2 in a glass tube. The tube was closed and heated for 20 minutes at 90°C in a water bath, and then allowed to cool down at room temperature. The reaction was then neutralized adding small volumes of 1 M sodium hydroxide solution. The solution was mixed after each addition until a volume of 125 μL was obtained. After neutralization, the pH of the hydrolyzed extract was adjusted to 8.2. Hydrolysed extracts were submitted to periodate oxidation and B2 toxin was indirectly quantified through conversion into NEO. It was assumed that the molarity of B2 toxin hydrolyzed was equal to the molarity of the NEO present in fraction 2 after hydrolysis.

5.2.7. LC-FLD analysis

The LC system consisted of a Hewlett-Packard/Agilent Model 1050 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. The PST oxidation products were separated using a reversed-phase *Supelcosil* LC-18, 150 x 4.6 mm id, 5 μm column (Supelco) equipped with a guard column *Supelguard Supelcosil* C18, 20 x 4.0 mm id, 5 μm (Supelco). The column was kept in an oven at 30°C. The mobile phase gradient used 2 mobile phases: A (0.1 M ammonium formate, pH=6) and B (0.1 M ammonium formate in 5% acetonitrile, pH=6). The elution gradient consisted of 0-5% B in the first 5 min, 5-70% B in the next 4 min and back to 0% B in the next 5 min. Flow rate was 1 mL min^{-1} and the injection volumes were 50 μL and 100 μL, for the oxidation products of peroxide and periodate reaction, respectively. The excitation and emission wavelengths for fluorimetric detection were set at 340 nm and 395 nm, respectively.

5.2.8. Performance and quality control

Recovery experiments of the analytical procedure were carried out using clam tissues at two concentration levels, following the methodology described in Quevauviller and Morabito (2000). PST-free clam matrices were used to assess recoveries. The matrices were spiked in triplicate with the addition of a toxin mixture including C1+2, B1, GTX2+3, STX, dcSTX and dcGTX2+3 to produce two concentration levels. The spiked level I contained toxin concentrations ranging from 20 ng g^{-1} (dcSTX) to 142 ng g^{-1} (C1+2), while spiked level II from 82 ng g^{-1} (dcSTX) to 567 ng g^{-1} (C1+2). Intervals of the

mean recoveries for the quantified PSTs were: 71-74% (C1+2), 97-98% (B1), 87-104% (GTX2+3), 90-93% (STX), 77-114% (dcSTX) and 85%-107% (dcGTX2+3). For the NEO, a recovery of 97% was obtained in matrices spiked in triplicate with 227 ng g^{-1} (level II). Repeatability values in terms of relative standard deviation were from 1 to 18%. Instrumental detection limits (nmol L^{-1}) were 3.9 (C1+2), 4.0 (B1), 4.0 (STX), 4.9 (dcSTX), 8.2 (dcGTX2+3), 8.5 (GTX2+3) and 31 (NEO). Evaluation of linear ranges for PSTs and instrumental limits of detection are described in Botelho et al. (2010a). The quality control of the results was obtained through the use of the certified reference materials C1&2, STX-e, dcSTX, GTX5-b (B1), dcGTX2&3, GTX2&3-b and NEO-b, from the Institute for Marine Biosciences, National Research Council Canada.

Figure 5.1 presents chromatograms illustrating the toxin separation in two standard mixtures of C1+2, GTX2+3, B1 and STX after peroxide oxidation (a), of dcGTX2+3 and dcSTX after peroxide oxidation (b), and in a selected cockle sample (sub-cellular particulate fraction P3-lysosomes) after peroxide oxidation of the C18-cleaned extract (c).

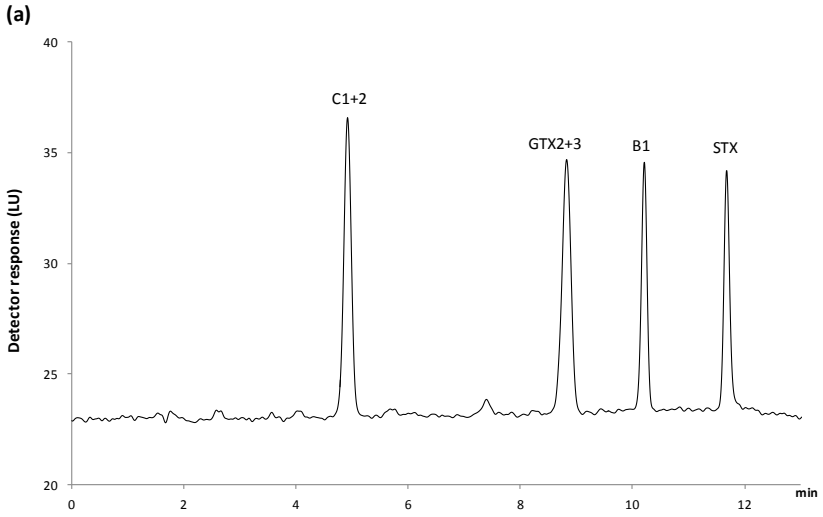

Figure 5.1. Chromatograms obtained for two standard mixtures of C1+2, GTX2+3, B1 and STX after peroxide oxidation (a), of dcGTX2+3 and dcSTX after peroxide oxidation (b), and for a selected cockle sample (sub-cellular particulate fraction P3-lysosomes); quantification of dcGTX2+3, C1+2, dcSTX, GTX2+3 and B1 after peroxide oxidation of the C18-cleaned extract (c).

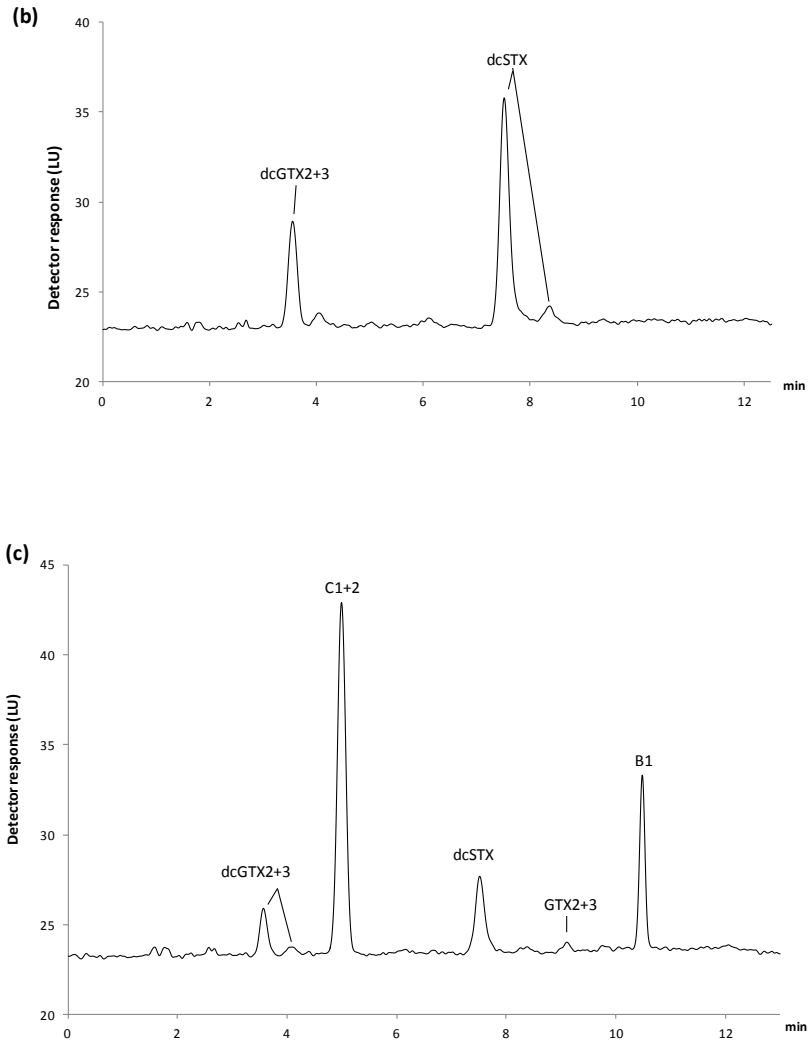

Figure 5.1. (cont.).

5.2.9. Calculation and statistical analysis

The content of each toxin quantified in the cytosolic fraction was estimated by the difference between the quantity in the digestive gland and the sum of toxin quantities in the four particulate cellular fractions. This estimation avoids errors associated with the direct determination of toxin concentrations in the cytosolic fraction due to the small mass in the supernatant fraction after the sequential fractionation. All quantities of toxins in digestive gland and sub-cellular particulate fractions are based on 1 g of digestive gland (wet weight).

Student's t-test was used to search differences in toxin quantities between each sampling date. The significance level for statistical analyses used was α=0.05. Statistical analyses were performed using Statistica 6.0 (Statsoft).

5.3. Results

5.3.1. Toxins in the digestive gland

Figure 5.2 shows the content of the quantified toxins in digestive glands of cockles per g wet weight (ww), sampled at the algal bloom peak (day 0) and in the post-bloom period (days 8, 12, 14, 19, 21 and 25). On day 0, the mean quantity of C1+2 (174 nmol) was one order of magnitude above B1 (43 nmol) and dcGTX2+3 (25 nmol), and two orders of magnitude above dcSTX (9.0 nmol), GTX2+3 (3.6 nmol) and STX (1.6 nmol). The quantity of toxins in digestive glands decreased with time, following the decline of *Gymnodinium catenatum* bloom. Despite the predominance of C1+2, B1, and dcGTX2+3 with respect to the other toxins, the decrease rate in the digestive gland varied among toxins. Mean quantities of the minor toxins STX and GTX2+3 decreased 92 and 80% ($p<0.05$) respectively, in the first eight days, and subsequently values remained close to the detection limits (Figure 5.2). Significant ($p<0.05$) decreases were also found for dcGTX2+3 (60%), dcSTX (55%) and C1+2 (50%) and, in the subsequent four days, additional mean losses ($p<0.05$) were 57, 49, and 61%, respectively. The content of B1 decreased by only 12% in the first eight days, then fell sharply by 50% between days 8 and 12 ($p<0.05$).

The quantities of B2, which were calculated indirectly through its hydrolysis into NEO, exceeded up to three times those of B1. The estimated quantities of B2 in digestive glands of selected samples for the days 0, 8, 12, 14, 19, 21, and 25 were (Figure 5.2): 159, 86, 38, 26, 10, 0.48, and <0.40 nmol, respectively. This decrease followed the tendency registered for C1+2 and dcGTX2+3, but was faster than the variation seen for B1.

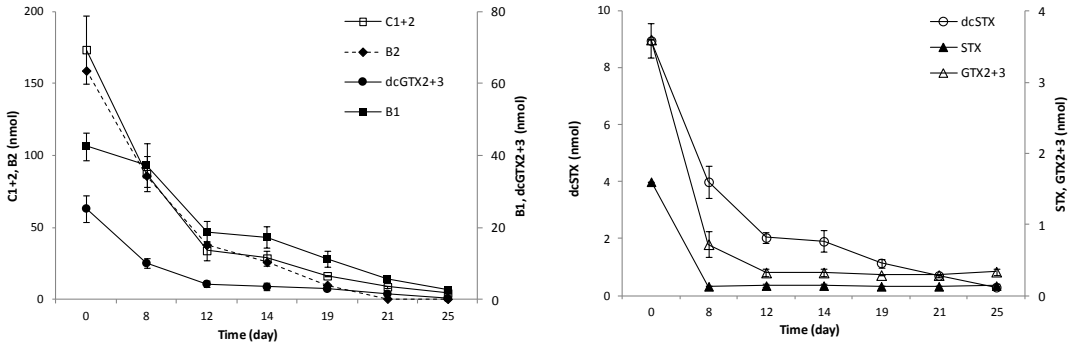

Figure 5.2. Quantities (nmol) of the toxins C1+2, B1, B2, dcGTX2+3, dcSTX, GTX2+3 and STX in composite samples of digestive glands of the cockle *Cerastoderma edule* exposed to a bloom of *Gymnodinium catenatum* (day 0) and the subsequent 25 days under post-bloom conditions; mass unit: one g wet weight of digestive gland; mean values (n=3; ±SD) for C1+2, B1, dcGTX2+3, dcSTX, GTX2+3 and STX, and individual values of selected samples for B2.

5.3.2. Toxins in sub-cellular particulate fractions

The nuclei+debris fraction (P1) appeared as the particulate component of the cell with the highest proportion of the quantified toxins (up to 23%). The fractions mitochondria (P2), lysosomes (P3) and microsomes (P4) accounted to less than 11, 9, and 9% respectively.

As observed for the whole digestive gland, C1+2, B1 and dcGTX2+3 were the major toxins present in the four particulate fractions (Figure 5.3).

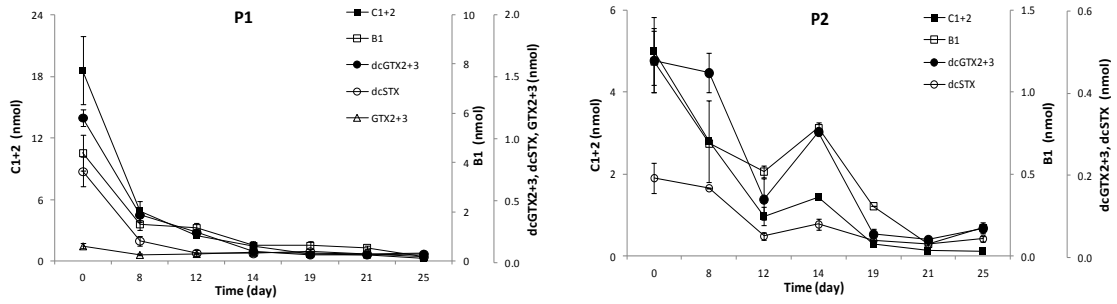

Figure 5.3. Quantities (nmol) of the toxins C1+2, B1, dcGTX2+3, dcSTX, GTX2+3 and STX in the sub-cellular particulate fractions (nuclei+debris - P1, mitochondria - P2, lysosomes - P3, microsomes - P4) of digestive glands of the cockle *Cerastoderma edule* over the 25 days under post-bloom conditions; mass unit: one g wet weight of digestive gland; mean values (n=3; ±SD).

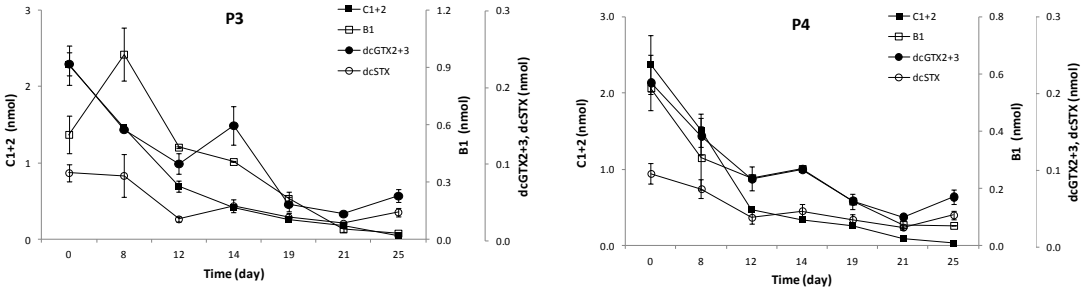

Figure 5.3. (cont.)

On the basis of one g ww of digestive gland, the mean quantities of toxins in P1, P2, P3, and P4 fractions on day 0 were: C1+2 (19, 5.0, 2.3, and 2.4 nmol, respectively), B1 (4.4, 1.2, 0.55, and 0.55 nmol), dcGTX2+3 (1.2, 0.40, 0.23, and 0.19 nmol) and dcSTX (0.73, 0.16, 0.087, and 0.084 nmol). Different decrease patterns were observed among pellets and toxins over the next 25 days. Toxin GTX2+3 was only quantified in the P1 fraction (0.12 nmol), and was relatively constant over the 25 days of observations (0.053-0.070 nmol). The sharper toxin decreases ($p<0.05$) were found between day 0 and day 8 in P1 (between 56% for GTX2+3 and 77% for dcSTX). Subsequently, decreases were not statistically different. A similar pattern was registered in the P4 fraction, although reduction was significant ($p<0.05$) only for C1+2 (36%), dcGTX2+3 (37%), and B1 (44%) between day 0 and day 8.

The mean quantities of toxins registered in the P2 and P3 fractions varied less regularly in the first 19 days. In P2, the contents of dcGTX2+3 and dcSTX showed a smoother variation ($p>0.05$) between day 0 and day 8 (6 and 13% respectively) than C1+2 (44%) and B1 (42%) with $p<0.05$. After 14 days under post-bloom conditions, an increase of 119% of dcGTX2+3 ($p<0.05$) and 51% B1 ($p>0.05$) was observed. In P3, between day 0 and day 8, the quantities of C1+2 and dcGTX2+3 decreased 37%, dcSTX remained constant, while the quantity of B1 increased 77%. The decrease of C1+2 and dcGTX2+3 and the increase of B1 were statistically significant ($p<0.05$). Between days 12 and 14, the quantity of dcGTX2+3 increased 51% ($p<0.05$). Values of STX were below the detection limit in the four particulate fractions.

5.3.3. Toxins in the cytosolic fraction

Between 63 and 97% of the quantified toxins in the digestive gland were estimated to be stored in the cytosolic fraction. The most abundant toxins were C1+2, B1, and dcGTX2+3 (Figure 5.4).

During the first eight days of post-bloom conditions, the mean quantities (nmol, on a basis of one g ww of digestive gland) diminished 48% for C1+2 (145 to 76), 63% for dcGTX2+3 (25 to 9.4), and 82% for GTX2+3 (3.4 to 0.60). Those differences were significant ($p<0.05$). Over the same period of time, no statistical differences were observed for dcSTX (7.0 to 3.2 nmol) and B1 remained relatively constant (30 nmol). The discrepancy between the variation with the time of B1 and each of the other toxins is clearly shown in figure 5.4. The quantity of STX diminished abruptly between day 0 (1.6 nmol) and day 8 (<0.1 nmol).

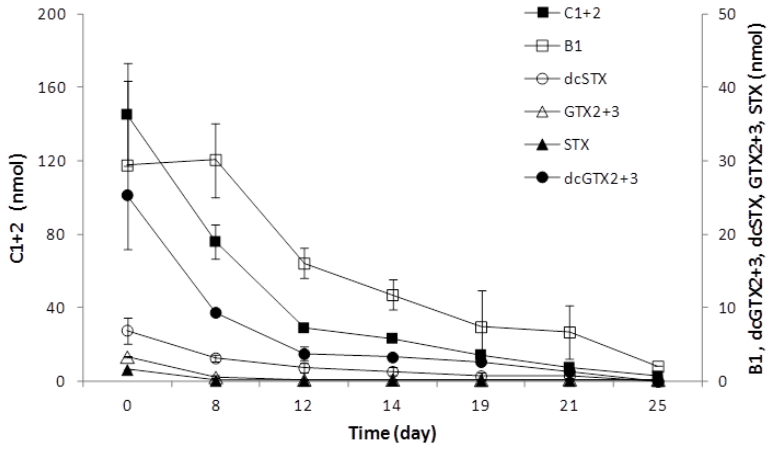

Figure 5.4. Estimated quantities (nmol) of the toxins C1+2, B1, dcGTX2+3, dcSTX, GTX2+3 and STX in the cytosolic fraction of digestive glands of the cockle *Cerastoderma edule* over the 25 days under post-bloom conditions; mass unit: one g wet weight of digestive gland; mean values (n=3; ±SD).

