PHYSIOLOGICAL EFFECTS AND BIOTRANSFORMATION OF PARALYTIC SHELLFISH TOXINS IN NEW ZEALAND MARINE BIVALVES

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Abstract

Although there are no authenticated records of human illness due to PSP in New Zealand, nationwide phytoplankton and shellfish toxicity monitoring programmes have revealed that the incidence of PSP contamination and the occurrence of the toxic *Alexandrium* species are more common than previously realised (Mackenzie et al., 2004). A full understanding of the mechanism of uptake, accumulation and toxin dynamics of bivalves feeding on toxic algae is fundamental for improving future regulations in the shellfish toxicity monitoring program across the country. This thesis examines the effects of toxic dinoflagellates and PSP toxins on the physiology and behaviour of bivalve molluscs. This focus arose because these aspects have not been widely studied before in New Zealand.

The basic hypothesis tested was that bivalve molluscs differ in their ability to metabolise PSP toxins produced by *Alexandrium tamarense* and are able to transform toxins and may have special mechanisms to avoid toxin uptake. To test this hypothesis, different physiological/behavioural experiments and quantification of PSP toxins in bivalves tissues were carried out on mussels (*Perna canaliculus*), clams (*Paphies donacina* and *Dosinia anus*), scallops (*Pecten novaezelandiae*) and oysters (*Ostrea chilensis*) from the South Island of New Zealand.

Measurements of clearance rate were used to test the sensitivity of the bivalves to PSP toxins. Other studies that involved intoxication and detoxification periods were carried out on three species of bivalves (*P. canaliculus*, *P. donacina*, *P. novaezelandiae*), using physiological responses (clearance and excretion rate) and analysis of PSP toxins in the tissues over these periods. Complementary experiments that investigated other responses in bivalves fed with the toxic cells were also carried out. These included byssus production, and the presence of toxic cells in the faeces of mussels, the siphon activity and burrowing depth in clams and the oxygen consumption in scallops.

The most resistant species to PSP toxins were the mussel, *Perna canaliculus* and the clam, *Dosinia anus*. Both species fed actively on toxic dinoflagellates and accumulated toxins. The intoxication and detoxication rate of the mussel was faster than the other

species of bivalve studied (*P. donacina* and *P. novaezelandiae*) which confirm mussels as a good sentinel species for early warning of toxic algal blooms.

The clearance rate of the clam, *Paphies donacina* decreased when fed on *Alexandrium* species but the effect of the PSP toxins on this physiological response was not confirmed. Over the detoxification period of 8 days, this clam did not detoxify which suggests that its ability to retain high level of toxins for an extensive period may be critical for public health management.

The scallop, *Pecten novaezelandiae* was clearly the most sensitive species to the PSP toxins and the clearance rate was significantly lower in the presence of the toxic dinoflagellate *A. tamarense*. Although the clearance rate was low, the scallops still fed on the toxic dinoflagellate and accumulated PSP toxins in the tissues. The scallops detoxified slowly which would affect the market for this bivalve in the presence of a toxic algal bloom. This bivalve would retain PSP toxins for longer period of time than other species such as mussels.

The oyster, *Ostrea chilensis*, had erratic clearance rate and did not respond clearly to any of the variables tested over the time. Oysters accumulated more toxins than the sensitive species, but they had been exposed to two more days of feeding with *A. tamarense*; therefore this species may actually have a similar intoxication responses to *P. novaezalandiae* and *P. donacina*.

The results from this thesis suggest further directions for the aquaculture sector and ongoing research in this field, which in future will lead to a better selection of suitable species for culture as well as species for monitoring of PSP toxins. In the future, research that integrates field and controlled laboratory studies will expand to other species of interest and a more complete record will in time be available in order to manage more efficiently the negative effects that harmful algal blooms may have in New Zealand.

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Chapter 1

General Introduction

1.1 Harmful algal blooms (HABs): a global overview

The microscopic planktonic algae of the world's oceans are critical food for filter-feeding bivalve molluscs (oysters, mussels, scallops, clams) as well as for the larvae of commercially important crustaceans and finfish. In most cases, the proliferation of planktonic algae (algal blooms) is beneficial for aquaculture and wild fisheries. In some situations, however, algal blooms can have a negative effect, causing severe economic losses to aquaculture, fisheries and tourism operations and having major environmental and human health impacts (Shumway, 1990; Hallegraeff, 1993; Rodríguez Rodríguez et al., 2011) .

Among the ~5,000 species of extant marine phytoplankton (Sournia et al., 1991), some 300 species can at times occur in such high numbers that they discolour the surface of the sea, while only ~80 species have the capacity to produce potent toxins that can find their way through fish and shellfish to humans (Hallegraeff, 2003). While harmful algal blooms, in a strict sense, are complete natural phenomena that have occurred throughout recorded history, in the past three decades the public health and economic impacts of such events have increased in frequency, intensity and geographical distribution (Hallegraeff, 1993).

There are different types of harmful algal blooms resulting in several effects in the environment and human health. Some species of microalgae produce harmless water discolorations; however, blooms can grow so dense that they cause indiscriminate kills of

fish and invertebrates through oxygen depletion. Several groups of microalgae produce potent toxins that are accumulated through the food chain and ultimately transferred to humans, causing a variety of gastrointestinal and neurological illnesses. The illnesses associated with these toxins include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), ciguatera (fish toxin) and neurotoxic shellfish poisoning (NSP) (Hallegraeff, 1993).

Paralytic shellfish poisoning is caused by ingestion of shellfish with accumulated PSP toxins and affect the central nervous system of mammals. These neurotoxins are among the most potent phycotoxins found in the marine environment and they act by reversibly blocking sodium conductance in nerve and muscle membranes (Evans, 1964). The PSP toxins are produced by various species of dinoflagellates such as *Alexandrium* spp., *Pyrodinium bahamense* and *Gymnodinium* spp. The PSP-producing dinoflagellates occur in both temperate and tropical waters such as North America, Europe, Japan, South America, Australia, New Zealand, South East Asia and India (Shumway, 1990).

Diarrhetic shellfish poisoning was first described in the 1980s (Yasumoto et al., 1980). This illness is easily confused with gastroenteritis and general stomach upsets associated with eating contaminated shellfish (Shumway, 1990). Toxins associated included okadaic acid, dinophysistoxin-1 and dinophysistoxin-3 which are lipid-soluble. It has been found that okadaic acid is a potent inhibitor of protein phosphatases 1 and 2A, key phosphatases in the cytosol of mammalian cells. This inhibition is rather specific and other cellular phosphatases and a variety of kinases are unaffected by okadaic acid (Cohen et al., 1990). The dinoflagellates associated with these toxins are *Dinophysis* spp. and the benthic dinoflagellate *Prorocentrum lima*. The global distribution of DSP includes Gulf of Mexico, Japan, Europe, Chile, Thailand, Canada (Nova Scotia), Australia and New Zealand (Hallegraeff, 2003).

Amnesic shellfish poisoning was first recognised in 1987 in Prince Edward Island, Canada, where it caused two deaths and 129 cases of acute human poisoning following the consumption of blue mussels (Bates et al., 1989; Wright et al., 1989). The symptoms include abdominal cramps, vomiting, disorientation and memory loss (amnesia). The causative toxin was domoic acid produced by the diatom *Pseudo-nitzschia* spp. Domoic acid is a potent neurotoxin. It belongs to a class of excitatory neurotransmitters called

kainoids that bind to specific receptor proteins in neuronal cells causing continual depolarisation of the neuronal cell until cell rupture occurs (Zaczek and Coyle, 1982). Shellfish containing more than 20 μg domoic acid per gram of shellfish meat are considerate unfit for human consumption. The detection of domoic acid in seafood products have been mainly reported in U.S., Canada, Europe (e.g Scotland), Australia, Japan and New Zealand (Wright, 1995; Hallegraeff, 2003).

The ciguatera poisoning (fish toxin) was first recorded in the 17th century by Spanish expeditionary groups exploring the Pacific Basin (Ragelis, 1984). The disease follows consumption of fish that have accumulated ciguatoxins derived from the benthic dinoflagellate *Gambierdiscus toxicus* through the marine food chain (Lewis and Holmes, 1993). The consumption of contaminated fish causes a wide array of gastrointestinal and neurological symptoms. The most potent sodium-channel toxin known, P-CTX-1, is the major toxin found in carnivorous fish, where it typically contributes about 90% of the total lethality and poses a health risk at levels ≥0.1 ppb (μg kg^{-1}). Annually, cases are reported in tropical and subtropical regions of the Pacific Basin, Indian Ocean and Caribbean. Isolated outbreaks occur sporadically but with increasing frequency in temperate areas such as Europe and North America (Ting and Brown, 2001).

Neurotoxic shellfish poisoning is caused by the consumption of molluscan shellfish contaminated with brevetoxins which causes a mild gastroenteritis with neurological symptoms (Shumway, 1990). Brevetoxins are a group of more than ten natural neurotoxins produced by the marine dinoflagellate, *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*) (Duagbjerg, 2001). Blooms of *Karenia brevis* are considered endemic to the Gulf of Mexico, particularly off the southwestern coast of Florida. There are numerous other *Karenia* spp. found in the Gulf of Mexico and around the world (particularly New Zealand) regularly associated with blooms. Most produce brevetoxins, gymnodimine, karlotoxins, and other potent natural toxins. These toxins activate voltage-sensitive sodium channels causing sodium influx and nerve membrane depolarization. No fatalities have been reported (Watkins et al., 2008).

Finally, cyanobacterial toxin poisoning causes an often acute and potentially fatal condition in humans and other animals from drinking freshwater that contains high

concentration of cells (PSP toxins). More than 55 species, belonging to 30 genera, have been shown to be toxin producer (Conberg et al., 2004).

1.2 Transformation of PSP toxins in marine bivalves

PSP toxins are a group of 21 closely related tetrahydropurines. The general structure for PSP toxins is shown in Figure 1.1 and Table 1.1. The first PSP toxin chemically characterised was saxitoxin (STX). The various PSP toxins significantly differ in toxicity with STX being the most toxic (Shimizu, 1987). They make up three subgroups: (1) carbamoyl (STX, neoSTX, and gonyautoxins (GTX1-4)); (2) N-sulfocarbamoyl (GTX5-6, C1-4) and (3) decarbamoyl (dc-) (dcSTX, dcneoSTX, dcGTX1-4). Other rare PSP toxin analogues may occasionally be found in dinoflagellates and in contaminated seafood (presumably as metabolites), but these are not usually quantitatively important and their relative toxicities are not known (Fast et al., 2006).

Table 1.1: Structure of the PSP derivatives (Cembella et al., 1993) and their relative toxicities.

Carbamoyl		N-sulfacarbamoyl		Decarbamoyl				
R4:OCONH2		R4: OCONHSO3		R4:OH				
Toxin	Relatively Toxicity	Toxin	Relatively toxicity	Toxin	Relatively toxicity	R1	R2	R3
STX 1	1	(B1) GTX5	0.06	dcSTX	0.51	H	H	H
Neo	0.92	(B2) GTX6	0.06	dcNeo	-	OH	H	H
GTX1	0.99	C3	0.01	dcGTX1	-	OH	H	OSO_3^-
GTX2	0.36	C1	0.01	dcGTX2	0.15	H	H	OSO_3^-
GTX3	0.64	C2	0.01	dcGTX3	0.38	H	OSO_3^-	H
GTX4	0.73	C4	0.06	dcGTX4	-	OH	OSO_3^-	H

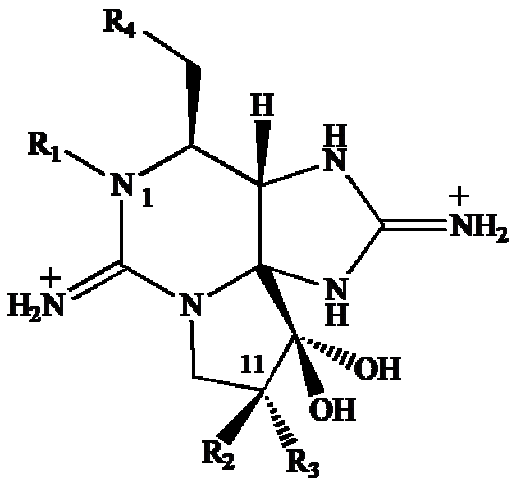

Figure 1.1: The general structure PSP toxins (Cembella et al., 1993)

Transformation of PSP toxins between the dinoflagellate and the shellfish have been previously reported (Oshima et al., 1990; Bricelj et al., 1991; Cembella et al., 1993; Choi et al., 2003) and have indicated that bivalves usually have lower proportions of N-sulfocarbomyl toxins and higher proportions of carbamoyl toxins than the toxigenic dinoflagellates that were ingested. Several kinds of transformation have been documented for PSP toxins. Epimerisation between α and β-epimers appears to take of place easily and it is not believed to be enzymatically mediated. Reduction, acid hydrolysis or enzymatic hydrolysis have also been demonstrated (Cembella et al., 1993, 1994; Oshima, 1995a; Murakami et al., 1999a,b), and in some cases within different organs of the same organism. Choi et al. (2003) demonstrated that the scallop *Chlamys nobilis* and the green-lipped mussel *Perna viridis* exposed to *Alexandrium tamarense* differ in their depuration kinetics, biotransformation and tissue distribution of PSP toxins. A significant fraction of GTX5 was detected in the mussels but not in the scallops and in both species the toxin profile differed from the toxic algae that had been consumed.

The reductive toxin transformations are characterised by a decrease in the N-OH toxin groups (GTX1, GTX4, Neo) which is accompanied by an increase in the N-H toxin group (GTX2, GTX3, STX) as well as by the elimination of the sulfate group at C11 atom (GTX1, GTX4 to Neo, GTX2, GTX3 to STX). These reductive eliminations are mainly observed in homogenates of digestive tissues. Hydrolytic cleavages occur in toxins bearing

a N-sulfocarbamoyl moiety (C1, C2, C3 C4, GTX5, GTX6) which can be non-enzymaticaly converted to their carbamate counterparts (GTX2, GTX3, GTX1, GTX4, STX, Neo) at pH values ≤ 7 (Hall and Reichardt, 1984; Oshima, 1995a). Hydrolytic cleavages are not very effective in tissues homogenates. This was demonstrated in Elke et al. (2007).

Epimerisation of the β-epimers (C2, GTX3, GTX4) primarily produced by dinoflagellates are usually transformed to their thermodynamically more stable α-epimers (C1, GTX2, GTX1) after their uptake by shellfish (Oshima, 1995a; Bricelj and Shumway, 1998). This transformation is common in almost all tissues of shellfish. Enzymatic hydrolysis of N-sulfocarbamate and carbamate toxins to decarbamates is probably restricted to a few bivalvia species (*Mactra chilensis*, *Peronidia venulosa*, *Protothaca staminea*) where it is usually observed in the digestive tissues (Elke et al., 2007).

1.3 Physiological responses of marine bivalves to PSP toxins

Toxicity of different shellfish in any given area is highly variable as well as the physiological and behavioural responses against toxic microalgae. Previous studies on the clearance rate of bivalves have found that the toxic dinoflagellate *Alexandrium* either inhibited (Shumway and Cucci, 1987; Bardouil et al., 1993; Lesser and Shumway, 1993), enhanced (Shumway and Cucci, 1987; Lesser and Shumway, 1993), or had no effect.

In general, bivalve species with nerves insensitive to PSP toxins (e.g., the mussel, *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 (e.g. the oyster, *Crassostrea virginica*), are highly sensitive to PSP toxins and exhibit physiological and behavioural mechanisms to avoid or reduce their exposure to toxic cells (Bricelj and Shumway, 1998).