5.4. Discussion

The methodology used herein followed the procedures currently used to separate organelles and the cytosolic fraction that are operationally defined by differential centrifugation (Campbell et al., 2005; Raimundo et al., 2008). Consequently, the results obtained may contain potential artefacts such as breakage or clumping of particles, leakage of soluble constituents from organelles, and overlap among sub-cellular fractions, as reported in Wallace et al. (2003). Under these circumstances, the partitioning of paralytic shellfish toxins among the cell compartments in digestive glands of cockles exposed to a bloom of *Gymnodinium catenatum* and to post-bloom conditions indicates that assimilated toxins were mainly stored in the cytosolic fraction. These results are in line with previous work related to the partitioning of hydrophilic toxins (Mauriz and Blanco, 2010). As the biomass of toxic algae decreased, the quantities of toxins in soluble and particulate sub-cellular fractions

diminished, either reflecting the progressive lower ingestion of toxic dinoflagellate cells under post-bloom conditions, or enzymatic and oxidative processes resulting in the elimination or biotransformation of the assimilated toxins.

5.4.1. Reduction of toxin content under post-bloom conditions

The dominance of C1+2 followed by B1 and dcGTX2+3 in the whole and cell compartments of digestive gland of cockles exposed to the algal bloom is in accordance with the toxin composition reported for this algal species (Band-Schmidt et al., 2010; Botelho et al., 2012). During the period of abundant biomass of this dinoflagellate, high toxin contents in the sub-cellular compartments and in whole digestive gland should primarily reflect the assimilation of the major toxins produced by the algae. The results of this study are in line with the predominance of those toxins in other bivalve species exposed to this dinoflagellate species, during both the uptake and depuration phases (Botelho et al., 2010a; Botelho et al., 2012; Samsur et al., 2007).

As the abundance of toxic cells diminished or became negligible in the lagoon, the quantity of toxins in cockles decreased within 8 to 12 days, according to the compounds. This variation was observed in the four sub-cellular particulate fractions and in the estimated cytosolic fraction. The decreases found at sub-cellular level are in agreement with laboratory studies showing that depuration rates of PSTs in whole bivalve tissues are relatively fast (Blanco et al., 2003; Botelho et al., 2010a; 2012; Yu et al., 2007). Under post-bloom conditions, the weight of elimination or transformation of the ingested toxins should progressively counterbalance the toxin uptake and became the dominant phase of the bioaccumulation process.

The nuclei+debris fraction is considered the most operational fraction obtained by the differential centrifugation approach, since it contains cell membranes, intact cells besides nuclei (Bonneris et al., 2005). Nevertheless, toxin contents in this fraction decreased markedly between day 0 and day 8, most likely due to weak associations of toxins with fraction components. A similar explanation may be considered for the rapid release of toxins in the microsomes fraction (P4). The decrease rate of toxin contents was slower for dcGTX2+3 and dcSTX in the mitochondrial fraction (P2) during the first days of post-bloom conditions, and for B1 in the whole digestive gland and cytosolic fraction. An increase of B1 content was found in the lysosomes fraction (P3). The maintenance of

these elevated values may reflect the slow apparent depuration rate of B1 relatively to other toxins, as shown in the laboratory (Botelho et al., 2010a, 2012).

5.4.2. Toxin profiles in sub-cellular fractions

The possible interconversion among toxins is better visualised by plotting toxin profiles for each separate cell compartment (Figure 5.5). The contribution of C1+2 to the toxin profile of the cytosolic fraction showed an opposite trend to B1 when toxic algal cells was still elevated (first 12 days): C1+2 accounted for 79% to the total toxins on day 0 and decreased to 61% on day 12, while the contribution of B1 increased from 16 to 32%. A similar pattern was observed for all the separate pellets: C1+2 accounted for 74-53%, and B1 for 17-37%. As observed for the cytosolic fraction, the proportion of B1 increased after 12 days to: up to 43% in nuclei+debris, 46% in lysosomes, 40% in mitochondria and 36% in microsomes fractions. Two major explanations are proposed for the opposite time-courses of C1+2 and B1: (i) high solubility of C1+2 relative to B1 (Samsur et al., 2006), which facilitates elimination and consequently the progressively lower proportion of C1+2 in the toxin profile; (ii) enhancement of B1 resulting from biotransformation of other assimilated toxins, namely B2. To the best of our knowledge, conversion of B2 into B1 has not been evidenced in shellfish, mainly due to the lack of certified reference material to confirm the presence of B2. Despite that analytical incertitude to quantify B2, the accentuated decrease of B2 in digestive gland between day 0 and day 8 (approximately 50%) may have contributed to the less pronounced decrease registered for B1 (Figure 5.2). Conversion of B2 into B1 would explain the singularity of B1 pattern, in comparison to the other quantified toxins, under depuration after bloom cessation. This time variation of B1 was clearly marked in the sub-cellular particulate fraction P3. In fact, B1 concentrations in lysosomes, between day 0 and day 8, showed a clear increase meaning that input exceeded elimination. The approach followed in the current work is, however, insufficient to elucidate the mechanisms behind biotransformation of toxins related to B1 (Fast et al., 2006).

5.4.3. Interconversion of toxins in mitochondrial and lysosomal fractions

During the period at which toxin content in digestive glands was elevated, the mitochondrial fraction (P2) presented slower decreases of dcGTX2+3 and dcSTX than C1+2 and B1. These differences may be

related to the enzymatic conversion of C1+2 into dcGTX2+3 and B1 into dcSTX (Cho et al., 2008; Lin et al., 2004; Sullivan et al., 1983). This hypothesis is reinforced by the two relations found over the 25 days of observations between the two pairs of toxins: dcGTX2+3 *versus* C1+2 (y=0.098 ln$x$ + 0.22, r=0.933) and dcSTX *versus* B1 (y=0.098 $e^{1.68x}$, r=0.894). The interconversion among toxins may be associated with enzymatic activities existing in the mitochondrial fraction, as compounds ingested by organisms are transformed into simpler molecules during the energy production in this organelle (Voet and Voet, 1990). Concomitantly with toxin interconversion, elimination process proceeds since

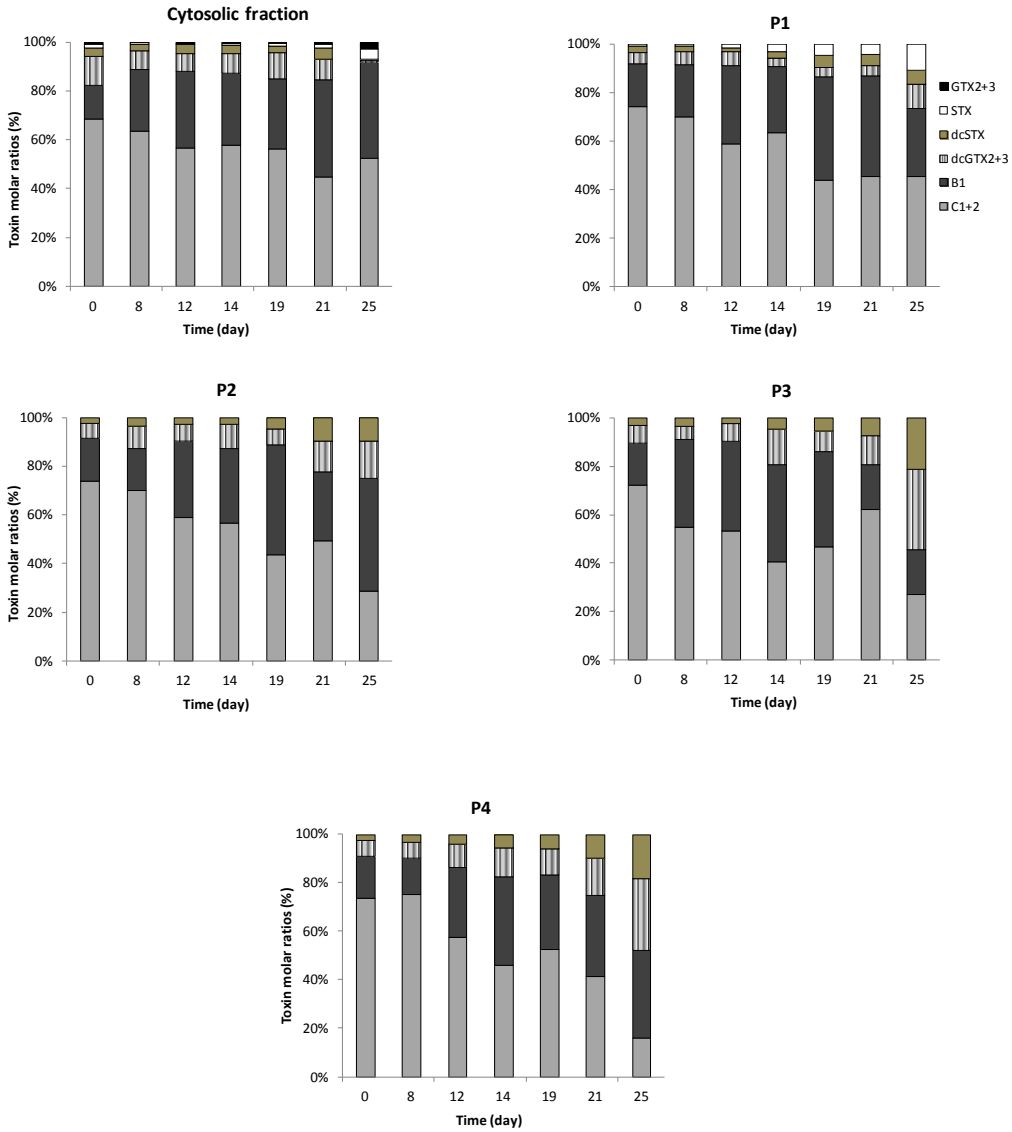

Figure 5.5. Toxin profiles expressed as molar ratios (%) between the quantity of each toxin and the quantity of all the quantified toxins in the particulate fractions nuclei+debris - P1, mitochondria - P2, lysosomes - P3, and microsomes - P4 and in the cytosolic fraction of digestive glands of the cockle *Cerastoderma edule* over the 25 days under post-bloom conditions; mass unit: one g wet weight of digestive gland; mean values (n=3).

organisms are under natural depuration conditions. Previous work showed the export of dcSTX as faeces or pseudo-faeces of the clam *Ruditapes decussatus* under depuration conditions after being exposed to *G. catenatum* (Botelho et al., 2012). In this study, the absence of B1 and C1+2 in faeces reinforced the importance of biotransformation in comparison to elimination processes.

The accentuated increase of B1 in the lysosome fraction (P3), and the plateau of dcSTX values on day 8, pointed to interconversion processes, as proposed for the mitochondrial fraction (P2). A plausible explanation for the increase of B1 is the possibility that this compound is a metabolic product of degradation of other toxins (Oshima, 1995b). In particular, the abundant toxin B2 may be reduced to B1 due to their similar chemical structures. Since contents of B2 largely exceeded B1, conversion of small quantities of B2 may be sufficient to enhance B1. In addition, C1+2 may also be converted into B1 through the cleavage of 11-hydroxysulfate, as proposed conversion of GTX2 and GTX3 into STX (Oshima, 1995b).

These transformations are associated to the major role of lysosomes in the intra-cellular digestive process of materials acquired through vesicular transport (Voet and Voet, 1990). Along this process undesirable compounds, such as toxins, may be incorporated in the lysosomes and subsequently degraded in order to protect the cell from cytotoxic effects (Moore, 1988; Owen, 1972; Viarengo et al., 1987). Svensson et al. (2003) pointed to the uptake and storage of okadaic acid within the lysosomal system in order to protect the blood cells in mussels. The results found in the present work, although dealing with hydrophilic compounds, may also be interpreted as the removal by lysosomes of toxins present in the cytosol. Besides enzymatic activity in the lysosomes, the possibility that other processes occurring in the cytosol have contributed to the toxin elimination should not be excluded.

5.5. Conclusions

The study of the sub-cellular partitioning of paralytic shellfish toxins in digestive glands of cockles exposed to a bloom of *G. catenantum* showed the presence of toxins in the sub-cellular particulate fractions constituted mainly by nuclei+debris, mitochondria, lysosomes and microsomes. The largest quantities were estimated in the cytosolic fraction. Under post-bloom conditions, the

variation of toxin contents and toxin profiles emphasises the differential elimination of these compounds. Interconversion among toxins and exchanges between insoluble and soluble cellular fractions are the most plausible reasons. In particular, the non-uniform decrease of B1 contents in the lysosomal fraction points to the conversion of other toxins, like B2 and C1+2, into B1 following the sub-cellular digestive processes of the ingested food.

Acknowledgements

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6

6. Identification of seasonal and multi-annual trends of bivalve toxicity by PSTs in Portuguese estuarine and coastal waters

Highlights

- Toxicity by PSTs in bivalves from estuarine and coastal waters of Portugal was examined.
- Irregular multi-annual variations over 18 years considering 4 species and 7 areas.
- Peak toxicities in autumn/winter and occasionally in summer.
- Episodes of mussel and cockle toxicities connected in three estuarine systems.
- Toxicity episodes of cockle and wedge clam connected despite toxin biotransformation.

Botelho, M.J., Vale, C., Ferreira, J.G. (submitted). Identification of seasonal and multi-annual trends of bivalve toxicity by PSTs in Portuguese estuarine and coastal waters. Estuarine, Coastal and Shelf Science.

Abstract

Temporal and spatial trends of paralytic shellfish toxins (PSTs) in bivalves from Portuguese estuarine and coastal waters, and connectivity of bivalve toxicity among the harvest areas, were examined using long-term monitoring data from the national biotoxin monitoring programme (BMP). Data from the period between 1994 and 2012 were chosen for commercial bivalve species sensitive to PSTs, and for production areas exhibiting recurrent episodes of bivalve toxicity: mussels (*Mytilus* spp.), and cockles (*Cerastoderma edule)* from the Aveiro, Mondego, Óbidos, and Formosa estuarine systems, and wedge clam (*Donax trunculus*) and surf clam (*Spisula solida*) from the coastal areas Aguda, Comporta, and Culatra. Bivalve toxicity data point to an irregular multi-annual variation of PSTs episodes, most likely associated with the inter-annual variability of favourable oceanographic conditions triggering the bloom formation of *Gymnodinium catenatum*, which is the species responsible for PSP in Portuguese waters. Episodes in the southern coast of Portugal were less recurrent. However, the values above the PST regulatory limit (elevated toxicity) displayed a seasonal signal with a peak between autumn and early winter and, in some years, an additional enhancement in summer. A connectivity index of bivalve toxicity was defined among the surveyed areas, on the basis of the number of weeks per month that bivalves showed elevated toxicity values. High connectivity was obtained among Aveiro, Mondego and Óbidos, suggesting the import of *G. catenatum* cells from blooms formed or reaching the coastal waters adjacent to these estuaries. During episodes of elevated toxicity, toxin profiles in mussels and cockles were dominated by N-sulfocarbamoyl compounds, which are the major toxins produced by toxic dinoflagellates. Connectivity was also registered between cockles from Formosa and wedge clams from the adjacent area of Culatra, although the toxin profile of wedge clams suggests a greater influence of transformation or elimination of ingested toxins than in the cockles.

Keywords: Harmful algal blooms; Bivalves; Paralytic shellfish poisoning; *Gymnodinium catenatum;* Monitoring programmes

6.1. Introduction

Paralytic shellfish toxins (PSTs) are a broad group of neurotoxins produced by species of harmful marine dinoflagellates belong to the genera *Alexandrium*, *Pyrodinium* and *Gymnodinium* (Wiese et al., 2010). The mechanism of action of PSTs is the blockage of the conductance of nerve signals by interfering with the sodium channels of excitable cells (Kao, 1966). The better studied PSTs are included in the following groups: carbamate (saxitoxin-STX, neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), N-sulfocarbamoyl (B1, B2, C1 to C4), and decarbamoyl (dcGTX1 to dcGTX4, dcSTX and dcNEO). As higher performance laboratory instruments became available, analytic methodologies improved and the chemical structure of other PST toxins was progressively identified (Llewellyn, 2006).

During blooms of species that produce PSTs, bivalves concentrate toxins in their tissues from the large volumes of water cleared when feeding. Most of the *Alexandrium* species have a carbamate-dominated toxin profile (Cembella et al., 1987; Chou et al., 2004), while N-sulfocarbamoyl toxins are dominant components in *G. catenatum* (Ordás et al., 2004; Band-Schimdt et al., 2005; Botelho et al., 2012). Decarbamoyl toxins are generally less abundant in those dinoflagellates. Nevertheless, bivalves may modify the toxin composition of the causative dinoflagellate by differential uptake and depuration kinetics of individual toxins (Botelho et al., 2010a; Yu et al., 2007) in conjunction with metabolic interconversion of the assimilated toxins (Shimizu and Yoshioka, 1981; Sullivan et al., 1983; Kotaki et al., 1985; Oshima, 1995b). Ingested toxins and those resulting from interconversion contribute in different proportions to bivalve toxicity (Oshima et al., 1990; Samsur et al., 2006).

Human consumption of contaminated bivalves by PSTs may result in neurological and gastrointestinal illnesses (Gessner and Middaugh, 1995). The first paralytic shellfish poisoning (PSP) event was reported in 1927 near San Francisco, USA, caused by *Alexandrium catenella* (Sommer and Meyer, 1937). The association of *G. catenatum* blooms with PSP episodes was firstly reported in NW Spain, Pacific coast of Mexico, Australia, Japan (Hallegraeff et al., 2012), and Portugal (Franca and Almeida, 1989). Due to the potential severity of the symptoms, since the 1970s to 1980s several countries run national monitoring programmes of PSTs in commercial bivalves to alert the consumers about bivalve toxicity episodes (Mons et al., 1998; Batoréu et al., 2005). As monitoring programmes

were implemented in various countries, it became apparent that *Gymnodinium catenatum* is a cosmopolitan species (Band-Schimidt et al., 2010). Hallegraeff et al. (2012) have proposed two possible mechanisms for *G. catenatum* blooms: (i) high abundance of cells derived from inocula by local cyst beds or motile cells (autochthonous case), triggered by organic enrichment associated to rainfall events (e.g., Tasmanian estuaries); and (ii) the allochthonous case of *G. catenatum* blooms that are inoculated from offshore, and build up during upwelling relaxation (e.g., NW Spanish Rias). In Portuguese coastal waters, the record of *G. catenatum* blooms has been also associated with category (ii), i.e. relaxation of coastal upwelling that occurs recurrently in the western region, and slow currents or inshore eddies that contribute to the maintenance of this species near the coast (Moita el al., 2003; Pitcher et al., 2010).

The toxicity of bivalve species by PSTs and the presence of toxic phytoplankton species in Portuguese coastal and estuarine waters have been monitored since the 1980s. Using the national biotoxin monitoring database, overviews of the commercial bivalve contamination associated with diarrhetic shellfish poisoning, amnesic shellfish poisoning and paralytic shellfish poisoning were presented in Sampayo et al. (1997) and Vale at al. (2008). These overviews focused on the incidence of maximum toxicity values for several commercial species and on the most affected production areas. The main objective of the present work is to examine whether the occurrence of PST episodes in Portugal between 1994 and 2012 exhibited multi-annual or seasonal variation signal. The hypothesis was tested for mussels, cockles, wedge clams and surf clams regularly sampled in transitional and coastal waters of Portugal. In addition, the connectivity of toxicity episodes among more regularly surveyed areas was examined.

6.2. Bivalve toxicity and data analysis

6.2.1. Biotoxin monitoring programme

Bivalve toxicity data used in the present work were obtained from the Portuguese biotoxin monitoring programme (BMP) started in 1994 by the Portuguese Institute of Marine Research (IPIMAR) and subsequent Governmental Institutes (Sampayo et al., 1997; Vale et al., 2008). The monitoring of bivalve toxicity is accomplished by the surveillance of toxic algal blooms producers of DSP, PSP and ASP (Anon., 2004a; Sampayo et al., 1997; Vale et al., 2008). Presently, the BMP covers

38 production areas located in coastal lagoons, estuaries and open coastal areas where commercial bivalves are currently harvested (Figure 6.1).