Nevertheless, there are some discrepancies between the in vitro assay of nerve sensitivity to STX or TTX applied to several species of bivalves and the whole-organism response to toxic cells. For example, the northern quahog, *Mercenaria mercenaria*, is insensitive to STX based on the in vitro assay, but it is known to accumulate relatively low toxin levels during a major PSP outbreak (Twarog et al., 1972) and shows significant feeding inhibition and shell closure in the presence of a highly toxic *Alexandrium* isolate (Bricelj et al., 1991). In contrast, the surf-clam *Spisula solidissima* is highly sensitive to TTX, but shows no feeding or burrowing inhibition in response to dinoflagellate toxicities as high as 74 pg STX equiv. $cell^{-1}$ (Bricelj et al., 1996). Bricelj and Shumway (1998) showed that this surf-clam also achieve extremely high PSP toxicities in both field and laboratory studies and suggested that the nerve assay must be verified using alternative in vivo measures sensitivity, such as physiological and behavioural responses.

Measurements of feeding rate has provided a useful index to compare the toxin sensitivity and thus potential for toxin uptake of various species (Shumway and Cucci, 1987). For example, the maximum filtration rate of the scallop, *Chlamis nobilis* was double that of the clam, *Ruditapes philippinarum* when feeding on the algal mixture of *Alexandrium tamarense*. This clam may have reduced its potential toxicity by decreasing the uptake rate (Li et al., 2001). Li et al. (2002) showed a decline in the clearance rate of the clam *Rudithapes philippinarum* when exposed to a highly toxic *A. tamarense*, and also decreased absorption efficiency. Shumway and Cucci (1987) observed that the scallop *Placopecten magellanicus* exposed to toxic dinoflagellates may elicit vigorous swimming and clapping activity accompanied by the copious production of mucus and pseudofaeces, albeit without apparent significant reduction in clearance rate. Numerous literature studies have supported the wide range of responses of bivalves to toxic algal blooms and the species-specific effects of these blooms on the physiology, behaviour and rates of accumulation of PSP toxins in the tissues of the bivalves. Therefore, no generalities can be made.

1.4 Harmful algal blooms in New Zealand

New Zealand shellfish have been monitored for the presence of marine biotoxins since January 1993 when shellfish toxicity was first detected in New Zealand. Since 2002, the New Zealand Food Safety Authority (NZFSA) has been responsible for shellfish safety from harmful marine biotoxins. Although there are no authenticated records of human illness due to PSP in New Zealand, the nationwide phytoplankton and shellfish toxicity monitoring programme has revealed that the incidence of PSP contamination and the occurrence of the toxic *Alexandrium* species are more common than previously realised (Mackenzie et al., 2004).

Seasonal phytoplankton blooms in New Zealand commonly start with diatoms in early spring, followed by dinoflagellates in summer, with further late diatom blooms in autumn (Rhodes et al., 2001). Another species of dinoflagellate identified as a source of PSP is *Alexandrium catenella* which can bloom year around, but often appears after late summer storms in the Bay of Plenty, possibly due to the disturbance of sediments which trigger the germination of cyst beds (Rhodes et al., 2001). *Alexandrium tamarense* was first observed in a sample collected from Marsden Point, Bream Bay in April 1997. Since then this species has been observed in Northland, Hauraki Gulf and Houhoura. In April 2000, *A. tamarense* was observed off Moititi Island in Bay of Plenty and tentatively indentified in a sample from Port Gore, Marlborough Sounds in March 1999. In May 2003 it was observed in samples from Kennedy Bay on the eastern Coromandel coast. Cell numbers generally have been low. Mackenzie et al. (2004) indicated that no blooms of this species were encountered and there were no record of an association with any shellfish contamination event until that date.

In 1993 the first verification of shellfish contamination involving PSP toxins occurred in the Bay of Plenty, North Island (Smith et al., 1993). This was due to a bloom of *Alexandrium minutum* and the level of PSP toxicity reached >900 μg 100 g^{-1} in surf-clams *Paphies* sp. Other occurrences of low-level contamination with PSP toxin have been shown by Mackenzie et al. (1996a) (*A. minutum*) and Mackenzie et al. (1996b) (*A. ostenfeldii*).

Other marine biotoxins of public concern routinely found in New Zealand are domoic acid (ASP), and okadaic acid with its esteres DTX1, DTX2, DTX3 (DSP). Neurotoxic shellfish poisoning (NSP) cases may have occurred in 1993, however, no significant NSP toxicity has been detected since.

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Diarrhetic shellfish poisoning (DSP) is of great concern in New Zealand, in that low levels of okadaic acid can result in illness. Two cases of DSP were reported from the U.K. involving New Zealand greenshell mussels from Beatrix Bay in the Marlborough Sounds harvested at the end of 1993. There have been several DSP events tracked in non-commercial areas. The most prevalent one occurred in the non-commercial growing area in the Queen Charlotte Sound of the Marlborough Sounds where a long-term bloom of *Dinophysis acuta* has resulted in a six month closure. Okadaic acid has been found in the area in blue mussels and cockles, but not in scallops. The Akaroa Harbour on Banks Peninsula and the Timaru area, both in the South Island, have been also affected by DSP events (Trusewich et al., 1996).

In December 1994, several events of amnesic shellfish poisoning (ASP) were detected in three regions of the country; the Marlborough Sounds where greenshell mussels became toxic, Tauranga Harbour in the Bay of Plenty and, Doubtless Bay in Northland where scallops were most affected. In February 1995, the Biotoxin Management Board chose to increase the frequency of domoic acid testing, predominantly for scallops (Trusewich et al., 1996). Domoic acid is produced by *Pseudo-nitzschia* sp. blooms and several species are not toxic or produce extremely low concentrations of toxins per cell. Species identification is impossible under the light microscope, but whole DNA probes have been successfully merged into toxic algal monitoring programs including the New Zealand one (Rhodes et al., 2001).

1.5 Shellfish resources in New Zealand

Over the past 30 years aquaculture in New Zealand has grown from very small beginnings to a significant primary industry, currently estimated to be worth in excess of NZ$360 million annum, with a target of reaching $1 billion annum in sales by 2025. Around 66% of the aquaculture products are exported to 72 countries worldwide, and the industry is recognised as operating one of the strictest quality assurance programs for shellfish in the world (New Zealand Aquaculture farm facts, 2009). The main exported products are mussels, salmon and oysters, but many other shellfish are currently harvested and sold within New Zealand.

Greenshell^{TM} mussels (*Perna canaliculus*) are the major exported shellfish in New Zealand. The total exports of mussels in the year ending December 2008 were worth $NZ 204 million (The New Zealand Seafood Industry Council Ltd). The United States and European Union are the largest markets, taking around 30% each of the total exports, and the remainder goes largely to Asian nations and Australia. Mussels are produced in sheltered bays in longline culture at a number of locations in New Zealand. The most important growing area is within the Marlborough Sounds on the northern part of the South Island (Bondo et al., 2003).

Scallops (*Pecten novazelandiae*) are found around the coast in the sandbanks and mudbanks of sheltered bays from the low tide mark to about 50 metres depth. They lie on the seabed with the flat shell uppermost, often singly but sometimes grouped. The main dredging areas are Tasman Bay and Golden Bay, Marlborough Sounds, Coromandel coasts, and Northland coasts. They are best harvested before they spawn in the spring and early summer. New Zealand's scallop fishery is managed by strict quotas, which allow only a set quantity of scallop to be taken commercially each year. This Total Allowable Commercial Catch (TACC) was set at 841 metric tonnes for the 2008/09 fishing year (The New Zealand Seafood Industry Council Ltd).

The deepwater clam, tuatua (*Paphies donacina*) is an important handpicked resource of local people in New Zealand, especially in Pegasus Bay, Canterbury. It is found from low-tide to about 4 m, although juveniles may extend to the high-tide mark. This clam is subject to localised catastrophic mortality from erosion during storms, high temperatures and low oxygen levels during calm summer periods, blooms of toxic algae and excessive freshwater outflow (Cranfield and Michael, 2001).