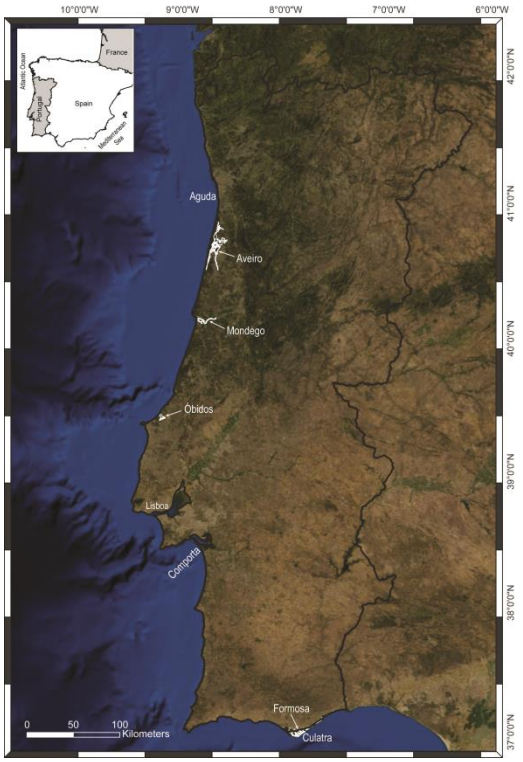

Figure 6.1. Harvesting areas of mollusc bivalves: Aveiro, Mondego, Óbidos, and Formosa (estuarine systems), and Aguda, Comporta and Culatra (open coastal areas).

The programme includes most of the commercial bivalve species harvested in coastal lagoons and estuaries (*Mytilus* spp., *Cerastoderma edule, Ruditapes decussatus, Venerupis pullastra, Scrobicularia plana, Ensis* spp*., Solen* spp*., Venus verrucosa, Ostrea* spp*.* and *Crassostrea* spp.) and in the coastal zone until 15 m depth (*Spisula solida, Donax trunculus, Ensis siliqua, Callista chione* and *Chamelea gallina*) (Oliveira et al., 2013). In estuarine systems, species were harvested from natural banks, except *Mytilus* spp., which was collected from intertidal substrates and *R. decussatus* and *Crassostrea* spp. from intertidal growth grounds. The episodes of DSP, PSP and ASP are alerted by bivalve toxicity values above the corresponding regulatory limit (RL). Every time a sample presents toxicity values exceeding the RL, the closure for bivalve harvesting is mandatory. According to the European Regulation, the sector regains the activity when two successive samples show values below RL (Anon., 2004b).

From 1994 to 2001 the bivalve toxicity by PST was determined by a mouse bioassay methodology (AOAC, 1990; Anon., 2005a). From 2002 to 2008 toxin concentrations were determined chemically using a LC method with pre-column derivatisation based on Lawrence et al. (1995) modified by Vale and Sampayo (2001). After 2009, PST determinations followed the methodology described in Anon. (2005b). After chemical determinations by these two methodologies, toxicity values were estimated multiplying toxin concentrations by the toxicity equivalence factor (TEF) of each individual compound quantified. Following the recommendations of the EU Reference Laboratory for Marine Biotoxins, after June 2010, different TEFs were used to estimate the bivalve toxicity (EFSA, 2009). For the purpose of data consistency in this study, all toxicity values originally determined using Oshima TEF (Oshima, 1995b) were recalculated using EFSA. Since 1940s the regulatory limit for PSTs is 800 µg STX di-HCl equivalents per kg of shellfish (Anon., 2004b; Sommer and Meyer, 1937). A detailed description of the BMP, location of the natural production areas of bivalves, analytical methodologies and toxicity calculations are described in Vale and Sampayo (2001), Vale et al. (2008), and Botelho et al. (2010b).

6.2.2. Formulas and calculations

6.2.2.1. Sampling representativeness

Sporadically, the BMP database contains, for the same production area and species, two toxicity values for the same week. Those situations correspond to an abrupt increase of bivalve toxicity values exceeding the RL. To better compare with other periods only the first value was considered, under the assumption that each toxicity value integrates one weekly period. Due to logistic problems bivalves were not sampled every week, sampling representativeness varying along the monitoring programme. The sampling representativeness on a monthly (SR_M, %) and yearly (SR_Y, %) basis was calculated for each bivalve species and production area using the following expressions:

$$SR_M = N_1 \times 100/4 \qquad (6.1)$$

Where N_1 is the number of samples collected monthly, and 4 is the number of weeks per month.

$$SR_Y = N_2 \times 100/52 \qquad (6.2)$$

Where N_2 is the number of samples collected annually, and 52 is the number of weeks per year.

On the basis of SR_M *or* SR_Y the confidence levels associated with the sampling periods (*CL*, scored from 0 to 4) were defined:

CL	SR_M *or* SR_Y (%)
4	>80
3	60-79
2	40-59
1	20-39
0	<20

6.2.2.2. Proportion of elevated toxicity samples

For each bivalve species and production area, the proportion of samples with elevated toxicity (TS_M and TS_Y, %) was calculated by dividing the number of samples presenting toxicity values above the RL per month (N_3) or year (N_4), by the number of samples collected in that period of time, month (N_1) or year (N_2):

$$TS_M = N_3 \times 100/N_1 \qquad (6.3)$$

$$TS_Y = N_4 \times 100/N_2 \qquad (6.4)$$

6.2.2.3. Connectivity Index

To assess the possible interconnection of PST episodes among two or three estuarine or coastal areas, a Connectivity Index for bivalve toxicity on a monthly basis (*CI*) was defined. The score of *CI* (0 to 4) was based on the number of weeks per month that bivalve species showed toxicity values above RL concomitantly in each area. For example, the maximum score of connectivity (4) was attributed to the month that areas exhibited four or three weeks of elevated toxicity values.

CI scores	*Number of weeks/month in each area*
4	four, three
3	three, two, one
2	two, one
1	one, none
0	none

6.3. Results

6.3.1. Data selection

After having associated each toxicity value with the corresponding week (section 6.2.2), the selection of the data was based on the following steps: (i) selection of surveyed areas with recurrent PST episodes (high TS_Y); (ii) choice of bivalve species considered as sentinel organism (mussel) or with economic value to the region (cockles and clams); (iii) selection of annual periods with moderate to elevated sampling frequency of bivalves (high SR_Y and *CL*).

6.3.1.1. Surveyed areas

Three areas in the coastal zone were selected (Figure 6.1): Aguda and Comporta in the west coast, and Culatra in the south. Since the abundance of bivalves within each area varied along the 18-year survey (Rufino et al., 2008), the coordinates of the harvesting sites have changed. Closure of bivalve harvest from the western areas has been influenced by the recurrent episodes of *G. catenatum* blooms (Moita et al., 1998, 2003; Vale et al., 2008), while episodes were less recurrent in the southern area. Four estuarine areas were chosen: Aveiro, Mondego and Óbidos in the west coast, and Formosa in the southern area. Whereas Mondego is a tubular estuary, Aveiro, Óbidos and Formosa have broad inner areas and narrow connections to the sea, are generally classified as lagoons (Bettencourt et al., 2004). All estuarine systems are mesotidal, and exchange large water volumes with the adjacent sea. The western systems considered in this study exchange water and seston with coastal waters with recurrence episodes of *G. catenatum* blooms, whereas the Formosa coastal lagoon imports less toxic cells of dinoflagellates. Its selection was due to the large quantities of cockles and clams produced and harvested (DGRM, 2012).

6.3.1.2. Bivalve species

Four bivalve species were chosen: mussel *Mytilus* spp., cockle *Cerastoderma edule*, wedge clam *Donax trunculus*, and surf clam *Spisula solida*. Mussels were collected from hard substrates in inlet channels of Aveiro, Mondego, Óbidos systems, and cockles from sandy inner areas of Aveiro, Mondego, Óbidos and Formosa. The wedge clam *Donax trunculus* and surf clam *Spisula solida* were harvested from open areas of western and southern coasts. Habitats and ecology of these species are described in Gaspar et al. (1999), Joaquim et al. (2008) and Rufino et al. (2010).

6.3.1.3. Years

The sampling representativeness (SR_Y, Eq. 6.2) of toxicity data, between 1994 and 2012, was examined to select the species and surveyed areas (Table 6.1). In general, sampling in the open coast (Aguda, Comporta and Culatra) had lower SR_Y than in estuarine areas (Aveiro, Mondego, Óbidos and Formosa), since sampling in coastal waters is highly dependent on the activity of commercial vessels. The period 1994-2002 showed lower SR_Y than the following years, particularly in estuarine areas.

Table 6.1. Annual sampling representativeness (%) between 1994 and 2012 in Aveiro, Mondego, Óbidos, and Formosa (mussel and cockle), Aguda (surf clam), Comporta (wedge clam) and Culatra (surf clam and wedge clam).

	Aveiro		**Mondego**		**Óbidos**		**Formosa**	**Aguda**	**Comporta**	**Culatra**	
Year	M	C	M	C	M	C	C	SC	WeC	SC	WeC
Annual Sampling Representativeness (SR_Y,%)											
1994	62	19	42	21	15	25	58	23	8	38	38
1995	50	52	35	31	25	35	87	6	4	21	38
1996	52	27	33	25	40	54	31	2	na	62	23
1997	54	40	60	58	40	50	na	4	na	21	6
1998	27	40	54	56	27	33	6	12	13	38	63
1999	27	44	33	33	10	17	40	12	4	31	12
2000	31	37	15	15	na	15	8	8	2	29	6
2001	6	4	8	4	na	na	6	2	na	2	4
2002	17	12	2	na	12	13	4	na	4	na	6
2003	87	73	25	na	67	63	4	na	13	2	19
2004	81	79	44	4	87	81	8	10	21	4	50
2005	87	85	54	31	79	31	27	31	50	15	38
2006	85	83	81	79	77	69	63	44	54	23	71

Table 6.1. (cont.)

	Aveiro		Mondego		Óbidos		Formosa	Aguda	Comporta	Culatra	
Year	M	C	M	C	M	C	C	SC	WeC	SC	WeC
Annual Sampling Representativeness (SR_Y,%)											
2007	50	58	73	71	69	63	81	48	56	38	54
2008	25	23	42	38	69	60	31	21	38	15	35
2009	65	17	62	2	19	2	69	35	na	19	40
2010	50	85	79	77	48	4	67	52	na	2	44
2011	92	92	31	6	73	37	60	29	4	15	44
2012	33	44	na	15	42	6	25	19	12	19	44

M=mussel; C=cockle; SC=surf clam; WeC=wedge clam

na=not applicable

6.3.2. Interannual variation of bivalve toxicity by PSTs

6.3.2.1. Estuarine areas

Figure 6.2 shows the annual proportion of samples (TS_Y, Eq. 6.4) of elevated toxicities from Aveiro, Mondego, Óbidos and Formosa for the period 1994-2012, as well as the corresponding confidence levels (*CL*). Values of TS_Y in Aveiro, Mondego and Óbidos were enhanced in two periods of time: 1994-1995 and 2005-2009. Confidence levels (*CL)* were lower in the first period (2±0.9) than in the second one (3±1.3). Between the two periods, PST concentrations in most mussel and cockle samples were undetected (bioassay) or below the limit of detection (chromatographic method). The hypothesis of PST episodes remaining undetected due to lack of samples could be valid for 1999-2002, but it is unlikely in 2003 and 2004 since *CL* reached 4±1.4. The most elevated values of TS_Y were registered in 2008, varying from 50% (Aveiro and Óbidos) to 82% (Mondego) for mussels, and from 21% (Aveiro) to 75% (Mondego) for cockles. These values indicate that more than half of the surveyed weeks in Mondego showed bivalve toxicity above the regulatory limit, and consequently the legal harvest was interrupted. Cockles from Formosa showed toxicity peaks in 1994, 1995 (as observed in the western estuarine systems), and 2009. In 1996, 2003 and 2004 only a few samples exhibited toxin concentrations above the limit of detection. The values of TS_Y for cockles from Formosa in 1994, 1995 and 2009 varied from 11 to 24%, meaning that less than a quarter of the surveyed weeks showed cockle toxicity.

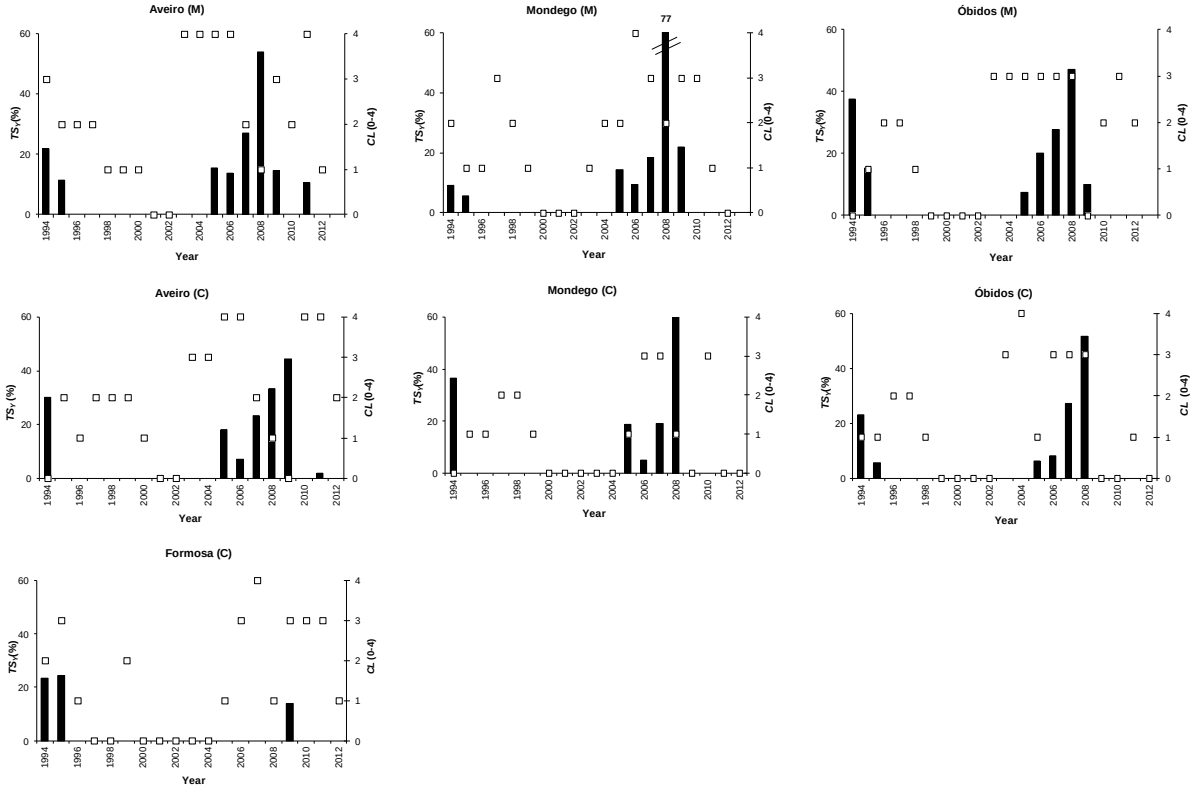

Figure 6.2. Annual proportion of elevated toxicity samples (bars, TS_Y) and confidence levels (squares, *CL*); mussels (M) and cockles (C) from Aveiro, Mondego, Óbidos and Formosa, between 1994 and 2012.

6.3.2.2. Open coastal areas

For the period 1994-2012, the annual proportion of samples (TS_Y, Eq. 6.4) with toxicity above the RL from Aguda, Comporta, and Culatra, as well as the corresponding confidence levels are shown in Figure 6.3. The multi-annual variation of bivalve toxicity in open coastal areas was less clearly defined than in estuarine areas. Indeed, elevated toxicity values were not registered in the two periods (1994-1995 and 2005-2009), but in several years depending on the harvesting area 1994, 2005-2007, 2009 and 2011 in surf clams from Aguda; 1994-1995, 2007-2008 in wedge clams from Comporta; and 1994-1995, 2008-2009 and 2012 in wedge and surf clams from Culatra. Elevated toxicity in 1994 or 1995 was found in the three coastal areas, while the episodes were less regular in subsequent periods. The confidence levels (*CL*) varied with the species and area: for surf clams 2±0.8 (Aguda) and 2±0.9 (Culatra); for wedge clams 1±0.0 (1994-1995) and 2±0.7 (2007-2008) for Comporta, and 2±0.6 for Culatra. The proportion of toxic weeks (TS_Y) for surf clam varied from 7% in Aguda to 60% in Culatra, while for wedge clam values ranged from 8% in Culatra to 50% in Comporta. The period 1996-2004 was not accounted due to sampling uncertainty (low *CL*). Although most of samples from

the open coastal areas presented undetected toxins, low PST values were quantified in a few samples, either through bioassays (1999 and 2000) or by chromatography (2003 and 2004).

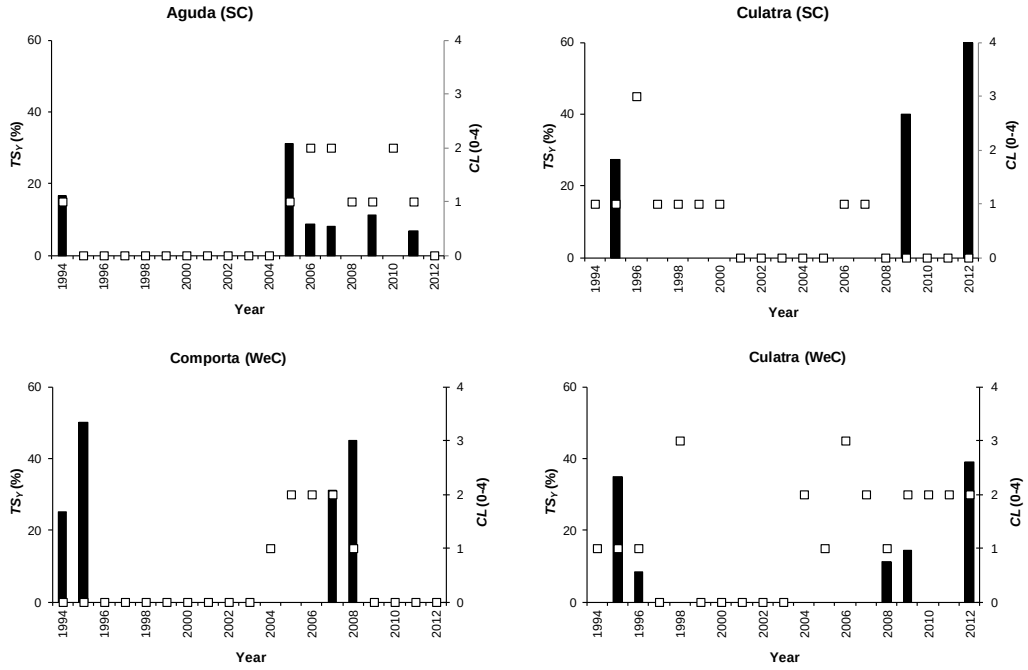

Figure 6.3. Annual proportion of elevated toxicity samples (bars, TS_Y) and confidence levels (squares, *CL*); surf clams (SC) from Aguda and Culatra, and wedge clams (WeC) from Comporta and Culatra, between 1994 and 2012.

6.3.3. Seasonal variation of bivalve toxicity by PSTs

The seasonal trend of PST toxicity was examined for the two periods 1994-1995 and 2005-2009 because it include the toxicity episodes of longer duration. The values of TS_M and *CL* for the seven surveyed areas are presented in the Figure 6.4. Mussels and cockles from Aveiro, Mondego and Óbidos showed elevated values of TS_M between October 1994 and January 1995, while the duration of toxicity episodes in surf clam and wedge clam was limited to November 1994 (Aguda), October 1994 and January 1995 (Comporta). Cockle toxicity episodes in Formosa were also registered in autumn/early winter of 1994 and 1995, while for wedge clam and surf clam from Culatra toxicity was only found in autumn/early winter of 1995. The pattern of enhanced TS_M values in autumn/early winter was also found for 2005, 2007 and 2009 in Aveiro, Mondego and Óbidos (Figure 6.5). Additional toxicity episodes were observed in June-August 2006 and 2008. The prolonged periods of toxicity between autumn 2007 and summer 2008 should be emphasised, with high *CL* score (3 to 4).