Oysters (*Ostrea chilensis*) are widespread throughout New Zealand and is the target of commercial fisheries in Foveaux Strait and the Nelson/Marlborough (Challenger) fishery area. Shellfish dredging in Tasman Bay, Golden Bay, and the Marlborough Sounds is a multi-species fishery with oysters, scallops, and green-lipped mussels caught together. In recent years, the industry has voluntarily restricted catch levels according to the biomass and distribution of the population estimated in the annual biomass survey, and the economics of catch per unit effort during the season. Estimates of the recruited biomass (= 58 mm) of oysters in both Tasman Bay and Golden Bay (made from surveys of oysters and scallops combined) show a general decline from 1998 to 2008 (Ministry of Fisheries, 20 OYS7 09).

1.6 Aims of the thesis

This thesis examines the effects of PSP producing dinoflagellates on the physiology and behaviour of bivalve molluscs. This focus arises because these aspects have not been widely studied before in New Zealand. A full understanding of the mechanisms of uptake, accumulation and toxin dynamics of bivalves feeding up on toxic algae is fundamental for improving future regulations in the shellfish toxicity monitoring program. The physiological and behavioural responses of mussels, clams, scallops and oysters to the presence of the toxic dinoflagellate, *Alexandrium tamarense* was investigated. The hypothesis tested was that bivalve molluscs differ in their ability to metabolise PSP toxins produced by *A. tamarense* and are able to transform toxins and may have special mechanisms to avoid toxin uptake. To test this hypothesis, different

physiological/behavioural experiments and quantification of PSP toxins in bivalve tissues were carried out.

Chapter 2, describes the general methods applied in this study, including acclimation of bivalves to laboratory conditions, culture of microalgae, measurements of clearance and excretion rates, and toxin analyses of tissues and dinoflagellates. Information about statistical analyses and standardisation of data presented throughout the thesis have also been included in this chapter. These general methods were applied in Chapters 3, 4, 5 and 6.

Chapter 3 compares the short-term effect of *Alexandrium tamarense* on the clearance rate of mussels, scallops, clams and oysters. Species-specific differences were demonstrated and feeding responses were related to the accumulation of PSP toxins in the tissues of the bivalves.

Chapters 4, **5** and **6**, investigates the rate of intoxication and detoxication of mussels, clams and scallops exposed to toxic and non toxic dinoflagellates. These experiments included measurements of clearance and excretion rates and quantification of PSP toxins in the tissues. The toxin profile of different tissues was also investigated and complementary experiments exploring other responses of bivalves to dinoflagellates species were also included in these chapters. This was the case for studying byssus production and the presence of dinoflagellates in the faeces of mussels (Chapter 4), behaviour studies on clams (Chapter 5) and oxygen consumption of scallops (Chapter 6).

Chapter 7 is the general discussion and combines the results from Chapters 3, 4, 5 and 6. This allows the placement and discussion of the responses of bivalves from New Zealand in a world wide context. The conclusions present important information for future management of the shellfish resources in New Zealand exposed to a PSP toxic event.

Chapter 2

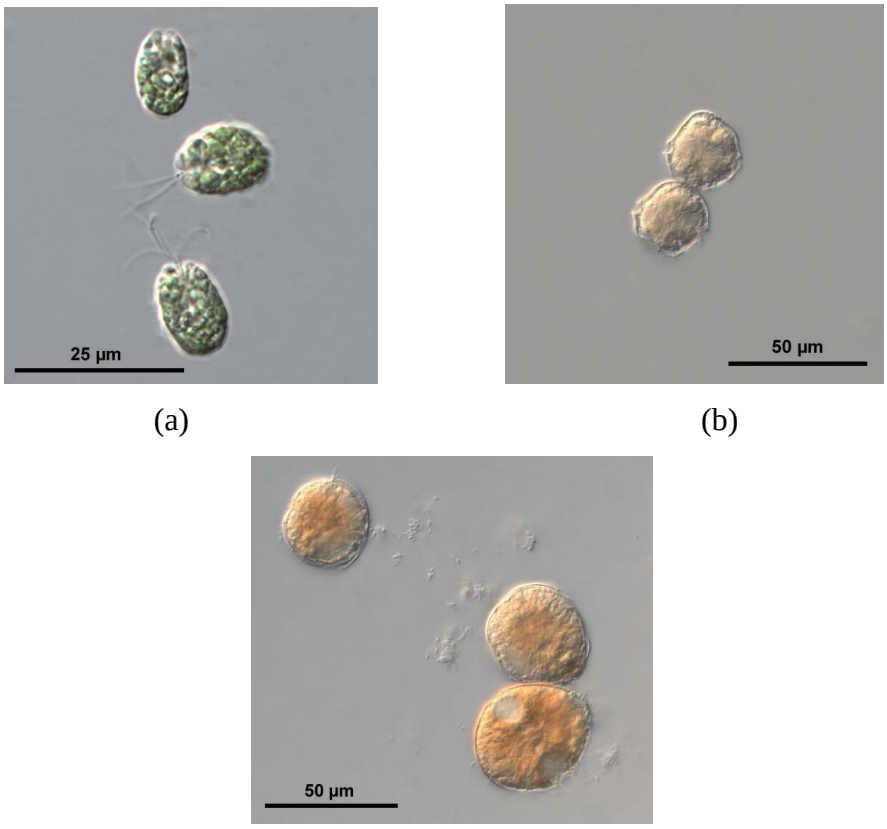
General methods

This chapter describes the methods applied in the subsequent chapters, including details on the growth and maintenance of algal cultures, laboratory acclimation of bivalves and the procedures for determining clearance rate and excretion rate. The protocol for extraction of PSP toxins from dinoflagellates and tissues and quantification by HPLC has also been included. The statistical analyses and standardisation of data used throughout the thesis are explained. These general methods were applied in Chapters 3, 4, 5 and 6.

2.1 Growth and maintenance of algal cultures

Three microalgae were cultured for feeding the bivalves. *Tetraselmis* sp. is a nontoxic green marine flagellate (9.9 ± 15.7 μm) commonly used as a live food for bivalves, crustaceans and rotifers in hatcheries. This microalgae was mostly used for feeding the bivalves before the start of experiments (acclimation period) and as a mixture with dinoflagellate cells (intoxication and detoxification periods). *Tetraselmis* sp. (Figure 2.1a) was cultured in a nutrient-enriched filtered seawater medium (f /2; Guillard, 1975) under 24 h continued light, 19°C and with constant aeration (10 L plastic bottles). The other two microalgae used in this study were the dinoflagellates *Alexandrium tamarense* (CAWD121) and *Alexandrium margalefii* (CAWD10). Both microalgae were supplied by Cawthron Institute in Nelson, New Zealand and cultures of these dinoflagellates were established at the University of Canterbury in Christchurch. *Alexandrium* species were grown in a nutrient-enriched filtered seawater medium (GP: Loeblich and Smith, 1968) under a 12 h light/dark cycle, 19°C and without aeration in 2 L flasks. Changes of the medium and subcultures were carried out every 3 weeks.

The dinoflagellate *A. tamarense* (Figure 2.1b) was isolated from Marsden Point, New Zealand (1997) where it is usually observed as single cells or occasionally couplets. It has been described as a PSP toxin producer (Mackenzie et al., 2004). Cells of this toxic dinoflagellate (size 28.4 ± 30.4 μm) were supplied to the bivalves during an intoxication period with the expectation that the bivalve tissues would become toxic. The dinoflagellate *A. margalefii* (Figure 2.1c) is a medium sized species (37.5 ± 40.0 μm) which was isolated from Marsden Point in 1993. This species is free of PSP toxins (Mackenzie et al., 2004) and was supplied for feeding the control bivalves and during detoxification periods. All species of microalgae were harvested during the exponential growth phase and were offered to the bivalves as living microalgae.



(a) (b)

(c)

Figure 2.1: Microalgae cultivated for feeding bivalves during experiments (a) *Tetraselmis* sp. (b) *Alexandrium tamarense* (c) *Alexandrium margalefii*. Photos were taken from cultures held at the University of Canterbury.

The number of cells in the cultures was determined indirectly using a Turner AquaFluor^{TM} Handheld fluorometer. For each species of microalgae a dilution set was prepared (different concentrations) and measured in the fluorometer. The same set was also counted using a Sedgwick-Rafter chamber. Plotting results from both procedures, a direct relationship was obtained (Table 2.1).