The variation of toxicity episodes of wedge clam (Comporta) and surf clam (Aguda) was in line with the observations in the estuarine areas, although of shorter duration. This shorter period cannot be associated with poor sampling representativeness, since high *CL* was obtained in autumn and winter. Toxicity of cockles (Formosa), wedge clam and surf clam (Culatra) were above the regulatory limit in August 2009, and toxin concentrations were generally below the limit of detection between 2005 and 2008.

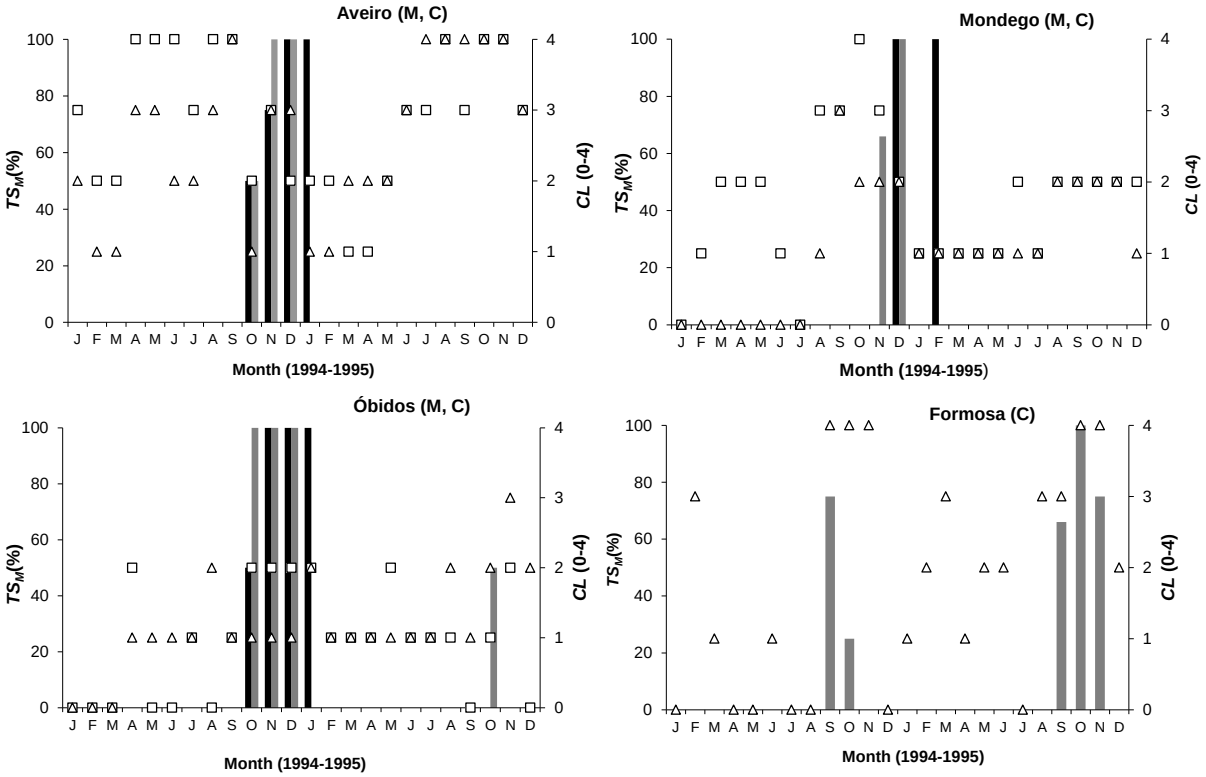

Figure 6.4. Monthly proportion of elevated toxicity samples (black and grey bars, TS_M) and confidence levels (squares and triangles, *CL*); mussels (M) and cockles (C) from Aveiro, Mondego and Óbidos; cockles from Formosa; surf clams (SC) from Aguda and Culatra; wedge clams (WeC) from Comporta and Culatra; period: 1994-1995; black bars and squares represent mussels and surf clams; grey bars and triangles represent cockles and wedge clams.

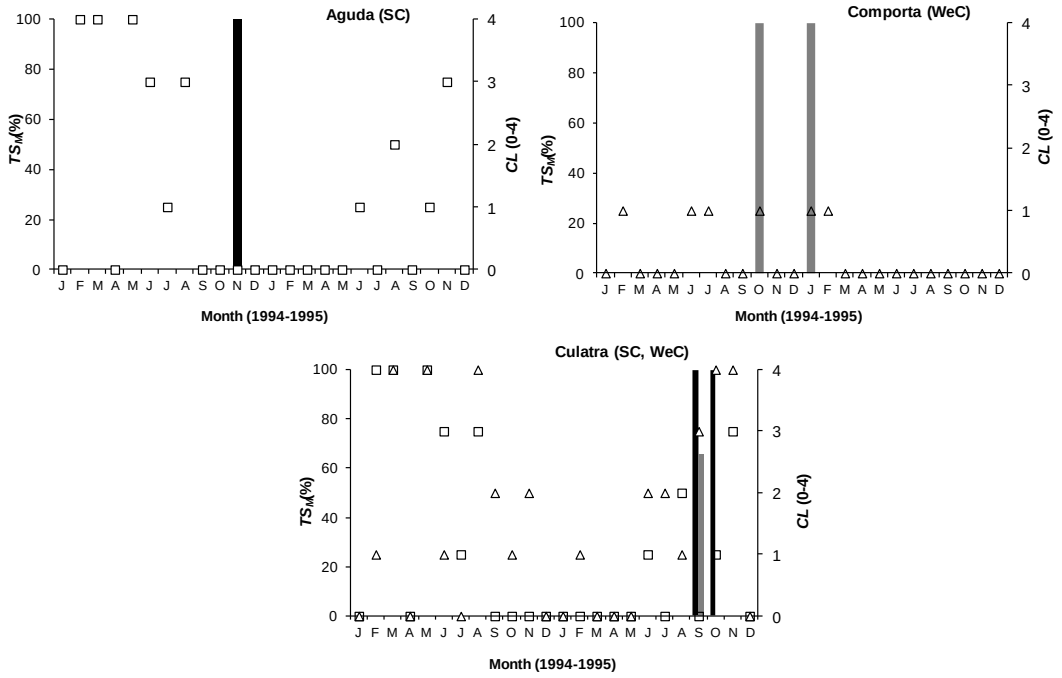

Figure 6.4. (cont.)

6.3.4. Molar proportion of the toxins C1+2 and B1 in bivalves

The toxins C1+2 and B1 are the major components of the toxin profile of *G. catenatum*, the current algae responsible for PST in the Portuguese coastal areas (Sousa et al., 1995; Negri et al., 2007; Botelho et al., 2012). To search whether toxins in bivalves collected in the BMP mirror the composition of that algal species, the molar proportion of C1+2 plus B1 (N-sulfocarbamoyl toxins) relative to the total quantified PSTs was calculated (in %) for mussels, cockles, surf clams and wedge clams from selected areas, between 2007 and 2011.

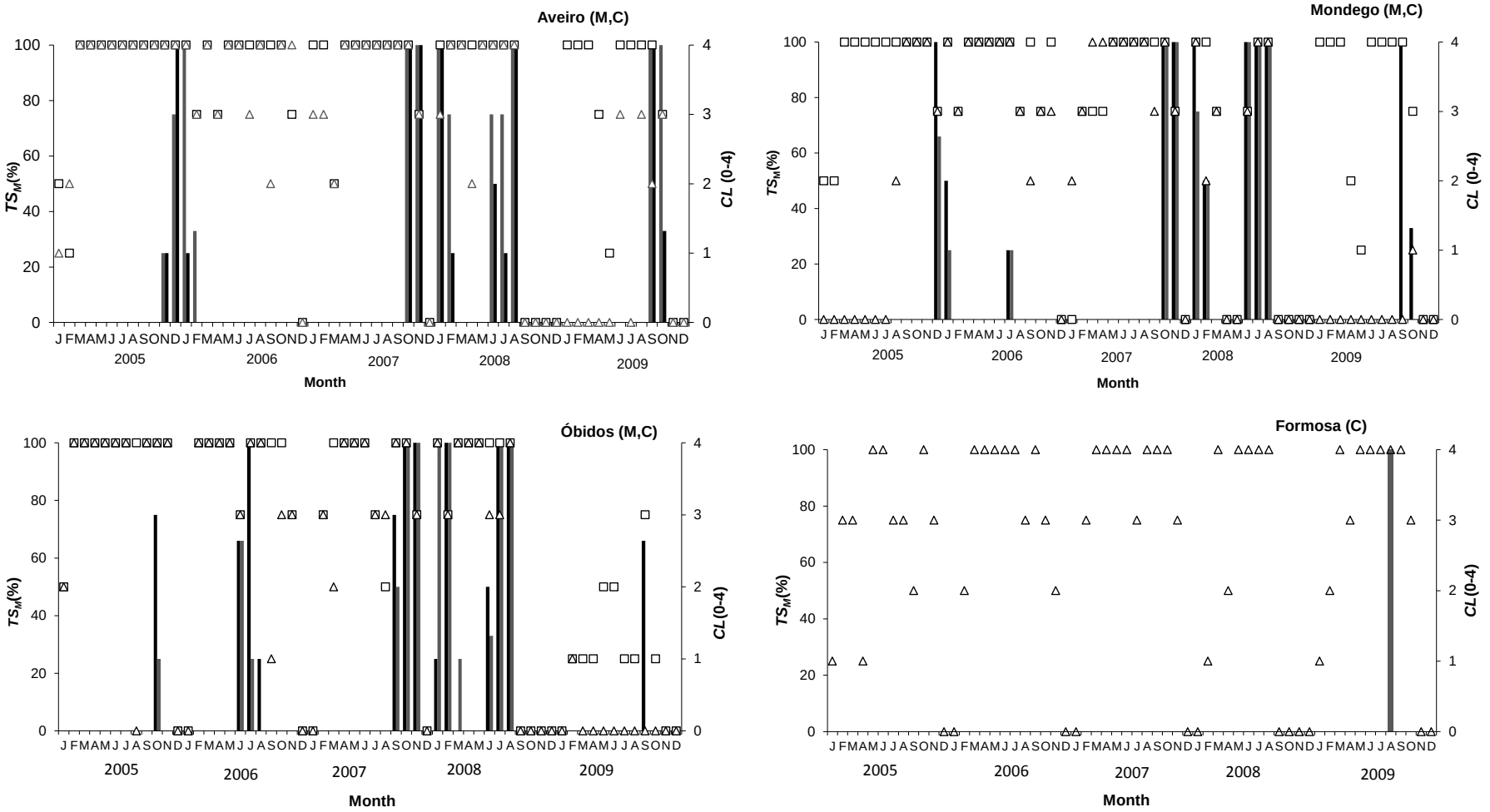

Figure 6.5. Monthly proportion of elevated toxicity samples (black and grey bars, TS_M) and confidence levels (squares and triangles, *CL*); mussels (M) and cockles (C) from Aveiro, Mondego and Óbidos; cockles from Formosa; surf clams (SC) from Aguda and Culatra; wedge clams (WeC) from Comporta and Culatra; period: 2005-2009; black bars and squares represent mussels and surf clams; grey bars and triangles represent cockles and wedge clams.

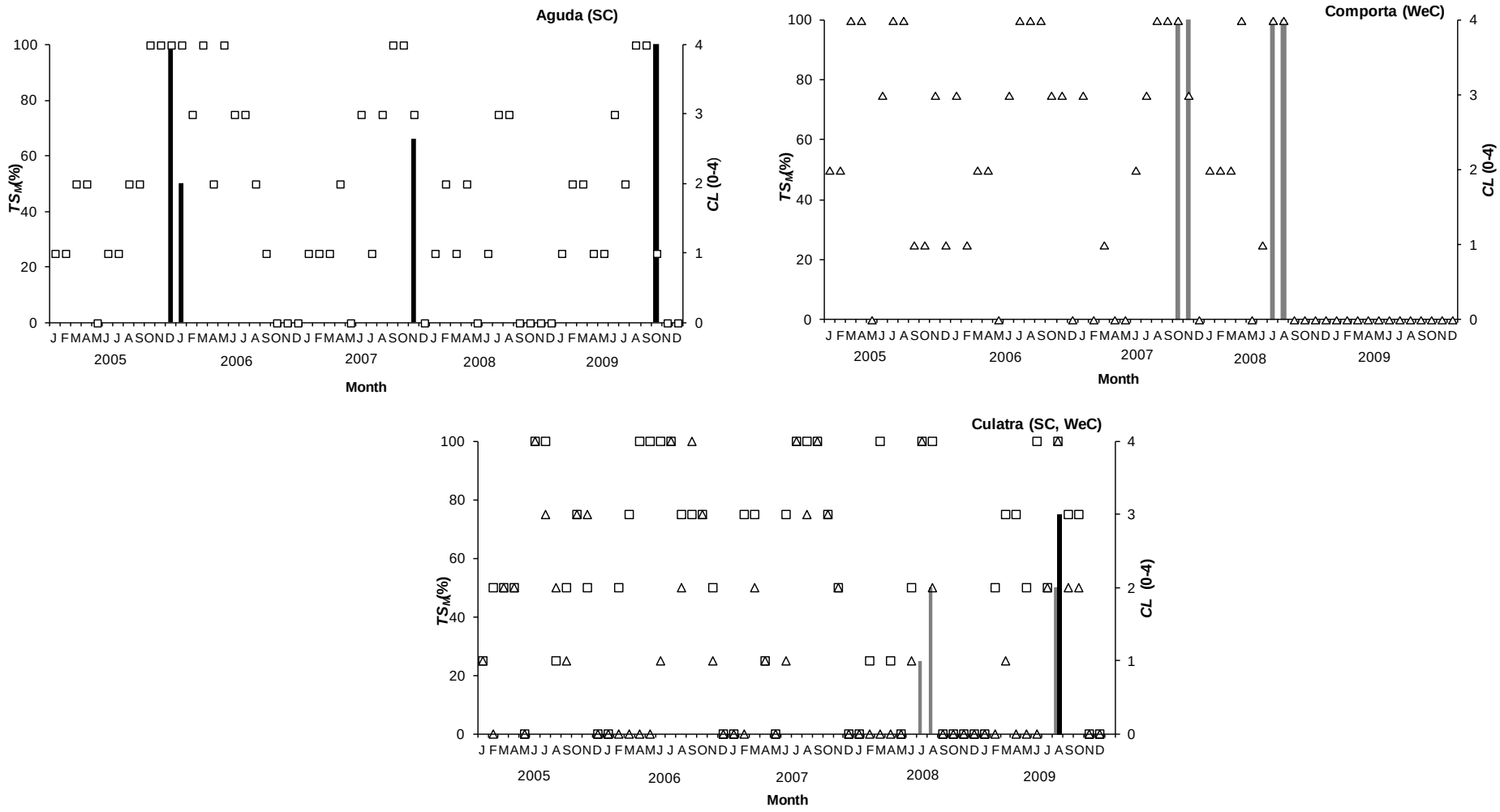

Figure 6.5.(cont.)

Figure 6.6 shows the N-sulfocarbamoyl proportion for mussels and cockles from Aveiro, Mondego, Óbidos and Formosa, and for wedge clams from Comporta and Culatra. These values were calculated for samples that displayed toxicity above the regulatory limit. Significant differences among the surveyed years are marked by asterisks. Considering the year 2008, medians of N-sulfocarbamoyl proportions in mussels and cockles from Óbidos (72 and 65%, respectively) were significantly ($p<0.05$) lower than from Aveiro (76 and 72%) and Mondego (78 and 73%). Statistical differences were also registered for cockles between Óbidos (60%) and Aveiro (71%) in 2007, but no significant differences among those areas were found in the other years. Proportions in cockles from Formosa (61-66%) were significantly lower than from Aveiro and Mondego (71-78%). The 25^{th}-75^{th} percentile interval of C1+2 and B1 in wedge clams from Comporta and Culatra was 71-78% (Figure 6.6). The concentrations of C1+2 and B1 in surf clams were below limits of detection, the N-sulfocarbamoyl values differing substantially from wedge clams, mussels and cockles that accounted for up to approximately 80% of the quantified PSTs (not shown in Figure 6.6).

6.4. Discussion

This work examines bivalve toxicity by PSTs along 18 years in Portuguese transitional and coastal waters. Toxin concentrations were obtained from the biotoxin monitoring programme database over that period using three methodologies: mouse bioassay (AOAC, 1990) between 1994 and 2001, chemical determination described in Vale and Sampayo (2001) from 2002 to 2007, and the chemical method recommended in the European Regulations (Anon., 2005b) until 2012. After adopting chemical determinations as routine procedure in the programme, toxicity values were estimated by multiplying individual toxins quantified in the whole bivalve tissues by the corresponding toxicity factor (Oshima, 1995a; EFSA, 2009). Depending on the bivalve species, toxicity values above the regulatory limit continue for days or weeks after the algal bloom (Bricelj and Shumway, 1998; Vale et al., 2008). These situations tend to prevail when elimination rates of ingested toxins are slow. Furthermore, the transformation of N-sulfocarbomoyl compounds in decarbomoyl analogues may represent enhanced toxicity since these compounds have higher toxicity factors (Artigas et al., 2007; Botelho et al., 2012). Bivalve toxicity values reported in this study thus reflect the balance between toxin uptake by filtration of *Gymnodinium catenatum* cells and elimination of the

ingested toxins or metabolic inter-conversion of toxins (Shimizu and Yoshioka, 1981; Oshima, 1995b; Bricelj and Shumway, 1998).

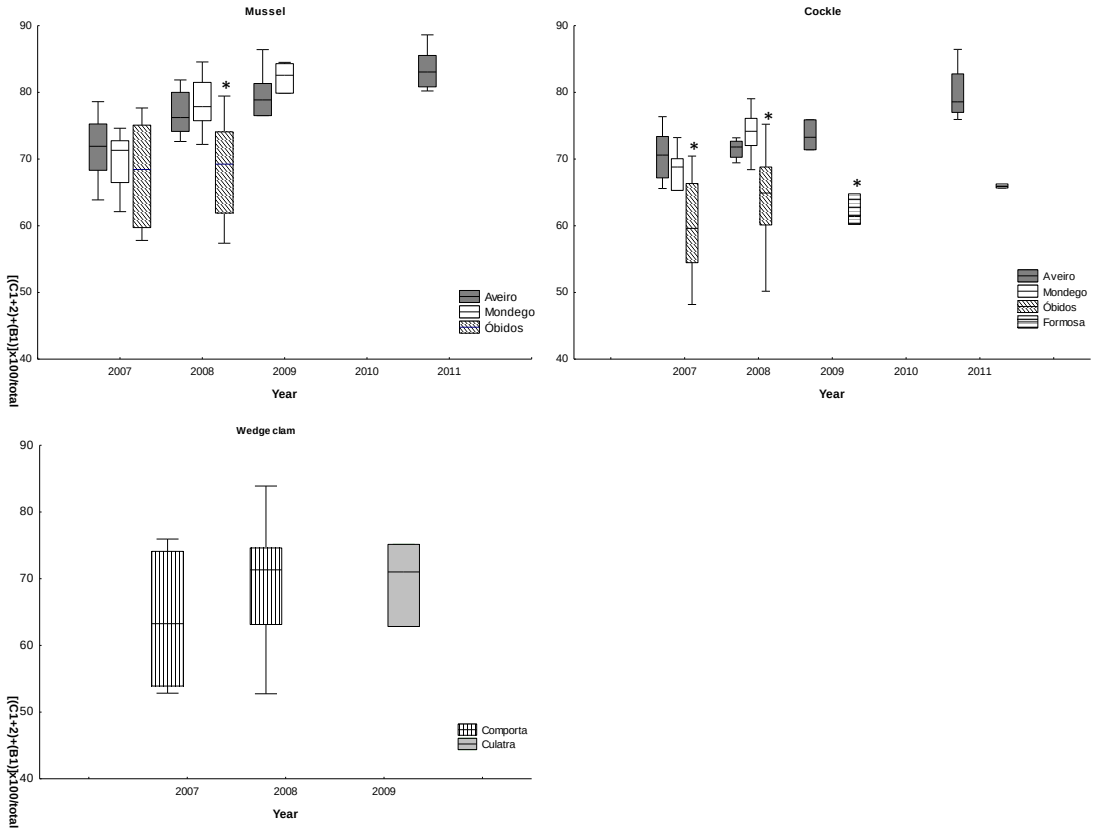

Figure 6.6. Median, 25th and 75th percentile, minimum and maximum molar proportions of (C1+2)+B1 to the total quantified PSTs (%), between 2007 and 2011; proportions calculated to elevated toxicity samples; mussels and cockles from Aveiro, Mondego, Óbidos; cockles from Formosa; wedge clams from Comporta and Culatra; n varied from 3 (wedge clams, Culatra, 2009) to 23 (mussels, Aveiro, 2008); * p<0.05.