Table 2.1: Equations relating fluorometer readings and Sedgwick-Rafter cell counts to estimate the concentration of microalgae in cultures (x = number of cells; y = fluorometer reading).

Tetraselmis sp.	*A. tamarense*	*A. margalefii*
$y= 0.0059x^{1.2}$	$y= 0.9729x^{0.9}$	$y= 1.8129x^{0.8}$
$R^2= 0.99$	$R^2= 0.99$	$R^2= 0.99$

2.2 Laboratory acclimation of the bivalves

All bivalves studied were brought to the laboratory immediately after collection. The main collection sites were close to Christchurch; South New Brighton: 43º32’47.2’’S, 172º44’51.5’’E (clams), Banks Peninsula: 43º39’53.6’’S, 173º4’38.1’’E (mussels) and Lyttelton Harbour: 43º36’7.1’’S, 172º43’9.9’’E (oysters). Scallops were collected in Ketu Bay, Malborough Sound (40º59’5.8’’S, 173º59’15.8’’), five hours driving north from Christchurch and special transportation was arranged to avoid stress from the trip. More details about the transportation of the scallops are given in Chapter 5.

All bivalves were transported in seawater from the collection sites and then placed in aquaria with circulating sea water (15°C and salinity of 30ppt). These aquaria had a capacity of 60 L for mussels, clams and oysters, and 800 L for scallops. All the epibionts living on the shells were removed carefully using a knife. From the second day,

Tetraselmis sp. was supplied every day in keeping with the requirements of each species. Bivalves were acclimated to laboratory conditions for at least 2 weeks before the start of any experiments.

At the start of the experiments, randomly selected acclimated bivalves were placed in smaller aquaria or vessels with non-circulating seawater, but constant aeration for 24 hours. The amount of water in each aquarium varied according to the requirements of each species, number of animals in the aquaria and the response to tests on them (more details are given in the following chapters). The general experimental conditions were 15°C, salinity of 30ppt, constant aeration and a change of seawater every 2 days. Bivalves were kept in these conditions, for the duration of the experiments and on certain days, individuals were taken from these aquaria or vessels to measure the physiological responses.

2.3 Physiological measurements

2.3.1 Clearance rate

Clearance rate is defined as the volume of water cleared per unit time (L h^{-1}) and was measured in a static system containing filtered seawater and constant aeration (Coughlan, 1969). A single bivalve was placed in a vessel one hour before the start of the experiment (Figure 2.2).

To start the experiment, a known quantity of algae was supplied to the bivalves (initial concentration). This concentration of food was below the level of pseudofaeces production (pseudofaeces were not observed in any of the experiments carried out) therefore it was possible to estimate that clearance rate was equal to filtration rate and both terms have been reported throughout the thesis as the same feeding response.

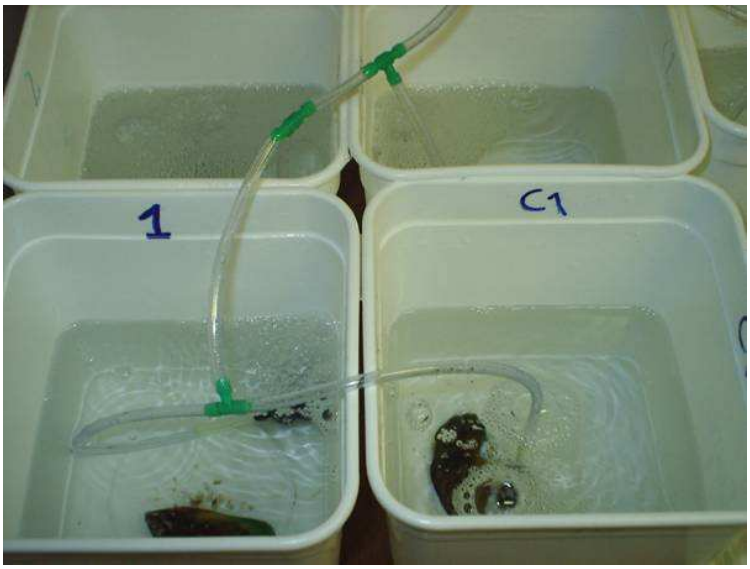


Figure 2.2: Individual vessels used for measuring the clearance rate in mussels. They contained 2 L of filtered seawater and had constant aeration.

To test for any sedimentation of the cells during the feeding measurements, a control vessel without a bivalve present was also maintained. The decrease of microalgal density resulting from bivalve filtration was determined every 30 min for three hours by fluorescence measurements. The water samples (3 mL) were taken from the middle part of the column of water using a pipette that was moved across the container.

Every 30 min, three replicates from the seawater were taken and read in the fluorometer. The average of the reading was converted to number of cells using previously equation calculated (Table 2.1). Six measurements were taken along the 3 hours and five clearance rates were calculated using the equation of Coughlan (1969):

$$CR = \frac{(\ln C_1 - \ln C_2)V}{t}$$

Where

CR = Clearance rate (L h^{-1})

C_1 = number of cells at time 0

C_2 = number of cells at time 1

V = experimental volume (litres)

t = time between measures C1 and C2 (hours)

The clearance rate of one individual under certain conditions was determined using the average of the five clearance rates calculated.

2.3.2 Excretion rate

Excretion rate is defined as the total proportion of absorbed energy eliminated as metabolic residues (ammonia) by excretion organs (μg NH4-N h^{-1}). This physiological response was immediately measured after the clearance rate experiments.

The excretion rate was measured in covered glass vessels using filtered seawater. Bivalves were maintained individually and a control vessel without a bivalve was maintained for the same time. Bivalves were left undisturbed for a period of time (1 to 2 hours) and seawater samples from each vessel then analysed by the Solórzano (1969) method:

$$ER = \frac{28 \cdot X \cdot V}{t}$$

Where

ER = excretion rate (μg NH4-N h^{-1})

X = ammonia excreted (NH4-N)

V = experimental volume (litres)

t = time (hours)

The analysis of ammonia is based on the spectrophotometric determination of the indophenol blue complex formed by the reaction of ammonia with phenol and hypochlorite, in alkaline pH.

Dilutions of known concentrations of ammonia were prepared and the reagents (phenol, hypochlorite and alkaline solutions) added. Phenol was always added first and the sample homogenised before addition of the next reagent. The same procedure was applied to the seawater samples. After the addition of the last reagent, all samples were covered with parafilm and placed in the dark (black box) for ~16 hours.

After incubation, the amount of ammonia in the sample was directly compared with the optical density of the color (blue) in the sample (Figure 2.3). A dark blue color indicated higher concentrations of ammonia. Calibration curves and the seawater samples were read in the spectophotometer at a wavelength of 640 nm. The concentration of excreted ammonia was used to calculate the excretion rate following the equation of Solórzano (1969) as described previously.

Figure 2.3: Dilutions series showing the different colours produced by known concentrations of ammonia. Dark blue represents high concentration and light blue represents lower concentration.

2.4 Analysis of PSP toxins

2.4.1 Extraction of PSP toxins from dinoflagellates

The same dinoflagellate culture used for the intoxication experiments of the shellfish was analysed to determine the PSP toxin profile. Samples of dinoflagellates were taken periodically over the three year period to detect any change in the toxin profile of *Alexandrium tamarense*. In the experiments described in Chapter 3, triplicate samples were taken on day 2 of each experiment. In the intoxication and detoxification experiments described in Chapters 4, 5 and 6, triplicate samples were taken on day 10. The total number of samples analysed of *A. tamarense* was 24 and the results were described in Chapter 3.

The number of cells in the culture was counted using a manual fluorometer calibrated previously with a Sedgwich-Rafter chamber (Table 2.1). The extraction of the PSP toxins followed the protocol in Wade et al. (2008) used by staff at the Cawthron Institute, Nelson with some modifications. An aliquot of the culture (14 ml) was gently centrifuged at 2500g for 5 min and the supernatant removed by pipetting carefully without disturbing the pellet. The pellets were stored frozen at -80°C until they were used in the extraction procedure. For the extraction the pellets were defrosted and 500 µL of 0.05M AcOH (acetic acid) added. Cells were ruptured by sonication (on ice) using a micro-tip cell disrupter for 2 min. Samples were centrifuged at 3000g for 10 min (4°C) and the supernatant (containing the toxins) transferred to ultrafiltration vials (10,000 Da molecular filter, Millipore Ultrafree-MC) and centrifuged (4ºC at 13000g for 30 min). The final filtrate was analysed by HPLC.

The total amount of toxins in the dinoflagellates was given by the sum of all toxins quantified in the run and is expressed as pg STX-diHCL equiv. $cell^{-1}$.

2.4.2 Extraction of PSP toxins from bivalve tissues

For the different experiments, bivalves were killed and the separated contaminated tissues were placed in 50 mL falcon tubes and frozen at -80°C until further extraction of the toxins. The extraction of the PSP toxins followed the protocol used by the Cawthron Institute (Wade et al., 2008) with some modifications. The tissues were defrosted and 0.1M HCl (Hydrochloric acid) was added in a 1:1 proportion (1 mL of HCl per 1 g of tissue) and homogenised by ultraturrax. To avoid transformation of PSP toxins during this procedure, samples were kept on ice. Next, the pH of the homogenate was measured (pH paper) and adjusted to pH 3-4 using either 1.0M HCl or 1.0M NaOH (Sodium hydroxide) as required followed by centrifugation of the mixture (3000g for 10 min at 4°C). The supernatant was passed through a cartridge column (Sep-Pack C18, Waters) which was pre-washed with 10 mL of methanol, air-dried for 1 min followed by 10 mL of water, and air-dried again for 1 min. To the dried cartridge was added 3 mL of the acid extract, allowing the first 1.5 mL to drain to waste and collecting the next 0.5 mL into an ultrafiltration vial (10,000 Da molecular filter, Millipore Ultrafree-MC). The ultrafiltration vials were centrifuged at 13000g for 30 minutes (4°C). The last clean up step is important for eliminating proteins. The final filtrate was analysed by HPLC.

The total amount of toxins in the tissues of bivalves was given by the sum of all toxins quantified in the run and is expressed as μg STX di-HCl equiv. 100 g^{-1} of wet tissue. Throughout this thesis, the quantification of toxin body burden of PSP toxins was always referred to 100 g of wet tissues of the bivalve.

2.4.3 Quantification of PSP toxins by HPLC with postcolumn oxidation

A rapid liquid chromatography assay with postcolumn oxidation was used for quantificating PSP toxins profiles. The assay followed the protocol in Wade et al. (2008) used at the Cawthron Institute with some modifications (see Table 2.2). The HPLC system involved two separate runs using a different column for each run: one for C1-C2 toxins and the other for GTX1-5, dcGTX2-3, Neo, dcNeo, STX and dcSTX toxins. C toxins were eluted from a column Phenomenex Synergy 4 μ Hydro RP-80A 250 x 4.6 using tetrabutyl ammonium phosphate (pH 5.8) for 10 min (1 mL min^{-1}). GTX/STX toxins were eluted

using a programmed gradient of four mobile phases over 40 min on a column Phenomenex Synergy 4 μ Polar RP-80A 250 x 4.6. The mobile phases included acetonitrile, ammonium phosphate (pH 6.9), sodium heptanesulphonic acid and Milli Q water at a combined flow rate of 1 mL min^{-1}.

The eluent from both runs was oxidised in a post-column reaction at 80°C, using NaIO4, to produce a fluorescent derivates. After this reaction, toxins were detected using a fluorescence detector (excitation 330nm, emission 390nm). All mobile phases and solutions were made up using Milli Q water and were filtered (0.45 μm) before use. The columns required care, especially the GTX/STX column where precipitation of buffer could readily take place. To avoid precipitation, after the daily runs, the system was washed with 95% Milli Q water and 5% acetonitrile. To start the analysis of a new batch of samples, the columns were each conditioned for 2 hours and a blank run followed by the standards mix. If the chromatogram of the standard mixture containing all toxins was properly separated, the batch of samples was injected.

The system was calibrated using the following standards from The Certificated References Materials Program (CRMP) operated by the National Research Council of Canada (NRC); C1, C2, dcGTX2, dcGTX3, dcSTX, dcNeo, GTX1, GTX2, GTX3, GTX4, GTX5, NEO and STX. Each PSP toxin standard was injected individually in a dilution series for correlating the peak area with the concentration of the toxin. This correlation was significant ($R^2 \geq 0.98$) for all the toxins analysed (data not shown). The linear relationship between the concentration of the toxin and the peak area allowed the mixing of different ratios of toxins in one solution leading to rapid identification of toxin identity in the HPLC chromatogram.

Table 2.2: Conditions for the analysis of PSP toxins by HPLC.

Parameter	Description/composition	Flow rate
Column C toxins	Phenomenex Synergy 4 μ Hydro RP-80A 250 x 4.6	1.0 mL min^{-1}
Injection volume	10 μL	
Mobile phase A	2mM Tetrabutyl ammonium phosphate, pH 5.8	
	Run isocratilly with stop time 10 min	
Column GTX/STX	Phenomenex Synergy 4 μ Polar RP-80A 250 x 4.6	1.0 mL min^{-1}
Injection volume	25 μL	
Mobile phase A	Milli Q water	
Mobile phase B	Acetonitrile HPLC grade	
Mobile phase C	50mM Ammonium phosphate, pH 6.9	
Mobile phase D	50mM Sodium heptanesulphonic acid	
Gradient	Start: A 67%, B 0%, C 11%, D 22%	
	12 min: A 67%, B 0%, C 11%, D 22%	
	13 min: A 30%, B 10%, C 11%, D 22%	
	34 min: A 67%, B 0%, C 11%, D 22%	
	Stop time 40 min	
Oxidising reagent	7mM Periodic acid in 50 mM potassium phosphate buffer, pH 7.8	0.4 mL min^{-1}
Acidifying reagent	0.5M Acetic acid	0.4 mL min^{-1}
Reaction post column	10m Teflon tubing (0.5mm ID) at 80ºC in oven	
Detector	Excitation 330nm, emission 390nm	
Column	Room temperature	

Two standards mixtures were prepared: one contained C toxins and the other contained the GTX/STX toxins. Separation of toxins in the chromatograms was successful for C1 (4.6 min), C2 (5.7 min), GTX4 (8.7 min), GTX1 (9.9 min), dcGTX2 (11.8 min), GTX3 (13.1 min), GTX2 (16.3 min), dcSTX (23.3 min) and STX (23.7 min). Toxins dcGTX3 (10.3 min) and GTX5 (10.6 min), eluted close to each other and separations were observed when the pH of the mobile phase C (Table 2.2) was between 6.9 and 7.0. Unfortunately, separation of these two toxins was not consistent during the trial runs, and it was decided to express them as a combination of the two toxins eluting at 10.6 min (dcGTX3 + GTX5). The same situation occurred with dcNEO (22.5 min) and Neo (22.4 min) and they were also expressed in the toxin profile as a combination that had eluted at 22.9 min (dcNeo + Neo). Due to the separation problem, the relative toxicity of these combinations was not possible to determine, therefore dcGTX + GTX5 was calculated using the relative toxicity of dcGTX3 (0.3766) and dcNeo + Neo was calculated using the relative toxicity of Neo (0.9243) (see Chapter 1, Table 1.1).

Each of the standard mixtures was diluted 10-fold and an aliquot injected at the beginning, during, and at the end of each batch of samples. Due to the complexity of this mixture (nine peaks), after every three samples, an injection of the GTX/STX standards mixture was made. For C toxins, an injection of the standard mixture was made after every five samples because of the easy identification of the two peaks. Chromatograms from the standards mixture and samples were compared using Chromeleon (Version 6.5) which allowed the integration and identification of the different peaks in the run. PSP toxins in the samples were quantified based in the known concentration of toxins present in the standard mixture. Some dilution of concentrated samples was required and corrections were made later in the calculations. Also, the total amount of toxins in the bivalves was standardised based on the wet weight of the tissue under study, or in the case of *Alexandrium tamarense*, by the number of cells collected in the sample.