6.4.1. Uptake and biotransformation/elimination of toxins

The PST episodes were more recurrent in the west coast of Portugal, where blooms of *G. catenatum* have been reported (Moita et al., 1998, 2003; Vale et al., 2008). The elevated proportion of C1+2 plus B1 in mussels, cockles and wedge clams is in line with the predominance of these toxins in *G. catenatum* cells isolated from blooms occurring on the Portuguese coast (Sousa et al., 1995; Negri et al., 2001; Botelho et al., 2012). The toxin profiles therefore point to the high influence of the uptake phase when abundant *G. catenatum* cells are present in coastal and estuarine waters.

The most favourable conditions to trigger *G. catenatum* blooms in coastal waters have been described in various works for the geographic regions where blooms of this species have been

reported (Fraga et al., 1988; Hallegraeff et al., 1995; Band-Schmidt el al., 2010; Bravo et al., 2010). The review by Hallegraeff et al. (2012) points to the existence of two possible mechanisms for the bloom dynamics of *G. catenatum*: autochthonous bloom formation by local cyst beds, as occurs in Tasmanian estuaries; and allochthonous bloom build-up during upwelling relaxation, as reported in Iberian coast and Spanish Rias. In the western coast of Portugal, the record of *G. catenatum* blooms have been associated with conditions under relaxation of coastal upwelling and slow currents or eddies where this species is retained (Moita et al., 2003; Pitcher et al., 2010). The coastal oceanography of the west Iberian Peninsula is characterised by seasonal wind-driven coastal upwelling, which can be considered to be a northern extension of the NW Africa upwelling system (Wooster et al., 1976). A strong high pressure system over the North Atlantic in summer causes alongshore northerly winds off Portugal and Galicia, which force upwelling events from April through September (Hallegraeff et al., 2012).

Toxicity episodes registered in surf clams from Aguda (NW coast) resulted from exposure to *G. catenatum* blooms, although C1+2 and B1 concentrations, which are dominant in the algal cells, were below the limit of detection in surf clams. The explanation for those low concentrations was given by experiments *in vitro* that showed the transformation of C1+2 and B1 into their decarbamoyl analogues in a few hours (Artigas et al., 2007). Toxicity of surf clams expressed thus mainly the toxicity of decarbamoyl toxins, either present at low proportion in the ingested cells of *G. catenatum* and mainly resulted from inter-conversion after uptake. Surf clam from the south-eastern coast (Culatra) exhibited shorter and less frequent toxicity episodes than the western areas, which is in line with the weak influence of upwelling, caused by occasional westerly winds (Fiúza, 1983).

6.4.2. Import of *G. catenatum* cells to estuaries

Toxin concentrations in mussels and cockles from Aveiro, Mondego and Óbidos (Figure 6.1) seem to be mainly affected by the coastal algal blooms. Most likely, the toxic cells generated offshore that are displaced towards the coast tend to be tidally advected into those estuarine systems (Fraga et al., 1988). The import of toxic cells and their maintenance or development under high salinity conditions is facilitated by the elevated tidal influence (mesotidal systems) and low freshwater inputs (Bettencourt et al., 2004). Consequently, wild mussels from the inlet channel and cockles from inner areas are quickly exposed to the toxin-producing species imported from adjacent coastal waters. The

possibility of organic nutrients available in estuarine environments promoting the increase of toxic cells should not be excluded, since *G. catenatum* exhibits the capacity to utilize organic forms of nutrients (Doblin et al., 1999; Oh et al., 2002; Yamamoto et al., 2004). This hypothesis has been considered for Óbidos lagoon to explain the longer duration of mussel toxicity episodes with respect to the adjacent coastal waters (Pereira et al., 2011). Despite the high water volume exchanged daily between Formosa and the adjacent sea (Falcão and Vale, 2003), the toxicity episodes in this lagoon were rarer, in line with the sporadic *G. catenatum* blooms in the adjacent coastal zone.

The toxicity episodes in the three western estuarine systems were documented through toxin composition of the mussel *Mytilus* spp. or the cockle *Cerastoderma edule*. By using the same bivalve species, biological factors such as filtration rate and metabolism associated with elimination and biotransformation of the assimilated toxins are likely to have a minor influence on the assessment of toxicity episode duration. The longer episodes registered for mussels in comparison to cockles are in line with the previous review of toxicity episodes in Aveiro and Óbidos (Vale et al., 2008). Those differences may be related to feeding rates of bivalve species (Bricelj and Shumway, 1998; Ward et al., 2013). The duration of toxicity episodes varied among the surveyed estuarine systems, which most probably reflects the quantity of toxic cells in each zone. The availability of those cells may be influenced by water circulation and residence time inside the systems. Shorter toxicity periods registered in the Mondego (tubular estuary) in comparison to Aveiro and Óbidos (coastal lagoons) are probably related to those morphological characteristics. Import of toxic cells from the adjacent sea between October and January tends to be slow in the Mondego, due to the increase of river discharges in this season. This effect is of less impact in Óbidos and Aveiro since these systems are broader and receiving lower freshwater inputs (Bettencourt et al., 2004).

6.4.3. Unpredictability of bivalve toxicity episodes on decadal scale

A salient aspect of this study is the prolonged period (1996-2004) of low bivalve toxicity by PSTs. The reviews of Sampayo et al. (1997) and Vale et al. (2008) have already mentioned the low toxicity for this period of time. The lack of toxicity episodes in estuarine systems cannot be attributed to poor sampling representativeness, since high confidence levels were often observed during this period (Figure 6.2). Toxin concentrations in the whole tissue of mussels, cockles, surf clams and wedge clams were often undetected (bioassay) or below the limit of detection, suggesting that *G. catenatum* did

not bloom or if cells flourished offshore they were not transported to the surveyed coastal areas (Figueiras et al., 1994; Pitcher and Boyd, 1996). This observation is in line with previous works pointing to massive *G. catenatum* blooms off the western Portuguese coast in 1976, 1985, 1994 and 1995, contrasting to lack of blooms for the years in between (Estrada et al., 1995; Moita et al., 2003; Amorim et al., 2004). In the southern coastal waters, *G. catenatum* cells were not observed in 2006 and 2007, but abundances increased occasionally in 2008 (Brito et al., 2012). Those studies on algal dynamics suggest the interruption of *G. catenatum* blooms on an inter-annual scale. The bivalve toxicity data between 1994 and 2012 is in line with that irregular occurrence.

The prolonged episodes of toxicity during 2007/2008 in Aveiro, Mondego, Óbidos (estuarine systems) and Comporta (coastal areas) are also of interest. Because the toxicity was concomitantly registered in the three estuarine systems along 160 km of coastline, it is tempting to admit that those episodes resulted from the import of toxic cells from the coastal area. The sedimentary record of *G. catenatum* cysts in the Iberian coast is consistent with the evidence of non-periodic low-density blooms (Amorim and Dale, 2006; Smayda and Trainer, 2010). Inter-annual variation of *G. catenatum* blooms have also been reported in other coastal systems such as Galicia, Spain (Estrada, 1995) and Mexico (Mee et al., 1986; Cortés Altamirano et al., 1996). Explanation for this puzzling inter-annual variation has not been provided so far. Mismatches between observed and expected *in situ* bloom behaviour of *G. catenatum* suggest that unrecognized upwelling systems factors that fall within the physical-chemical-biological domain are more important than life cycle strategies (Bravo et al., 2010; Smayda and Trainer, 2010).

6.4.4. Seasonality of toxicity episodes

The toxicity episodes in the western coast of Portugal were frequently manifested during autumn and early winter (Figure 6.4). The signal of these episodes remained for three to four weeks per month in autumn 2007 and winter 2008. Moreover, mussel and cockle toxicities exceeded the regulatory limit in June-July of 2008, providing an additional signal of toxicity. Along the 18 years of survey, the summer toxicity episodes were much less regular than in the autumn. The seasonal occurrence is related to *G. catenatum* blooms generated in the Iberian coast (Estrada et al., 1995; Sousa et al., 1995; Vale et al., 2008), triggered by upwelling events from April through September. Commonly *G. catenatum* blooms have been observed by the end of summer when

upwelling-favourable winds relax (Fraga, 1996; Hallegraeff et al., 2012). Lack of regularity in the appearance of the toxicity episodes has been observed in other geographical regions. For example, most blooms in Mexico were reported in March-April, but occasionally in other periods of the year (Band-Schimidt et al., 2010). The analysis of the PST data in Portugal indicates that the autumn signal may occasionally appear in summer.

6.4.5. Connectivity of PST episodes

Toxin profiles in bivalves exposed to blooms of *G. catenatum* may vary with the time, as differential elimination of individual compounds occurs or assimilated toxins are inter-converted (Samsur et al., 2007; Botelho et al., 2012). This work examined only the toxin profile in periods of elevated toxicity. Mussels and cockles from Aveiro, Mondego and Óbidos showed toxin profiles dominated by N-sulfocarbamoyl toxins, and therefore toxicity values approximately express the individual toxins ingested with the toxic cells. In contrast, surf clams showed profiles dominated by decarbamoyl toxins resulted from biotransformation of N-sulfocarbamoyl toxins associated with the ingested cells of *G. catenatum* (Artigas et al., 2007). Consequently, toxicity values of surf clams are based on the concentration and toxicity factors of the transformed toxins. Despite bivalve specificity, possible interconnection of toxicity episodes among the surveyed areas were searched through the connectivity index (*CI*) defined in the section 6.2.2. The interconnections were searched for the periods 1994-1995 and 2005-2009 for the following possibilities (Figure 6.7): Aveiro-Mondego-Óbidos (using both mussel and cockle), Aguda-Aveiro (surf clam *versus* mussel) and Culatra-Formosa (wedge clam *versus* cockle).

The scores of *CI* for Aveiro-Mondego-Óbidos (mussel) reached 3 to 4 in December 1994 and between October 2007 and August 2008, while mean values in the remaining years were below 1. The short distance between Aveiro-Mondego and Mondego-Óbidos (approximately 85 km) facilitates the quasi-simultaneous occurrence of toxicity episodes among these systems. The connectivity was evident in 2007-2008, when elevated toxicity persisted for a long period. These results reinforce the hypothesis of the import of *G. catenatum* cells from coastal areas to nearby estuarine systems. Mussels from inlet channels showed higher CI than cockles from the inner branches, which may be related to the proximity of sampling location to the sea, or to elevated feeding rate of mussels (Bricelj and Shumway, 1998). Possible effects of nutrient composition on cell proliferation in each system

were not evident from the connectivity index. However, the N-sulfocarbamoyl proportions calculated for mussels from Óbidos showed broader ranges than from Aveiro and Mondego. Furthermore, cockles from Óbidos and Formosa showed lower median values than from those two estuarine systems. The hypothesis of higher interconversion of assimilated toxins by *C. edule* in Óbidos and Formosa than in Mondego and Aveiro is less plausible, since the same was observed for *C. edule* from the same biogeographical area. Differences attributed to sampling are also unlikely, since confidence levels in those estuarine systems were 3 to 4 (Figure 6.5). The possibility of the toxin profile of *G. catenatum* differing among these systems, in response to specific environmental conditions such as different nutrient composition, should therefore not be excluded. In fact, laboratory studies showed that nitrogen and phosphorus availability and molar ratio may change PSTs profile in *A. fundyense*, and *G. catenatum* strains showed modifications of PSTs molar ratios growing under different culture media (Poulton et al., 2005; Band-Schmidt et al., 2005). Elevated concentrations of organic forms of nitrogen in Óbidos (Pereira et al., 2011) and Formosa (Falcão and Vale, 2003) allow hypothesising the production of different toxins by *G. catenatum* in these coastal lagoons.

Scores of CI for Aguda-Aveiro (surf clam versus mussel) reached 3-4 in November 1994, December 2005, January 2006, November 2007 and October 2009. By contrast, mean values were below 1 in other years. These elevated scores are based on the comparison between toxicity of N-sulfocarbamoyl toxins in mussels and toxicity of decarbamoyl analogues. Toxicity episodes in Culatra-Formosa (wedge clam versus cockle) were scarce, CI being 3-4 only in September, October and November 1995, and August 2009. Scores were also calculated on the basis of slightly different toxin profiles (Figure 6.6). Lower values of the N-sulfocarbamoyl ratio means smaller contribution of C1+2 and B1 to the toxin composition, which may be interpreted as toxin profile of wedge clam during toxicity episodes having a higher influence of transformation or elimination.

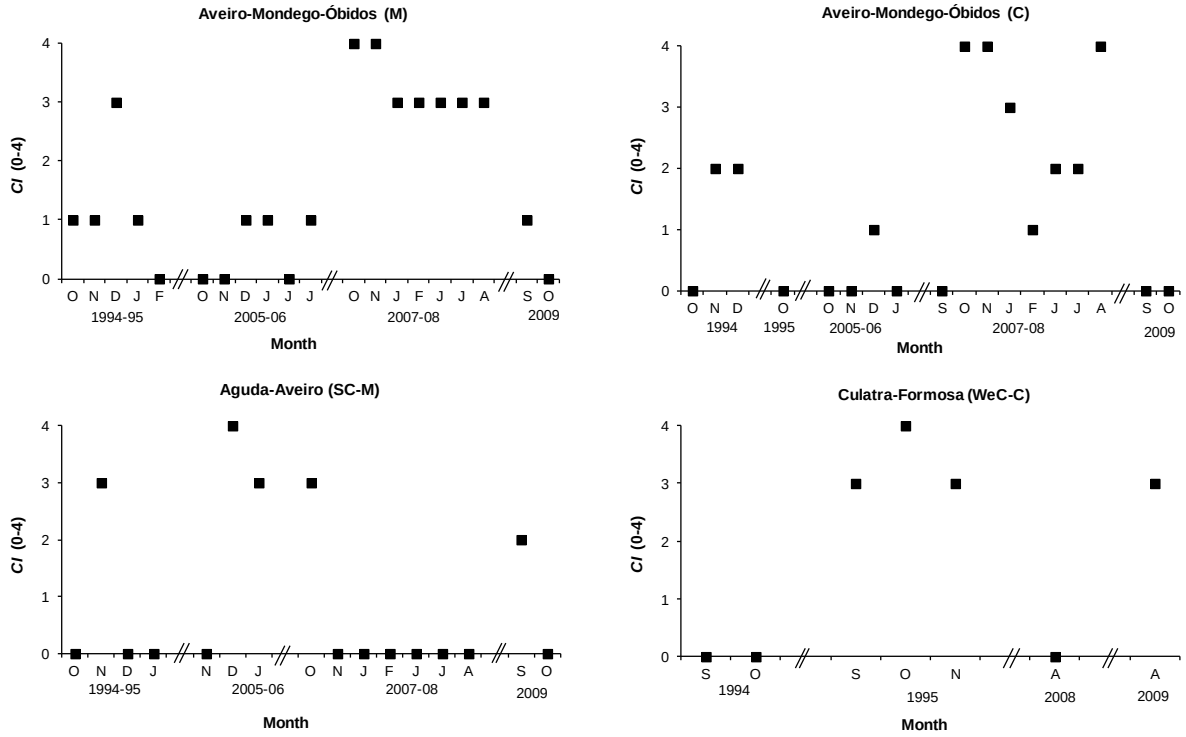

Figure 6.7. Connectivity Index (*CI*) for Aveiro-Mondego-Óbidos (mussel and cockle), Aguda-Aveiro (surf clam *versus* mussel) and Culatra-Formosa (cockle *versus* wedge clam), for the periods 1994-1995 and 2005-2009; *CI* scored from 0 to 4 based on the number of weeks per month with toxicity values above regulatory limit.

6.5. Conclusions

The analysis of toxicity by PSTs in bivalves from transitional and coastal waters of Portugal over 18 years pointed to an irregular multi-annual variation of toxic episodes. The elevated toxicity values displayed a seasonal signal with a peak in autumn/winter and occasionally in summer. The episodes of mussel and cockle toxicities were connected in three estuarine systems of the western coast, reflecting the occurrence and spatial distribution of *G. catenatum* blooms in the adjacent coastal waters. Connectivity was also found between cockles from the estuarine systems and wedge clams from the adjacent south coast, despite the different toxin profiles resulting from differential biotransformation or elimination of ingested toxins.

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7

7. Profiles of paralytic shellfish toxins in bivalves of low and elevated toxicities following exposure to *Gymnodinium catenatum* blooms in Portuguese estuarine and coastal waters

Highlights

- PST profiles in four bivalve species from Portuguese waters were examined.
- Toxin profiles varied between low and elevated bivalve toxicities in the period 2007-2012.
- Molar ratios R1=(C1+2):B1 were higher in elevated than in low cockle toxicities.
- R2=[(dcSTX)+(dcGTX2+3)]:[(C1+2)+(B1)] were higher in low wedge clam toxicities than in mussels and cockles.
- Changes in R2 are presumably due to biotransformation after exposure to bloom.

Botelho, M.J., Vale, C., Ferreira, J.G. (submitted). Profiles of paralytic shellfish toxins in bivalves of low and elevated toxicities following exposure to *Gymnodinium catenatum* blooms in Portuguese estuarine and coastal waters. Chemosphere.

Abstract

Profiles of paralytic shellfish toxins (PSTs) were examined in 405 composite samples of *Mytilus* spp., *Cerastoderma edule*, *Donax trunculus* and *Spisula solida* collected between 2007 and 2012 from natural production areas in two estuaries (Aveiro and Mondego), two coastal lagoons (Óbidos and Formosa), and three open coastal areas (Aguda, Comporta and Culatra). Toxin concentrations were obtained from the biotoxin monitoring programme database. Episodes of PST toxicity in Portugal have been associated with *Gymnodinium catenatum* blooms. Toxin profiles for each species showed no trend over the surveyed years. In general, profiles differ only slightly among areas, except for Óbidos. However, toxin profiles in bivalves varied between low and elevated toxicities, corresponding to below and above the PST regulatory limit, respectively. The ratio R1=(C1+2):B1, which were the main toxins produced by *G. catenatum* cells, decreased considerably between elevated and low toxicity cockles, indicating the elimination of C1+2 or conversion of compounds into B1. R2=[(dcSTX)+(dcGTX2+3)]:[(C1+2)+(B1)], which represents the ratio of minor to major toxins in *G. catenatum* cells, increased substantially in wedge clams (*Donax trunculus*) of low toxicity and less markedly in cockles (*Cerastoderma edule*) and mussels (*Mytilus* spp.). These differences are interpreted as the predominance of a biotransformation phase after exposure to the algal bloom. The toxin profile of surf clams (*Spisula solida*) was dominated by decarbamoyl compounds, reflecting intense biotransformation during exposure to blooms. The ratio R2 in low toxicity samples suggest that elimination of the produced decarbamoyl toxins is slower than biotransformation.