2.5 Statistical analyses and standardisation of the data

2.5.1 Repeated measures design

Repeated measures ANOVA can be used for many different types of analysis, including conventional tests of significance, univariate solutions with adjusted degrees of freedom, two different types of multivariate statistics, or approaches that combine univariate and multivariate tests. Repeated measures designs constitute one of the most commonly used classes of designs in a variety of applied fields (Keselman et al., 1998)).

Specifically, the conventional univariate method of analysis assumes that the data have been obtained from populations that have the well-known normal (multivariate) form, that the degree of variability (covariance) among the levels of the repeated measure variable conforms to a spherical pattern, and that the data conform to independence assumptions. However, many other ANOVA-type statistics are available for the analysis of repeated measures designs. Some will be insensitive to violations of the assumptions associated with the conventional tests or do not depend on the conventional covariance assumption (i.e., multisample sphericity). These ANOVA-type procedures include univariate tests with adjusted degrees of freedom, multivariate test statistics, statistics that do not depend on the conventional assumptions of multisample sphericity, and hybrid types of analyses that involve a combining of the univariate and multivariate approaches (Keselman, 2001).

In the usual repeated measure design, the same subject is repeatedly observed under several conditions, (e.g. time points or treatments). The subjects are assumed to be homogeneous with respect to sex, age, etc. In most cases where measurements are taken repeatedly on the same subject, different variances of the response variable may be observed. A characteristic feature of the repeated measures design is that the effect a treatment has during its period of application, its direct effect, may persist into the following period or periods. If the effect persists only into the immediately following period the effect is called the first-order residual effect or residual effect for short. If the effect lasts into the following two periods it is referred to as the second-order residual effect, and so on. The choice of repeated measure design must therefore take into account

the possibility of residual effects and must be made in a way that the treatments can be efficiently compared after allowing for the residual effects (Iqbal and Jones, 1994).

This is the case of the design of the experiments presented in this thesis. Physiological responses were measured on the same individual over a period of time. Two treatments (PSP and control groups) were designed in which 4 bivalves (replicates) from each treatment were studied over the time. It is important to point out that in the case of the PSP group, individuals were exposed to two different periods (a toxic and a non-toxic). Due to this, the first period may have influenced the responses of the second period which has been taken in account in the interpretation of the statistical results obtained.

In this thesis, all experiments testing physiological and behavioural responses were run using a repeated measures design. In contrast with this, toxin analyses were done on different individuals on each day of sampling.

For the statistical analyses, data were first examined for homogeneity of variance (Levene's test) and normality (Kolmogorov-Smirnov). Then, repeated measures ANOVA was used to test the effects of the different treatments (PSP and control group) and the effect of exposure time on the response of bivalves. The significance threshold $\alpha = 0.05$ was used for all hypothesis tested. When a test was judged to be significant, a post-hoc Tukey multiple comparison test was performed (Snedecor and Cochran, 1989). Analyses were done using STATISTICA 8 and a complete repeated measures ANOVA table was reported when appropriate.

2.5.2 Standardisation of the data

Physiological and behavioral responses were related to the dry weight of the bivalves. For this, wet tissues were weighed then dried for 72 hour at 60°C and re-weighted. Individuals from the control group were killed to calculate the dry weight, whereas it was not possible to directly determined the dry weight of the bivalves from the PSP group (tissues were frozen for PSP toxins analyses). The dry weight of the PSP group bivalves was therefore calculated using the wet/dry weight ratio of the control group which was constant for each

species (data not shown). The dry weight of the bivalves was presented as the mean ± 1 standard deviation. The length of the shell was also represented with this same notation.

The influence of body size on filtration rates has been widely reviewed in the literature (Jørgensen, 1976; Winter, 1978; Newell, 1979) and it is now well documented that the filtration rate (y) increases with increasing body size (X) in accordance with the general allometric equation

$$y = a \cdot x^{b}$$

where a is the intercept and b is the weight exponential. Therefore, it is necessary to introduce a weight correction to compare bivalves of different sizes. The same general allometric equation was used to describe the effect of the body size on excretion rate and oxygen consumption in bivalves.

In practice, the value of 0.75 represents an approximate mean of b for a variety of bivalves (Jørgensen, 1976; Widdows, 1978; Winter, 1978). In this study, 0.75 was the value of the weight exponent b used to correct the data (rate of clearance, excretion and oxygen consumption) of a standard-sized organism (1 g dry weight). The following equation was used:

$$PR_s = \left[\frac{W_s}{W_e}\right] \cdot PR_e$$

where PR_s is the physiological response of a standard-sized animal, W_s is its weight (g), W_e is the weight of the experimental animal (g), PR_e is the uncorrected rate of the physiological response of the experimental animal, and b is the weight exponent (Bayne and Newell, 1983).

Results plotting the physiological responses of bivalves to the different treatments are presented as means ± 1 standard error.

The total toxic burden of PSP toxins in the tissues of bivalves is presented in a scatter graph where the mean value of toxicity (μg STX di-HCl equiv. 100 g^{-1}) for each day is connected through the intoxication and detoxification periods. The toxin profile of each tissue studied is presented in a stacked graph where each toxin is given as a percentage of the total toxin body burden. The toxic profile of *Alexandrium tamarense* is included in the graph. Also, the total content of toxins in the tissue (μg STX di-HCl equiv. 100 g^{-1}) has been placed above each bar.

Chapter 3

The effects of PSP toxins on the clearance rate of New Zealand marine bivalves

3.1 Introduction

It is well known that the accumulation of PSP toxins in bivalves is species-specific and the differences in toxicity between species can be as much as 100-fold (Bricelj and Shumway, 1998). Physiological processes and bioconversions of PSP toxins are believed to be the major factors controlling the tissue levels of the toxins. Comparative studies on PSP toxin profiles often show significant differences between the causative dinoflagellates and contaminated bivalves, thus indicating active toxin metabolism in shellfish (Oshima et al., 1990; Cembella et al., 1994).

The ability of shellfish to accumulate toxins may be largely dependant upon filtration rates and the density and distribution of the toxic dinoflagellates as well as the individual species' capabilities for selective ingestion and/or absorption (Shumway et al., 1985). Sensitivity to PSP toxins in bivalves has been evaluated using three different types of responses. Firstly there are neurological responses, or *in vivo* block of the action potential of nerve fibers exposed to STX (Twarog et al., 1972; Kvitek and Beitler, 1991). Secondly there are physiological responses, primarily the inhibition of clearance (feeding)

rates elicited by toxic dinoflagellates (Bricelj and Shumway, 1998), and thirdly there are behavioral responses such as shell valve movements, inhibition of siphon retraction, and the ability to burrow in sediments in infaunal bivalves (Bricelj et al., 1996).

In general, bivalves species that are neutrally insensitive to PSP toxins (e.g. the mussel, *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 (e.g. the oyster, *Crassostrea virginica*), are highly sensitive to PSP toxins and exhibit physiological and behavioural mechanisms to avoid or reduce exposure to toxic cells (Bricelj and Shumway, 1998). However, there are some discrepancies between the *in vitro* assay of nerve sensitivity to STX or TTX and the whole-organism response to toxic cell. For example, the northern quahog, *Mercenaria mercenaria*, is insensitive to STX based on the *in vitro* assay, but is known to accumulate relatively low toxin levels during a major PSP outbreak (Twarog et al., 1972) and shows significant feeding inhibition and shell closure in the presence of a highly toxic *Alexandrium* isolate (Bricelj et al., 1991). In contrast, the surfclam *Spisula solidissima* is highly sensitive to TTX, but shows no feeding or burrowing inhibition in response to dinoflagellate toxicities as high as 74 pg STX equiv. $cell^{-1}$ (Bricelj et al., 1996). Bricelj and Shumway (1998) showed that this surfclam also achieved extremely high PSP toxicities in both field and laboratory studies and suggested that the nerve assay must be verified using alternative *in vivo* measures of sensitivity, such as physiological and behavioural responses. Filtration rates provide a useful index to compare the toxin sensitivity and thus potential for uptake of various species (Shumway and Cucci, 1987). They can also be used to compare the response within a species to variations in dinoflagellate cell toxicity (Lee, 1993; Bricelj et al., 1996).