Keywords: Paralytic shellfish poisoning; *Gymnodinium catenatum;* Bivalves; Toxin profiles; Biotransformation

7.1. Introduction

Paralytic shellfish toxins (PSTs) comprise a suite of more than 30 natural tetrahydropurine derivatives produced in the marine environment by toxic phytoplankton such as *Gymnodinium catenatum*, *Alexandrium* spp. and *Pyrodinium bahamense* (Wise et al., 2010). During blooms of these species, bivalve filter feeders such as mussels, cockles, and clams ingest toxic cells and tend to exhibit transient high toxin concentrations. PSTs are of great concern as they are potent neurotoxins that block the voltage-gated sodium channels in excitable cells, suppressing ion permeation (Kao, 1966). This action has long been documented as a potential cause of serious illness in consumers of contaminated bivalves (Sommer and Meyers, 1937). The better-known toxins are included in the following groups in decreasing order of toxicity: carbamate (saxitoxin-STX, neosaxitoxin-NEO and gonyautoxins-GTX1 to GTX4), decarbamoyl (dcGTX1 to dcGTX4, dcSTX and dcNEO) and N-sulforcarbamoyl (B1, B2, C1 to C4) (Oshima, 1995a; EFSA, 2009). Other compounds with substituent side chains such as hydroxyl, hydroxybenzoate, or acetate have been identified and structurally described (Wise et al., 2010).

Studies of PSTs in dinoflagellate cells collected in different regions of the world have pointed to strain-specific profiles (Oshima et al., 1993; Franco et al., 1994; Krock et al., 2007). However, stresses such as alterations in physico-chemical properties and physiology may change toxin profiles of dinoflagellate cells (Etheridge and Roesler, 2005; Poulton et al., 2005; Band-Schmidt et al., 2010). When bivalves ingest toxic algae the accumulated toxins are selectively metabolized and eliminated. The toxin profile in each bivalve species thus varies with time and differs progressively from the algae from which the toxins orginate (Oshima et al., 1990; Samsur et al., 2006). For example, mussels and oysters mirror the toxin composition of the causative plankton cells better than clams, which exhibit different proportions of ingested toxins (Bricelj and Shumway, 1998). Specific toxic profiles of bivalves results mainly from different uptake, elimination/retention or epimerization mechanisms (Cembella and Shumway, 1995; Yu

et al., 2007; Botelho et al., 2010a). In addition, the occurrence of hydrolysis, chemical and/or enzymatic transformation pathways (Shimizu and Yoshioka, 1981; Oshima, 1995a), as well as bacterial degradation processes (Donovan et al., 2009) may also induce specific PST profiles.

Although toxin concentrations are routinely measured in bivalves of toxicity below the PST regulatory limit in national monitoring programmes, comparison of toxin profiles for different toxicity values are scarce. Turner et al. (2014) reported the variability of occurrence of PSTs in bivalves from the United Kingdom and found no correlation between profiles and total PST content of shellfish. The present work compares PST profiles in bivalve molluscs from Portugal of toxicity below and above the PST regulatory limit caused by *G. catenatum* blooms. Differences were tested for 405 composite samples of *Mytilus* spp., *Cerastoderma edule*, *Donax trunculus* and *Spisula solida* from seven harvesting areas between 2007 and 2012.

7.2. Material and Methods

7.2.1. Bivalve toxicity database

Values of bivalve toxicity by PSTs used in the present study were obtained from the database of the Portuguese biotoxin monitoring programme (BMP). Between 2007 and 2012, toxicity values were obtained through chemical determinations, although two analytical methodologies were used sequentially in this period of time: from 2007 to 2008, PST concentrations were determined using a LC method with pre-column oxidation based on Lawrence et al. (1995) modified by Vale and Sampayo (2001); from 2009 to 2012, the toxins C1+2, B1, dcSTX, dcGTX2+3, GTX2+3 and STX were quantified using the official AOAC pre-column oxidation method by liquid chromatography with fluorescent detection (LC-FLD) (Anon., 2005b). In the first method, the concentrations of the so-called dcSTX, dcGTX2+3, GTX2+3 and STX were determined through the areas of chromatographic peaks that eventually included unresolved peaks, corresponding to other toxins like NEO and GTX1+4. To prevent misunderstanding, concentrations of those

toxins were designated by “dcSTX”, “dcGTX2+3”, “GTX2+3” and “STX”. Due to the use of different methodologies, molar proportions of C1+2, B1, dcSTX, dcGTX2+3, GTX2+3 and STX calculated in the period 2009-2012 (method A - AOAC, 2005) cannot be compared to values of 2007-2008 (method B - Vale and Sampayo, 2001).

Bivalve toxicity values were estimated in terms of µg STX di-HCl equivalents per kg, multiplying the toxin concentration by the toxicity equivalence factor (TEF) of each individual compound. After June 2010, toxicity values were estimated following the recommendations of the EU Reference Laboratory for Marine Biotoxins. This results in the duplication of the TEF used to dcSTX, from 0.5 (Oshima, 1995b) to 1 (EFSA, 2009). For the purpose of data consistency in this study, all toxicity values originally determined using Oshima TEF for dcSTX were re-calculated using EFSA. In the case of isomeric pairs as GTX2+3 and C1+2, the highest TEF was used for each pair. The regulatory limit (RL) for PSTs is 800 µg STX di-HCl equivalents per kg (Anon., 2004). Bivalve toxicity values were only reported in the BMP database if above 55 µg STX di-HCl equivalents per kg. This value was obtained from validation work. A detailed description of the BMP, location of the natural production areas of bivalves in Portugal, analytical methodologies used, and toxicity calculation are described in Vale and Sampayo (2001), Vale et al. (2008), and Botelho et al. (2010b).

7.2.2. Selection of bivalve species and harvesting areas

Four bivalve species were used in this study, taking into account their geographic distribution, abundance and commercial value. The mussel *Mytilus* spp. and cockle *Cerastoderma edule* were considered due to their abundance in the areas of Aveiro, Mondego and Óbidos. Cockles were also collected in the Formosa lagoon. Mussels were sampled in hard substrates of the inlet channels, and cockles in sandy areas of inner zones. The wedge clam *Donax trunculus* and the surf clam *Spisula solida* were chosen because they are commercially harvested from natural production areas in the west coast (Aguda and Comporta) and south coast (Culatra) (Figure 7.1). Habitats and ecology

are described in Gaspar et al. (1999) and Joaquim et al. (2008).

Coordinates of the harvested areas have changed slightly during the multi-annual surveyed period, as the abundance of bivalves varied with time (Rufino et al., 2008). The three systems Aveiro, Mondego and Óbidos are located in the central west coast, and Formosa in the southeastern area. In addition, there are morphological and hydrological differences among these systems. Whereas the Mondego is a tubular estuary, Aveiro, Óbidos and Formosa have broad inner areas and narrow connections to the sea, are generally classified as lagoons (Bettencourt et al., 2004). The western systems (Aveiro, Mondego and Óbidos) are characterised by recurrent episodes of bivalve toxicity by PST (Vale et al., 2008). The Formosa coastal lagoon was selected due to the large quantities of bivalves grown in the tidal flats (Ferreira et al., 2014).

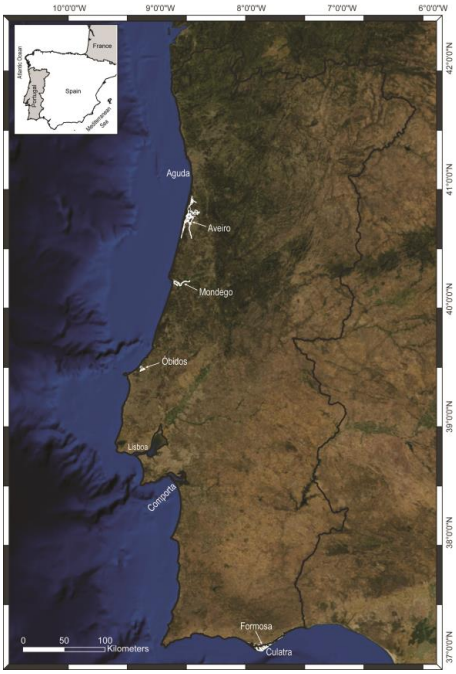

Figure 7.1. Harvesting areas of mollusc bivalves: Aveiro, Mondego, Óbidos, and Formosa (estuarine systems), and Aguda, Comporta and Culatra (open coastal areas).

7.2.3. Number of samples and bivalve toxicity values

Table 7.1 gives the number of samples of surf clams, wedge clams, cockles and mussels used in this work. This study is based on toxin concentrations of 405 composite

samples of bivalves (each one a pool of 30 specimens) obtained between 2007 and 2012 in seven harvest areas. The number of annual samples with toxicity below and above the PST regulatory limit, as well as the corresponding harvest area, are presented. Samples below the regulatory limit showed a total toxicity between 55 and 800 µg STX di-HCl equivalents per kg, while samples above this limit presented a broad variation of toxicity values for the various blooms and areas surveyed. Table 7.2 shows the median, minimum and maximum of PST toxicity ratios (total toxicity value/regulatory limit for PSTs) of surf clams, wedge clams, cockles and mussels collected between 2007 and 2012 in the corresponding harvest areas.

Table 7.1. Annual number of samples, between 2007 and 2012, with toxicity values below and above the regulatory limit for PSTs; surf clam (Aguda-AG, Comporta-CO and Culatra-CU), wedge clam (Comporta-CO and Culatra-CU), cockle (Aveiro-AV, Mondego-MO, Óbidos-OB and Formosa-FO) and mussel (Aveiro-AV, Mondego-MO and Óbidos-OB); 2007-2008 - method B; 2009-2012 - method A.

Annual number of samples												
Toxicity below RL												
Species	Surf clam			Wedge clam		Cockle				Mussel		
Area	AG	CO	CU	CO	CU	AV	MO	OB	FO	AV	MO	OB
2007	3	3	7	-	-	4	-	-	-	3	3	4
2008	-	-	-	9	3	20	-	-	-	18	6	-
2009	-	-	-	-	-	-	-	-	8	8	14	-
2010	-	-	-	-	-	62	3	-	3	21	3	-
2011	-	-	-	-	-	-	-	-	-	7	-	-
2012	-	-	-	-	3	-	-	-	-	-	-	-
Toxicity above RL												
Species	Surf clam			Wedge clam		Cockle				Mussel		
Area	AG	CO	CU	CO	CU	AV	MO	OB	FO	AV	MO	OB
2007	3	-	-	7	-	13	4	9	-	13	7	8
2008	-	7	-	9	-	7	8	19	-	17	13	14
2009	3	-	3	-	3	-	-	-	6	7	4	-
2012	-	-	3	-	3	-	-	-	-	-	-	-

7.2.4. Sampling of seston and determination of PSTs

In addition to bivalve toxicity data, samples of the toxin-producing algae were collected to assess the toxin profile in the cells ingested by bivalves. Between 5 to 10 litres of surface water samples were collected during a bloom of *Gymnodium catenatum*, from 23th June to 21th July 2008, in coastal waters adjacent to Óbidos (9 samples). Water samples for phytoplankton species identification were preserved in Lugol's iodine and the cells of *G. catenatum* identified under an inverted microscope after sedimentation. Water samples for toxin analysis passed through a GF/C glass filters (porosity 1.2 µm, 150 mm Φ), under light vacuum pressure (100 mmHg), and the material retained in the filters were frozen in 0.1 M acetic acid at -80^{o}C until analysis.

Toxins were extracted from the seston retained on filters by freeze/thaw cycle, followed by probe sonification in an ice bath for 30 s at 60% amplitude and 20 W (Vibra Cell, Sonics & Materials Inc.) (Botelho et al., 2012). Cell debris after probe sonification was examined under an inverted microscope and revealed full disruption of algal cells in selected samples. The pH of the extracts was adjusted to 6.5 with 0.2 M NaOH. The extracts were filtered (0.2 µm) and diluted to exactly 1 mL.

Table 7.2. Median and the interval of minimum and maximum of PST toxicity ratios (total toxicity value/regulatory limit) between 2007 and 2012; surf clam (Aguda-AG, Comporta-CO and Culatra-CU), wedge clam (Comporta-CO and Culatra-CU), cockle (Aveiro-AV, Mondego-MO, Óbidos-OB and Formosa-FO) and mussel (Aveiro-AV, Mondego-MO and Óbidos-OB); 2007-2008 - method B; 2009-2012 - method A.

Species	Area	Median toxicity ratio (minimum-maximum)			
		2007	2008	2009	2012
Surf clam	AG	1.7 (1.4-2.0)	-	2.0 (1.1-6.7)	-
	CO	-	6.0 (3.4-13)	-	-
	CU	-	--	3.1 (2.4-3.3)	2.3 (1.9-3.5)
Wedge clam	CO	23 (2.5-42)	7.0 (2.1-17)	-	-
	CU	-	-	2.9 (1.7-3.3)	3.6 (2.2-5.1)
Cockle	AV	9 (2.7-20)	3.9 (1.8-12)	-	-
	MO	22 (1.4-32)	3.4 (1.9-18)	-	-
	OB	8.9 (2.2-118)	2.8 (1.0-52)	-	-
	FO	-	-	1.9 (1.3-9.0)	-
Mussel	AV	23 (1.5-32)	5.0 (1.1-39)	11 (4.3-20)	-
	MO	11 (2.5-52)	5.0 (1.8-28)	1.5 (1.2-3.0)	-
	ÓB	40 (4.1-114)	33 (1.7-83)	-	-

The determination of PSTs was based on the AOAC pre-column oxidation method by LC-FLD (Anon., 2005b; Botelho et al., 2010b). Aliquots of seston extracts were used for oxidation of PSTs with peroxide and periodate oxidant prior to LC-FLD analyses. A similar procedure for both oxidations was followed, substituting the oxidant reagent by water in order to detect naturally fluorescent compounds. The quality control of the results was assured through the use of the certified reference materials C1&2, STX-e, dcSTX ,GTX5-b (B1), dcGTX2&3, dcNEO-b, GTX1&4-b, GTX2&3-b and NEO-b, from the Institute for Marine Biosciences, National Research Council Canada. Evaluation of linear ranges for PSTs and instrumental limits of detection are described in Botelho et al. (2010a). Instrumental detection limits (nmol L^{-1}) were 3.9 (C1+2), 4.0 (B1), 4.0 (STX), 4.9 (dcSTX), 8.2 (dcGTX2+3), 8.5 (GTX2+3), 25 (dcNEO), 30 (GTX1+4) and 31 (NEO). The LC system consisted of a Hewlett-Packard/Agilent Model 1050 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. The PST oxidation products were separated using a reversed-phase Supelcosil LC-18, 150 x 4.6 mm id, 5 µm column (Supelco) equipped with a guard column Supelguard Supelcosil C18, 20 x 4.0 mm id, 5 µm (Supelco). The column was kept in an oven at 30^{o}C. The mobile phase gradient used 2 mobile phases: A (0.1 M ammonium formate, pH=6) and B (0.1 M ammonium formate in 5% acetonitrile, pH=6). The elution gradient consisted of 0-5% B in the first 5 min, 5-70% B in the next 4 min and back to 0% B in the next 5 min. Flow rate was 1 mL min^{-1} and the injection volumes were 50 µL and 100 µL, for the oxidation products of peroxide and periodate reaction, respectively. The excitation and emission wavelengths for fluorimetric detection were set at 340 nm and 395 nm, respectively.

7.2.5. Statistical analyses

Prior to statistical analyses, toxin molar proportions were tested for normality and equality of variances. The Mann-Whitney U test was used to evaluate the existing

differences between toxin molar proportions in bivalves from selected areas. The significant tests were performed using the STATISTICA 6.0 Statistical Software System.

7.3. Results

7.3.1. PST composition in bivalve species

Median, maximum, minimum, 75^{th} and 25^{th} percentiles of molar fractions of the quantified paralytic shellfish toxins relatively to the total quantified toxins in samples of surf clams, wedge clams, cockles and mussels that have toxicity values above the PST regulatory limit are shown in Figure 7.2. The proportion of each toxin was calculated for those bivalve species, encompassing data from different harvest areas (Table 7.1) for the two periods of time: 2007-2008 (method B) and 2009-2012 (method A). The most striking aspect is the negligible contributions of C1+2 and B1 (<0.7%) to the toxin profile of surf clams. These contributions contrast to the median proportions of C1+2 and B1 in wedge clams (10 and 63%), cockles (39 and 32%) and mussels (57 and 40%). The molar fractions of dcGTX2+3 and dcSTX were significantly ($p<0.05$) higher in surf clams than in the other bivalve species. The disparity between the two groups of compounds was found in data obtained by both methods (A and B), although with different meanings between dcGTX2+3, dcSTX and “dcGTX2+3”, “dcSTX” (Figures 7.2a and 7.2b). The compounds GTX2+3 and STX had minor contributions to the toxin profiles of all species. The proportion of C1+2 was significantly ($p<0.05$) lower in wedge clams than in cockles and mussels, and the opposite was observed for B1.

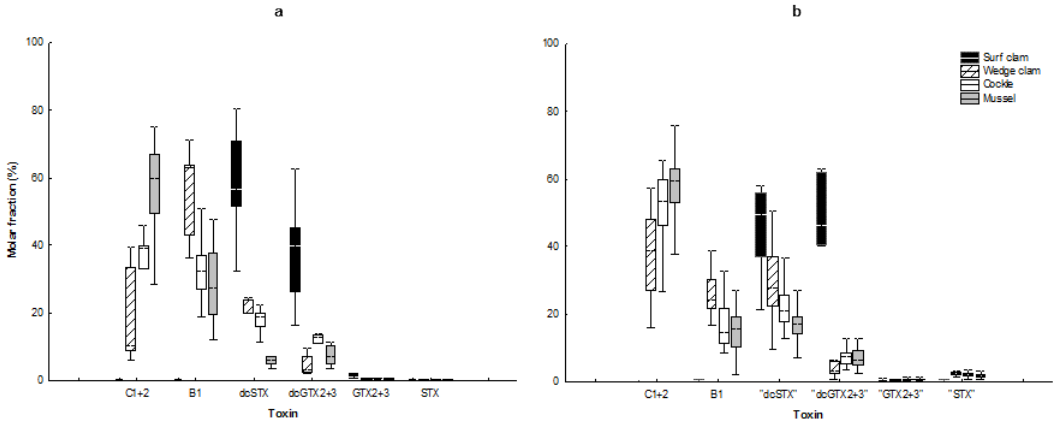

Figure 7.2. Median, maximum, minimum, 75th and 25th percentiles of molar fractions of quantified PSTs (%) in samples of surf clam, wedge clam, cockle and mussel presenting toxicity values above the PST regulatory limit; period of time: 2007 - 2012; methodologies used: (a) C1+2, B1, dcSTX, dcGTX2+3, GTX2+3 and STX (method A), (b) C1+2, B1, "dcSTX", "dcGTX2+3", "GTX2+3" and "STX" (method B).

7.3.2. Temporal and spatial variability of toxins

To assess whether variability of toxin proportions was influenced by blooms (temporal variation between 2007 and 2012) or harvest areas (spatial variation), significance tests were done for each species, considering two situations: bivalves from the same area exposed to different blooms, and bivalves exposed to blooms occurring in the same period at different areas.

The proportions of dcSTX and dcGTX2+3 in surf clams harvested in Culatra during two *G. catenatum* blooms, August-October 2009 and August-November 2012, were not significantly ($p>0.05$) different. Values of those toxins were also not statistically different ($p>0.05$) between samples collected in Aguda and Culatra during August-October 2009. Wedge clams from Comporta exposed to blooms in October-November 2007 and July-August 2008 showed different ($p<0.05$) proportions of the major toxins, C1+2, B1 and "dcSTX". Comparison of different blooms in Culatra was not carried out due to the low number of samples (Table 7.1). The proportions of all toxins quantified in cockles and mussels from Aveiro, Mondego and Óbidos showed no significant ($p>0.05$) differences between October-November 2007 and June-August 2008. Otherwise, proportions of C1+2 and B1 in cockles from Óbidos differed significantly ($p<0.05$) from

specimens from Aveiro and Mondego, both in 2007 and 2008 blooms. The proportions of C1+2 and B1 in mussels from Óbidos in the 2007 bloom differed significantly ($p<0.05$) from values found for Aveiro and Mondego.

7.3.3. PST composition in bivalves with different toxicity values

Figure 7.3 shows the median, maximum, minimum, 75^{th} and 25^{th} percentiles of the molar fractions of the toxins quantified in two sets of samples of surf clams, wedge clams, cockles and mussels that presented toxicity values above and below the PST regulatory limit. Elevated toxicity values were registered in summer or autumn and attributed to blooms of *G. catenatum* that reached the harvest areas (Moita et al., 2003). Bivalves with low toxicity values were collected in late winter and spring.

The toxins dcGTX2+3 and dcSTX were the major ones in surf clam samples of elevated and low toxicity. Furthermore, the molar proportions of each compound did not differ significantly ($p>0.05$) between the two sets of samples. The other toxins quantified, C1+2, B1, GTX2+3, and STX, remained as minor components, although significantly ($p<0.05$) higher in surf clams of low toxicity. Toxin proportions in samples of wedge clam analysed by method A showed considerable differences between low and elevated toxicities (n=3 and 6, respectively). The comparison using a more representative dataset (n=12 and 16 for the method B) pointed to no significant differences between low and elevated toxicity, except for “dcSTX” and “GTX2+3”. Cockles of low toxicity showed significantly ($p<0.05$) lower proportions of C1+2 than the elevated toxicity samples. Proportions of B1 varied inversely with the bivalve toxicity values, which is clearer defined considering method A. Significant ($p<0.05$) differences were also obtained for dcGTX2+3. In mussels, significant ($p<0.05$) differences were found for C1+2, dcSTX, GTX2+3 and STX (method A) and for C1+2, B1, "GTX2+3" and "STX" (method B).

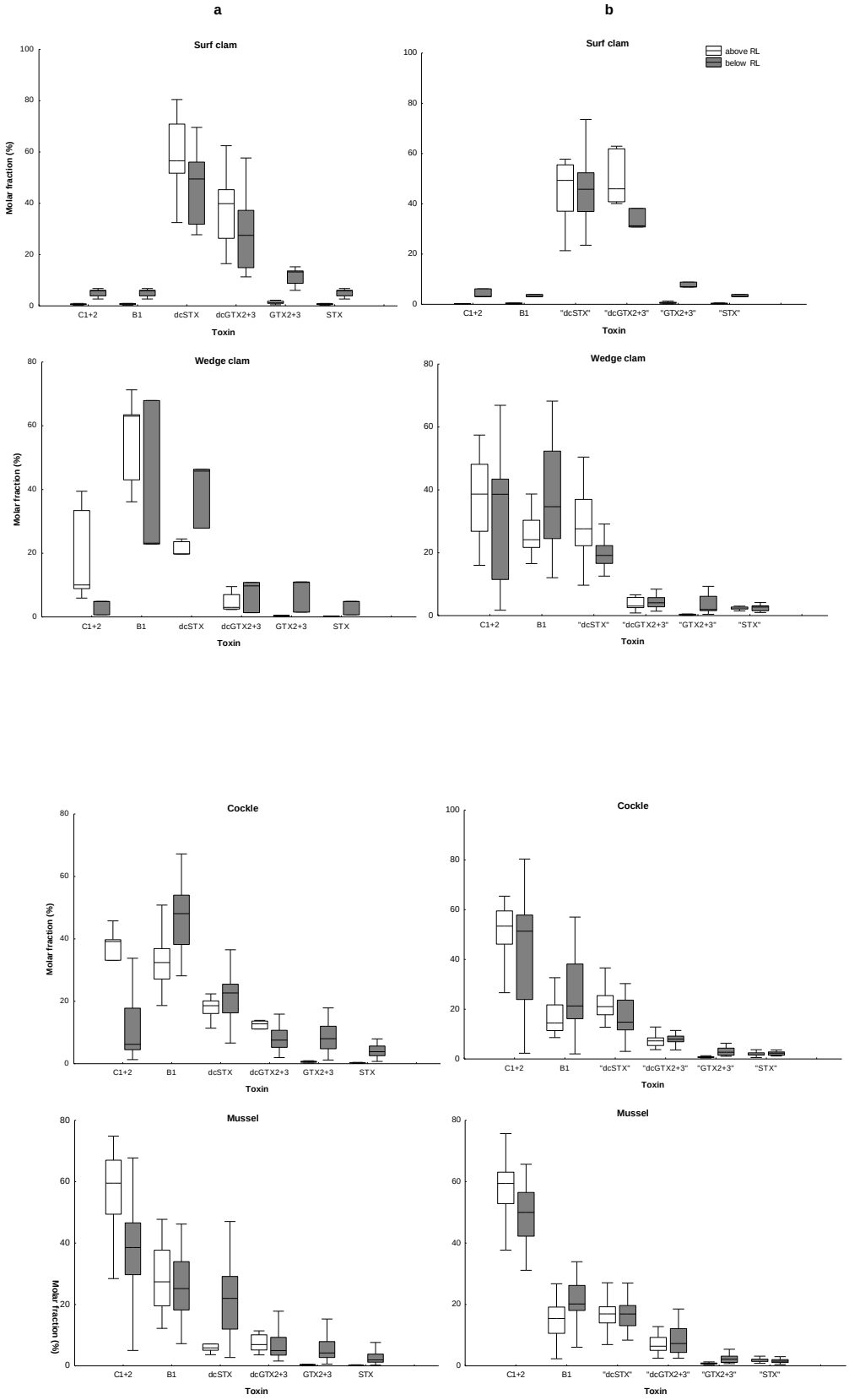

Figure 7.3. Median, maximum, minimum, 75th and 25th percentiles of molar fractions of quantified PSTs (%) in two sets of samples of surf clam, wedge clam, cockle and mussel presenting

toxicity values above and below the PST regulatory limit (RL) between 2007 and 2012; (a) C1+2, B1, dcSTX, dcGTX2+3, GTX2+3 and STX (method A); (b) C1+2, B1, "dcSTX", "dcGTX2+3", "GTX2+3" and "STX" (method B).

7.3.4. Toxin profile of PSTs in *Gymnodinum catenatum*

Between 28th June and 21st July 2008, the toxicity by PSTs of wild mussels from the NW coast (inlet of the Óbidos lagoon, Figure 7.1) was between 23 and 83 times greater than the corresponding regulatory limit (IPMA, database of the biotoxin monitoring programme). During this extreme event of mussel toxicity, paralytic shellfish toxins were quantified in cells of *G. catenatum* collected near the Óbidos lagoon inlet. Figure 7.4 shows the medians and the 75th and 25th percentiles of the molar proportions of the toxins C1+2, B1, dcGTX2+3 and dcSTX. The toxin profile was dominated by N-sulfocarbamoyl analogues, with the medians of the molar proportions of C1+2 (67%) and B1 (23%) exceeding the values found for decarbamoyl analogues dcGTX2+3 (5%) and dcSTX (4%) by one order of magnitude. The median ratio between toxins C1+2 and B1 was approximately 2.5.

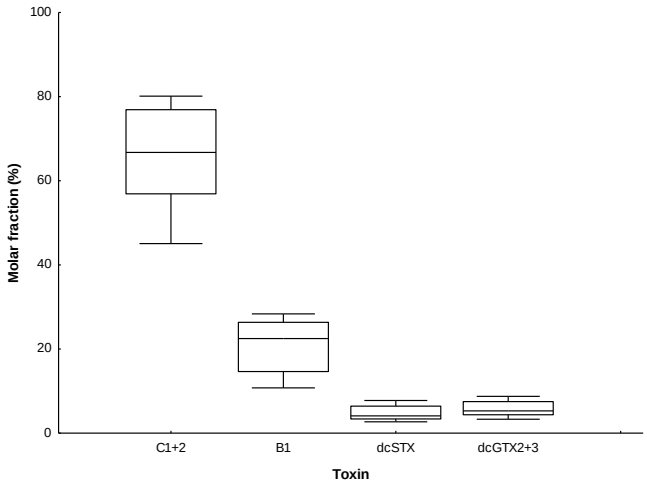

Figure 7.4. Median, maximum, minimum, 75th and 25th percentiles of molar fractions of toxins C1+2, B1, dcSTX, and dcGTX2+3 (%) quantified in seston samples (n=9) during a bloom of *G. catenatum* in coastal waters adjacent to Óbidos lagoon in 2008 (method A).

7.4. Discussion

The results of this study point to the differences in PST profiles among the species *Spisula solida, Donax trunculus, Cerastoderma edule, Mytilus* spp. collected in periods of elevated and low bivalve toxicity. Bivalve toxicity values are attributed to blooms of *G. catenatum* that reached the harvest areas (Moita et al., 2003). Most likely, the observed differences represent the variation of the balance between toxin uptake by filtration of the toxic cells, elimination of the ingested toxins, and metabolic inter-conversion of toxins (Shimizu and Yoshioka, 1981; Kotaki et al., 1985; Oshima, 1995b; Bricelj and Shumway, 1998). Since bivalves of low toxicity were collected a few months after the last *G. catenatum* bloom, toxin profiles in those specimens were probably dominated by elimination or biotransformation processes of the ingested cells during the prior bloom. Residual cells in the water column during winter and spring may also be filtered by the bivalve.

7.4.1. Toxin profiles of *G. catenatum* cells from the NW coast

The profile of PSTs in *G. catenatum* cells from the NW coast of Portugal collected during the bloom of 2008 agrees with the profile found for this species by Costa et al. (2010) in 2007. Moreover, N-sulfocarbamoyl and decarbamoyl compounds are the dominant toxins in cultivated cells isolated from Iberian strains of *G. catenatum* (Sousa et al., 1995; Ordás et al., 2004; Botelho et al., 2012). Different profiles have been reported for *G. catenatum* cells isolated from other areas in the world, such as Mexico, Australia, Singapore and Japan (Negri et al., 2001). The results of the current study reinforce the observations that *G. catenatum* cells in the Iberian coast are characterised by the high production of N-sulfocarbamoyl and decarbamoyl compounds. However, the possibility of slight variations in toxin composition should be considered. To search differences on profiles of *G. catenatum* cells from the NW coast, two ratios have been calculated: R1=(C1+2):B1, representing the proportion between the major toxins, and R2=[(dcSTX)+(dcGTX2+3)]:[(C1+2)+(B1)], the proportion between minor and major toxins.

These ratios were calculated for the current data (2008) and for the 2007 results (Costa et al., 2010). Values of R1 and R2 differed slightly in 2008 and 2007: 3.0, 3.9 and 0.10, 0.36 respectively. These differences may be related to the physico-chemical conditions associated with the algal bloom. Although *G. catenatum* blooms are triggered under similar oceanographic conditions in the NW coast of Portugal (Moita et al., 2008; Pitcher et al., 2010), different nutrient availability may exist, which could explain the modifications registered in the toxin ratios R1 and R2. Nutrient concentrations or composition have been shown that can modify the toxin profiles of cultivated *G. catenatum* cells (Band-Schmidt et al., 2010). Alterations in toxin ratios may also be explained by the different development stages of blooms due to changes in cell size, chain length and toxin leakage (Granéli and Flynn, 2006).

7.4.2. Alteration of major ingested PSTs by bivalves

The ratios R1 and R2 were also calculated for the bivalve species under two environmental conditions: (i) bivalves exposed to *G. catenatum* blooms in summer or autumn showing toxicity values above the PST regulatory limit, and (ii) bivalves under low abundance of toxic cells in winter/spring, and consequently presenting toxicity values far below the regulatory limit (Figure 7.5).

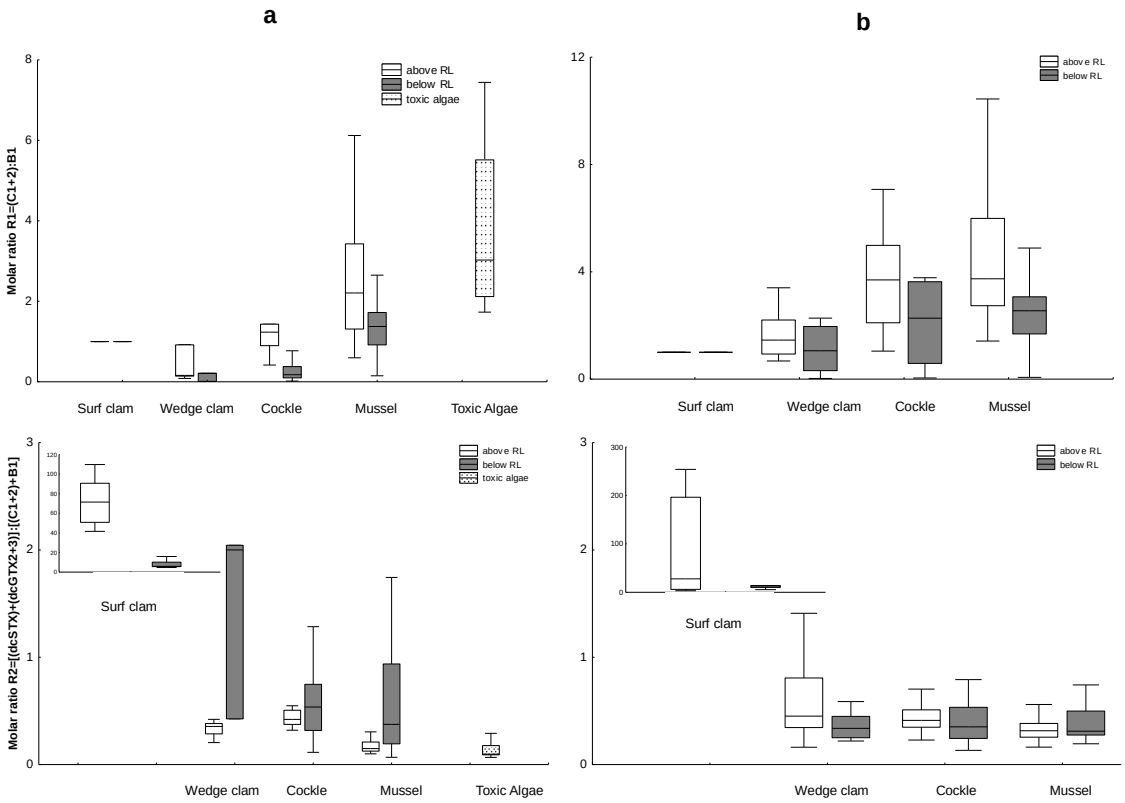

Figure 7.5. Median, maximum, minimum, 75th and 25th percentiles of the molar ratios R1=(C1+2):B1 and R2=[(dcSTX)+(dcGTX2+3)]:[(C1+2)+(B1)] calculated for samples of surf clam, wedge clam, cockle and mussel presenting toxicity values above and below the PST regulatory limit (RL), as well as for samples of seston during a bloom of toxic algae (*G. catenatum*) in coastal waters adjacent to Óbidos lagoon in 2008 (method A); period of time for bivalves: 2007 – 2012; methodologies used: (a) method A; (b) method B.

The ratios R1=(C1+2):B1 in surf clams could not be determined accurately because their concentrations were below the limit of detection in 90% of the samples. Medians of this ratio in wedge clams, cockles and mussels were lower than in *G. catenatum* cells collected in the NW coast during the blooms of 2007 and 2008 (Figure 7.5). Lower ratios in bivalves suggest the reduction of C1+2, most likely due to biotransformation to decarbamoyl toxins (Cho et al., 2008) or elimination, as well as the conversion of other compounds into B1 that counterbalanced its elimination or conversion. A plausible explanation for the increase of B1 is the possibility that this compound is a metabolic product of degradation of B2 due to their similar chemical structures (Oshima, 1995b). The ratios R1 in wedge clams were significantly ($p<0.05$) lower than in cockles and mussels with elevated toxicity. This difference points to more intense processes involving

those toxins in wedge clams during the period that was exposed to *G. catenatum* bloom than in cockles and mussels. The comparable ratio R1 registered in wedge clams during winter/spring, after a long period of natural depuration, supports this hypothesis. Conversely, the ratios in low toxicity (winter) cockle and mussels decreased significantly ($p<0.05$), which can be interpreted to mean that transformation or elimination processes involving C1+2 and B1 in those species are more prolonged than in wedge clams.

7.4.3. Prevalence of decarbamoyl derivates in surf clams

Since decarbamoyl derivates contribute approximately 10% to the toxin profile of *G. catenatum* cells (Figure 7.4), the ratio R2=[(dcSTX)+(dcGTX2+3)]:[(C1+2)+(B1)] may be considered as a footprint of PSTs biotransformation in bivalves. The differences among the four species, and between low and elevated bivalve toxicity, are shown in Figure 7.5. The most striking aspect is the extreme values of R2 for surf clams (median 72) in comparison to the other species analysed (0.2-0.4) and to *G. catenatum* cells (0.1). Those extreme values reflect the low concentrations of C1+2 and B1 in surf clams and their almost entire conversion into decarbamoyl analogues. The undetected values of these compounds in surf clams are explained by the rapid transformations of N-sulfocarbamoyl and carbamate toxins into to their corresponding decarbamate analogues (Artigas et al., 2007). Using in vitro experiments these authors showed that conversion occurs within a time-scale of one hour. In accordance, the profile of surf clams exposed to *G. catenatum* cells observed in the current work was dominated by dcSTX and dcGTX2+3, which reflects the rapid biotransformation of the ingested toxins. This type of profile persisted in periods of low toxicity, R2 in surf clams being significantly ($p<0.05$) different from other bivalve species (Figure 7.5). The lack of differences in the toxin proportion of surf clams among harvest areas is in line with the high biotransformation of the ingested toxins. Presumably, the broad difference registered in the ratio R2 between elevated and low toxicity of surf clams results from elimination of decarbamoyl derivates after blooms. Since inter-toxin conversion superimposes to other

steps of the bioaccumulation process, the major contributors to the toxin profile of surf clams stand independently of the abundance of toxic cells to which the specimens are exposed.

7.5. Conclusions

Differences of PST profiles between bivalves of elevated toxicity (exposed to *Gymnodinium catenatum* bloom) and bivalves of low toxicity (winter or spring with low abundance of toxic algae) are better illustrated through the molar ratios of R1=(C1+2):B1 and R2=[(dcSTX)+(dcGTX2+3)]:[(C1+2)+(B1)]. The ratio R1 decreased considerably between elevated and low toxicity cockles, indicating the elimination of C1+2 associated with the ingested toxic cells or conversion of compounds into B1. The ratio R2 increased considerably in wedge clams of low toxicity and less markedly in cockles and mussels. This change is interpreted as the predominance of a biotransformation phase after exposure to algal blooms. The toxin profile of surf clams is dominated by decarbamoyl compounds, reflecting intense biotransformation during exposure to blooms. This profile remains after that, although the ratio R2 decreased considerably, which may indicate the elimination of the produced dcSTX and dcGTX2+3.

Acknowledgements

We are grateful to our colleagues S.M. Rodrigues, P. Vale and P.R. Costa from the Laboratory of Marine Biotoxins, IPMA.

8

8. Final considerations

8.1. General discussion

This section presents an overview of the various topics dealt with in the chapters 3 to 7, carrying out an integrative discussion of the main outcomes. Linkage to the thesis objectives and the contribution to the scientific knowledge in the area are also highlighted.

First of all, it is important to point out that the present research has filled gaps on the knowledge of the pathways of individual paralytic shellfish toxins (PSTs) produced by the dinoflagellate *Gymnodinium catenatum* in commercial bivalves. Various studies have reported the uptake, elimination and biotransformation of toxins produced by *Alexandrium* species in other geographical areas of bivalve production (Bricelj et al., 1990; Sekiguchi et al., 2001). The research presented in this thesis was focused on PSTs produced by *G. catenatum*, the composition of which varies geographically and differs from the profile produced by *Alexandrium* species (Cembella et al., 1987; Hallegraeff et al., 2012). The accumulation was studied in commercial bivalve species harvested in estuaries, coastal lagoons, and inshore areas of Portugal, such as mussels (*Mytilus* spp.), cockles (*Cerastoderma edule*) and clams (*Ruditapes decussatus*, *Spisula solida*, *Donax trunculus*). Because of the specificity of each species, PST profiles differ among them. In addition, laboratory experiments were performed to study the uptake and depuration kinetics of the major toxins produced by that dinoflagellate (dcGTX2+3, dcSTX, C1+2 and B1), including the estimation of the corresponding rates.

Research question 1: Is it feasible to predict seasonal or inter-annual toxicity episodes by PSTs of bivalves produced and harvested in Portugal? Do these episodes vary with the geographical area? Is it possible to identify areas where toxicity episodes are more recurrent?

***Approach*:** In order to address this question, long-term data from the national monitoring programme (1994-2012) were examined in detail. Firstly, commercial bivalve species sensitive to PSTs were selected, areas with toxicity episodes were surveyed, and annual periods of moderate to elevated sampling frequency of bivalves were chosen. Finally, inter-annual and seasonal variations of

PST episodes were searched for.

In this study, PST episodes were considered when toxin concentrations in bivalves were above the regulatory limit defined for human consumption. As monitoring programmes of bivalve toxicity were implemented in various countries, *G*. *catenatum* was considered a cosmopolitan species (Band-Schmidt et al., 2010) and PSP became a relevant issue in various areas. Two possible mechanisms were proposed for *G*. *catenatum* blooms (Hallegraeff et al., 2012): "blooms derived from an inoculum by local cyst beds or (overwintering) motile cells, and are triggered by organic enrichment" (e.g., Tasmanian estuaries); and a second mechanism in which *G. catenantum* "blooms are inoculated from offshore and build up during upwelling relaxation" (e.g., Spanish rias). Despite the understanding of these mechanisms, occurrence of blooms and toxicity episodes are far from being regular or predictable. For example, inter-annual variation of *G*. *catenatum* blooms has been reported in various coastal systems such as Galicia in Spain (Estrada, 1995) and Mexico (Mee et al., 1986; Cortés Altamirano et al., 1996), without an explanation for this puzzling variation. It has been argued that unrecognized upwelling system factors could be more important than life cycle strategies of phytoplankton species (Bravo et al., 2010; Smayda and Trainer, 2010).

The two overviews of bivalve toxicity caused by *Dynophysis* spp., *Pseudonitzchia* spp. and *Gymnodinium catenatum* in the Portuguese coast for the periods 1986-1996 (Sampayo et al., 1997) and 1986-2006 (Vale at al., 2008) also showed irregularities in the incidence of maximum toxicity values. Various commercial species were considered in these works. This thesis presents a third overview covering the period between 1994 and 2012, and including toxicity values by PSTs in mussels, cockles, wedge clams and surf clams harvested in the main production areas in Portugal (**Chapter 7**). As observed in other regions of the world, an irregular multi-annual variation was registered over the surveyed 18 years. A salient aspect of this analysis is the prolonged period (1996-2004) of low toxicity by PSTs, despite the regular coastal upwelling in the Portuguese waters (Fiúza, 1983). The lack of PST episodes was already reported in this period by Vale et al. (2008). This observation is very much in line with the argument of the importance of unrecognized upwelling system factors relatively to life cycle strategies of *G. catenantum* (Bravo et al., 2010). To confirm this hypothesis further studies on algal bloom dynamic should be performed. This thesis also points to the connectivity of PST episodes along *circa* 150 km of the west coast of Portugal in autumn and early winter. The occurrence of high bivalve toxicity in the same weeks reinforces the hypothesis that

episodes in the west coast of Portugal are linked to the relaxation of coastal upwelling associated with slow currents or inshore eddies (Moita et al., 2003; Pitcher et al., 2010).

Research question 2: Could pronounced alterations in nutrient concentration and nutrient composition facilitate the development or maintenance of toxic algal blooms and consequently prolonged toxicity in bivalves?

Approach**:** This hypothesis was tested in a coastal lagoon with symptoms of eutrophication and located nearby a coastal area with recurrent toxic algal blooms.

During a "roundtable discussion" on the links between water quality, eutrophication, and HABs, a consensus was obtained with respect to the importance of nutrient concentration and nutrient composition as promoters of harmful algal blooms (Heisler et al., 2008). The possibility of a shift on nutrients prolonging the algal bloom was examined in a coastal lagoon with symptoms of eutrophication (Óbidos lagoon) permanently connected to a coastal area in the west of Portugal characterized by frequent upwelling episodes and bivalve toxicity (**Chapter 4**). High concentration ratios between phosphate and dissolved inorganic nitrogen and between phosphate and silicate were registered during summer in Óbidos lagoon, as result of high regeneration of phosphate in sediments and diffusion to the water column. This alteration may facilitate the development or maintenance of the bloom according to studies examining the linkages between the composition of the nutrient pool and shellfish toxicity (e.g., Hattenrath et al., 2010). Data on DST- and PST-derived toxicities in mussels from the lagoon along 2006 and 2009 were used. Between June and September 2006 (and less markedly in 2009), toxicity in mussels from the lagoon was higher and displayed a longer duration than in mussels from the adjacent coast. This occurred in the period that nutrient concentrations and composition shifted. Presumably, phytoplankton cells imported from the coastal waters, most likely associated with coastal upwelling, became exposed to a proportional enrichment of phosphorus relatively to nitrogen and silicon, as well as of organic forms of N and P. These conditions, coupled with high salinity due to negligible freshwater inputs in summer, may have favored the increase of cell densities and consequently bivalve toxicity. Although nutrient concentrations in estuaries are, in general, higher than in the coastal waters, linkage between nutrient composition and algae-produced toxins may not occur in most of estuaries. Negative effect such as high turbidity which limits light in the water column and low salinity being unfavorable to phytoplankton cells, may prevent that

association.

Despite specificities of estuaries and coastal lagoons, connectivity of bivalve toxicity episodes may be found between estuaries/lagoon and coastal waters. For example, connectivity was observed between toxicity of cockles from Ria Formosa (a coastal lagoon with negligible freshwater input permanently connected with the sea) and of wedge clam from the adjacent coastal area (**Chapter 3**).

Research question 3: Do toxin profiles of *G. catenatum* cells vary in the NW coast of Portugal? Does the profile change during the bloom?

Approach**:** To test those possibilities cells of *G. catenatum* collected during an event of mussel toxicity and high cell abundance were examined.

Cells of *G. catenatum* collected along a bloom in the NW coast differ slightly (**Chapter 7**). We have assumed that physico-chemical conditions may have been modified and the production of toxins has changed. However, other factors may be invoked, such as changes in cell size, chain length and toxin leakage (Granéli and Flynn, 2006). Despite that variability, toxin profiles of cells possibly ingested by bivalves are dominated by N-sulfocarbamoyl toxins, followed by the decarbamoyl analogues. This pattern is in agreement with previous studies (Sousa et al., 1995; Costa et al., 2010) and is characteristic in the Iberian coast (Camino-Ordás et al., 2004). A high proportion of C and B toxins in the toxin profile seems to be characteristic of the toxins produced by *G. catenatum* cells worldwide, except for Singaporean and Malay strains (Holmes et al., 2002; Mohammad-Noor, 2010), and some Andalusian strains (Camino-Ordás et al., 2004). Otherwise, the absence of NEO toxin in strains from the Iberian coast contrasts with those from the Gulf of California and the Mexican coast characterized by a elevated contribution of NEO to the toxin profile (Band-Schmidt et al., 2006).

Research question 4: How relevant is biotransformation of PSTs by bivalves during exposure and depuration phases? What can be learnt from toxin composition in faeces and pseudo-faeces produced by bivalves?

Approach**:** The response to this question was obtained from the results of a laboratory experiment with the clam *Ruditapes decussatus* including exposure to *G. catenatum* and subsequent depuration.

Regardless of connectivity of bivalve toxicity observed in this work, various studies have shown

that bivalves exposed to dinoflagellates exhibit different PSTs profiles from the toxin producers (Oshima et al., 1990; Bricelj et al., 1990). Metabolic interconversion of assimilated toxins achieved by enzymatic and chemical reactions in bivalve tissues (Shimizu and Yashioka, 1981; Oshima, 1995b), as well as to different uptake and depuration kinetics of individual PSTs (Yu et al., 2007; Botelho et al., 2010) are the major contributors to that difference. Different profiles between the algae-producing toxin and bivalve species were emphasized in a laboratory experiment with *G. catenatum* and *Ruditapes decussatus* (**Chapter 3**). The toxins dcSTX and dcGTX2+3 were minor compounds in the algae, but were predominant in clams as assimilated toxins are interconverted. Furthermore, an innovative aspect of those results was the toxin profile of the particulate organic matter (POM), which was dominated by dcSTX while the other compounds were below the limit of detection. Since POM consisted of faeces and pseudofaeces produced by the clams along the experiment, the compound dcSTX should result mainly from interconversion of other toxins. This compound was the major toxin released by the clams associated with faeces. Indeed, the non-quantified presence of the assimilated toxins (mainly C1+2 and B1) in POM showed the intense biotransformation of those toxins by *R. decussatus*. The results of this experiment highlight the relevance of biotransformation in the definition of toxin profiles in clams. *In vitro* experiments have demonstrated the transformation of assimilated toxins in other compounds, e.g., in the experiments referred in Artigas et al. (2007) and Fast et al. (2006).

Research question 5: Which toxins remain in bivalves during periods of low toxicity? Are the same toxins present in bivalves of low and high toxicity?

Approach**:** Comparison of toxin profiles in periods of low and elevated bivalve toxicity (data from the national monitoring programme, 2007-2012).

Monitoring programmes register toxin concentrations, toxicity and, by comparison to the PST regulatory limit, indicate the species and areas displaying values above and below the regulatory limit. However, comparison of toxin profiles for different toxicity values are scarce (Turner et al, 2014). Relationships between profiles and total PST content in bivalves were examined in bivalves from the United Kingdom and distinct profile types were identified. This thesis compares PST profiles in bivalves of toxicity below and above the PST regulatory limit caused by *G. catenatum* blooms in the period 2007-2012 (**Chapter 7**). The main production areas in Portuguese estuaries and coastal areas

were considered. Profiles differed between periods of elevated toxicity (exposed to *G*. *catenatum* bloom) and low bivalve toxicity (winter or spring with low abundance of toxic algae). This comparison was carried out for the species *Spisula solida*, *Donax trunculus*, *Cerastoderma edule* and *Mytilus* spp.. The observed differences reflect the variation of the mass balance including ingested toxic cells, differential toxin elimination, and metabolic interconversions. Despite the slight variability in toxins in the ingested cells, N-sulfocarbamoyl toxins, followed by the decarbamoyl analogues are the dominant compounds in all the analysed species, except for *S. solida*. Presumably, biotransformation is the most influencing factor of this pattern. By comparing the toxin molar ratios it seems that transformation or elimination processes involving C1+2 and B1 during winter and spring (low abundance of toxic cells in the water column), were more prolonged in cockles and mussels than in wedge clams. The calculated ratios [(dcSTX)+(dcGTX2+3)]:[(C1+2)+(B1)] (see Chapter 7) may be considered as footprints of PSTs biotransformation in bivalves. They pointed to the higher predominance of the biotransformation phase after exposure to algal blooms in wedge clams than in cockles and mussels. In addition, this approach highlighted the intense biotransformation of ingested toxins in surf clams since decarbamoyl toxins stand as the major contributors to the toxin profile independently of the abundance of toxic cells to which the specimens are exposed. In short, bivalves tend to retain some of the biotransformed toxins between seasonal blooms of *G*. *catenatum*, but not the toxins presented in the ingested toxic cells.

Research question 6: How are PSTs partitioned in bivalves at the sub-cellular level? Which cell components, soluble or insoluble fractions are important in the partitioning? Does sub-cellular partitioning of PSTs in tissues changes during depuration after exposure to *G. catenatum* bloom?

***Approach*:** This question was approached examining the sub-cellular partitioning of PSTs in digestive glands of cockles (*Cerastoderme edule*) exposed to a *G. catenatum* bloom and under post-bloom natural conditions.

The digestive gland of bivalves is the initial repository of ingested food, including xenobiotics and marine toxins. This organ contains by far the greatest proportion of the total toxin body burden in bivalves exposed to toxic algal blooms (Bricelj and Shumway, 1998). Enzymatic and other oxidation/reduction reactions are involved in the elimination or biotransformation of toxins in the digestive gland (Fast et al., 2006). The partitioning of PSTs among the sub-cellular fractions in

digestive glands of bivalves, exposed to toxic algae and during depuration, may provide a better knowledge of the distribution and linkage of PSTs in bivalve tissues. Different depuration rates for toxins may result from variations in partitioning and linkages. This approach was used in the present study to search for differences in the sub-cellular partitioning of C1+2, B1, dcGTX2+3, dcSTX, GTX2+3 and STX in digestive glands of the cockle *Cerastoderme edule*, after being exposed to a *G*. *catenatum* bloom and under natural depuration conditions (**Chapter 5**). The partitioning of PSTs among the cell compartments indicated that assimilated toxins were mainly stored in the cytosolic fraction, as other works related with the partitioning of hydrophilic toxins have reported (Mauriz and Blanco, 2010). However, the partitioning of toxins among cell organelles has been poorly documented, which limits the understanding of the mechanisms behind the release of toxins at sub-cellular level. This thesis presents the quantification of all toxins, except GTX2+3 and STX, in the sub-cellular particulate fractions (nuclei+debris, mitochondria, lysosomes and microsomes) of digestive glands of cockles. The toxin content decreased most markedly in nuclei+debris and microsomal fractions, during the first days of depuration (eight and 12 days respectively). Conversely, different patterns were observed among toxins in mitochondrial and lysosomal fractions. Interconversion among toxins and exchange between insoluble and soluble cellular fractions are the most plausible reasons for the observed patterns, as well for changes in toxin profiles among sub-cellular fractions. In particular, toxin interconversion may be associated with enzymatic activities existing in the mitochondrial fraction, as compounds ingested by the organisms are transformed into simpler molecules during the energy production in this organelle (Voet and Voet, 1990). In addition, the non-uniform decrease of B1 contents in the lysosomal fraction points also to the conversion of other toxins, like the abundant B2 and C1+2, occurring in the digestive process of materials acquired through vesicular transport (Voet and Voet, 1990). Along this process undesirable compounds, such as toxins, may be incorporated in the lysosomes and subsequently decomposed in order to protect the cell from cytotoxic effects (Moore, 1988; Owen, 1972; Viarengo et al., 1987).

8.2. Perspectives and future work

This study has identified a number of supplementary areas of research. These are discussed below:

- How can modelling be useful for prediction of the variation of the uptake, transformation and elimination of individual toxins in bivalve species exposed to *G. catenatum* blooms? Besides the selection of the best models, data on kinetics and interconversion of toxins in commercial bivalve species would be needed; this may be obtained from simulation experiments under controlled conditions. Outputs generated by the models should predict how toxin profiles vary on a timescale of days to weeks, and consequently provide results on the toxicity values with respect to the regulatory limits.

- Do assimilated toxins have effects on bivalves? At which life stage of development? Are larvae affected? Parameters such as survival and activity of early D-shape larvae under laboratory exposure to toxic algae could be examined to search for adverse effects on early life stage of bivalves, and subsequent effects on recruitment.

- Do toxins accumulated in bivalves have effects on other biological organization levels of the organism? A possible approach to these questions could be to examine biochemical responses to the assimilated toxins at cellular level. Selected biomarker parameters could be used to investigate the disturbances in cellular oxidative stress during exposure of bivalves to PSTs. This could be achieved by laboratory experiments or, in a less controlled manner with respect to environmental variables, by analysing natural populations of bivalves during blooms and subsequent periods.

8.3. Management applications

The study of paralytic shellfish toxins in bivalves carried out in this PhD thesis has potential applications on management of the harvesting and production of bivalves in Portugal relatively to the restrictions in commercialization during periods of bivalve toxicity values above the regulatory limit. The analysis of two decades of historical toxicity data of commercial bivalve species allowed the

identification of harvest areas presenting different incidences of toxicity episodes. This information is useful to the Administration, proving elements for risk analysis. It could be added to Marine Spatial Planning for a better documentation of potential areas for inshore aquaculture (Plano de Ordenamento do Espaço Marítimo, MAMAOT, 2011).

Furthermore, identification of coastal and estuarine areas with different vulnerabilities relatively to marine toxins, and consequently commercialization of the harvest or produced bivalves, provides key elements to private investors decide on long-term strategies of bivalve production in Portugal. Seasonal variation of bivalve toxicity allows establishing periods that temporary closure to commercialization is more likely to occur, and consequently commitment to supply bivalves during these periods should be taken with precautions. An important aspect for the bivalve production activity is the identification of species less vulnerable to accumulate toxins. However, with the exception of oysters that tend to exhibit low toxicity (not shown in this thesis), production techniques of other species that rapidly metabolise the ingested toxins still need improvements. Market requirements and competition among production areas is an important aspect of the strategy.

Monitoring programmes of marine toxins and HABs are designed at national level to alert producers, fishermen and consumers about bivalve toxicity. Benefits are evident, with an increased safety of available products and a consequent increase in consumer confidence. The cost of conducting this monitoring programme is low relatively to the revenue from bivalve production. Estimation of the annual revenue from two important production regions (Aveiro and Formosa/Culatra, including inshore and estuarine harvesting and production) was based on official data on quantities and wholesale prices of bivalves (DGPA, 2011). An approximate annual income of 2 700 K€ was estimated in 2010 considering the most important five commercial species: mussel, cockle, surf clam and wedge clam. The annual cost of the monitoring programme for those two areas may be estimated using the prices for the toxin analysis of IPMA (Despacho nº 23597/2006). Considering that five species are sampled on a weekly basis from each area, the annual cost of this programme is approximately 115 k€, which corresponds to less that 5% of the annual revenue. With this small fraction of the income, bivalve producers and fishermen can guarantee products in the market of low toxicity and, consequently, increasing the credibility of their activity. This value also suggests that producer organizations could organise the self-control of their products before

commercialisation at relatively low costs (Anon., 2004a). More expedite and cheaper methodologies should be used in the near future, encouraging producers to invest in self-control of toxins before export or sell products to the market.

The literature refers that severe eutrophication may result in HABs (Bricker et al., 2008; Heisler et al., 2008). However, most of the toxic algal blooms and bivalve toxicity in coastal areas are not related to eutrophication (Hallegraeff et al., 2012). Similarly, bivalve toxicity produced by blooms of *Gymnodinium catenatum* (producer of PSTs), *Dinophysis* spp. (producers of DSTs) in Portugal has not been related to eutrophication symptoms. An interesting question is whether occurrence of toxic algal blooms and bivalve toxicity will increase in the near future, and whether measures associated with Water Framework Directive (WFD) or Marine Strategy Framework Directive (MSFD) can reduce the frequency of those episodes. Nutrient concentrations and phytoplankton composition are quality elements for determination of Ecological Status in marine and estuarine waters (WFD, 2000/60/EC) and descriptors of environmental status (MSFD, 2008/56/EC). When values of these parameters do not comply with reference conditions or thresholds, measures should be taken to achieve good status. In Portuguese coastal waters, toxic algal blooms consume nutrients mainly from the upwelling of bottom water enriched in nutrients, and not to nutrient loads from the watershed. Moreover, toxicity episodes in estuaries and adjacent coastal areas are often connected, suggesting that toxic algal blooms formed in the coastal waters are advected to the estuaries. Under the present circumstances, possible measures to reduce the nutrient loads in river basins in the light of those directives should have negligible effects on toxic algal blooms causing bivalve toxicity in the Portuguese production areas.

However, the study in Óbidos lagoon, an eutrophic system, raises the possibility of nutrients from diffuse sources increasing the duration of high abundances of toxic dinoflagellate imported from the coastal waters. Climate change scenarios include the increase of runoff episodes being more intense and frequent, and enhancing the relevance of diffuse sources of nutrients to estuaries and coastal lagoons (Intergovernamental Panel on Climate Change, IPCC, 2014). Under these circumstances, a paradigm shift in bivalve toxicity could be hypothesized, whereby nutrients from land play a greater role in the onset and maintenance of blooms mainly in estuaries and coastal lagoons. If such scenario becomes realistic, measures to reduce nutrient inputs may be foreseen under the WFD in order to reduce the intensity and frequency of toxic algal blooms and therefore

bivalve toxicity episodes. The possibility of other toxin-producing species developing off the Iberian coast due to changes in temperature is also a very real possibility.

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