Toxic algal blooms present not only a public health hazard, but a major economic threat as well. The problems are more acute in some parts of the world than in others. The most effective means of controlling bivalve quality during outbreaks of toxic algae is either by a total closure during certain times of the year or by instituting a shellfish toxicity monitoring system. The latter approach has been carried out in many areas commonly plagued by such blooms (Shumway et al., 1988; Shumway, 1990). Bivalves differ in their ability to accumulate PSP toxins and such differences between co-occurring species are exploited to advantage in monitoring programs (Shumway et al., 1988; Bricelj and Shumway, 1998).

Since 1993, New Zealand has implemented a very successful monitoring program mainly based on cultivated mussels. However, because bivalves exhibit a wide range of responses and sensitivities to PSP toxins, it is necessary to investigate the effects of toxic dinoflagellates on the physiology of a diverse range of shellfish from New Zealand. The objective of this study was to expose five species of bivalves (mussels, clams, oysters and scallops) to the toxic dinoflagellate *Alexandrium tamarense* and measure clearance rate as an indication of their sensitivity to PSP toxins. Control bivalves were fed with a non-toxic species strain, *A. margaleffi*. Clearance rates at the end of the exposure period were measured and compared to the total content of PSP toxins in the bivalve tissues.

3.2 Methods

3.2.1 Collection of the bivalve molluscs

Mussels, *Perna canaliculus*, (dry weight 1.4 ± 0.3 g; shell length 72.8 ± 3.0 mm) were collected from subtidal culture ropes in Okains Bay, Banks Peninsula in August, 2007 (N = 50). Clams, *Paphies donacina* (dry weight 3.8 ± 0.8 g; length shell 86.2 ± 3.6 mm) and *Dosinia anus* (dry weight 1.5 ± 0.5 g; shell length 54.1 ± 5.6 mm) were collected at low tide by hand in the sand beach of South New Brighton in May and September, 2008 respectively (N =15 of each species). Adults of both species of clams were collected in the same place. Scallops, *Pecten novaezelandiae* (dry weight 3.6 ± 0.8 g; shell length 91.3 ± 0.4 mm) were collected in Ketu Bay, Marlborough Sounds by dredging in February, 2009 (N = 125). Oysters, *Ostrea chilensis* (dry weight 0.8 ± 0.2 g; shell length 64.3 ± 5.7 mm) were collected from a floating wharf in Lyttelton Harbour in July, 2009 (N = 20).

Bivalves were acclimated to the laboratory conditions in circulating seawater aquaria (non filtered) and were fed *ad libitum* with *Tetraselmis* sp. for two weeks. Mussels, oysters, and clams were placed in aquaria containing 60 L of circulating seawater, whereas scallops were placed in aquaria containing 800 L of circulating seawater. Before starting the experiments, bivalves were chosen randomly from these aquaria and placed individually in experimental vessels containing seawater with constant aeration.

3.2.2 Experimental design

Similar experiments were carried out for the five species of filter-feeding bivalves. The general design used was to place eight bivalves in individual vessels containing seawater, feed them with different microalgae and measure their clearance rates. First, they were fed with *Tetraselmis* sp. for 4 days and the clearance rate measured on the last day (day -1). From the next day (day 0), four replicates (four individuals) were exposed to the toxic dinoflagellate, *Alexandrium tamarense* (PSP group) and another four replicates (four individuals) were fed with the non-toxic dinoflagellate, *Alexandrium margallefii* (control group). Clearance rates were also measured under these conditions following the methodology describes in Coughlan (1969) as has been explained in Chapter 2 (2.3).

The general design was modified for each species of bivalve depending mainly of the size of the bivalves collected and the individual requirements of each species. Experiments differed in the volume of water used for maintaining bivalves and measuring clearance rates and the amount of algae used for feeding the bivalves and measuring clearance rates.

Mussels, clams and scallops were exposed to the *Alexandrium* species for 4 days, whereas oysters were exposed for 6 days. This was because of the large variability of clearance rates measured until day 4. It was decided to expose oysters for another two days in order to provide a more reliable estimate of the clearance rate. More details about differences between the experiments are given below and in Table 3.1.

Ostrea chilensis

Oysters were placed individually in vessels containing 2 L of seawater with constant aeration. For four days, oysters were fed with 2.5×10^7 cells day^{-1} $indiv.^{-1}$ of *Tetraselmis* sp. Clearance rate was measured on the last day using vessels containing 0.5 L of filtered seawater and the initial concentration for measuring the rates was 5×10^6 cells day^{-1} $indiv.^{-1}$ of *Tetraselmis* sp. Over the next six days, the PSP group was fed with 5×10^5 cells day^{-1} $indiv.^{-1}$ of *Alexandrium tamarense* and the control group was fed with 5×10^5 cells day^{-1}

indiv.$^{-1}$ of *A. margalefii*. Clearance rates were measured on days 0, 2, 4 and 6 and the initial concentration for measuring the rates was 2.5 x 10^5 cells L^{-1} of the *Alexandrium* species.

Pecten novaezelandiae

Scallops were placed individually in vessels containing 4 L of seawater with constant aeration. Clearance rates were measured using vessels containing 2 L of filtered seawater. For the first four days, scallops were fed with 2.5 x 10^7 cells day^{-1} indiv.$^{-1}$ of *Tetraselmis* sp. Clearance rates were measured on the last day using an initial concentration of 5 x 10^6 cells L^{-1} of *Tetraselmis* sp. Over the next four days, the PSP group was fed on 10^6 cells day^{-1} indiv.$^{-1}$ of *A. tamarense* and the control group fed on 10^6 cells day^{-1} indiv.$^{-1}$ of *A. margalefii*. Clearance rates were measured on days 0, 2 and 4 using an initial concentration of 5 x 10^5 cells L^{-1} of the *Alexandrium* species.

Paphies donacina

Clams were placed individually in vessels containing 2 L of seawater with constant aeration. Clearance rates were measured on the day before the start of the experiment and on days 0, 2 and 4 using vessels containing 1 L of filtered seawater. Over the first four days, clams were fed with 2.5 x 10^7 cells day^{-1} indiv.$^{-1}$ of *Tetraselmis* sp. Over the next four days, the PSP group was fed with 5 x 10^5 cells day^{-1} indiv.$^{-1}$ of *A. tamarense* and the control group was fed with 5 x 10^5 cells day^{-1} indiv.$^{-1}$ of *A. margalefii*. One day before the experiment, the clearance rates were measured using an initial concentration of 5 x 10^6 cells L^{-1} of *Tetraselmis* sp. On days 0, 2 and 4 the clearance rates were measured using an initial concentration of 2.5 x 10^5 cells L^{-1} of the *Alexandrium* species.

T**able 3.1:** Summary of the composition of the diets supplied to the bivalves over the experiments. The feeding diets correspond to the daily concentration of algae supplied to the bivalves. The clearance rate diets correspond to the initial concentration of algae used for measuring the clearance rate.

	Ostrea chilensis	***Pecten novaezelandiae***	***Paphies donacina***	***Perna canaliculus***	***Dosinia anus***
Feeding diets					
***Tetraselmis* sp.** cell day^{-1} indiv.$^{-1}$	2.5 x 10^7	2.5 x 10^7	2.5 x 10^7	5 x 10^7	2.5 x 10^7
A. tamarense					
nmol STX day^{-1}	175	350	175	350	175
cell day^{-1} indiv.$^{-1}$	5 x 10^5	10^6	5 x 10^5	10^6	5 x 10^5
A. margaleffi					
cell day^{-1} indiv.$^{-1}$	5 x 10^5	10^6	5 x 10^5	10^6	5 x 10^5
Volume vessel					
Liters	4	2	1	4	2
Clearance rate diets					
***Tetraselmis* sp.** cell day^{-1} indiv.$^{-1}$	5 x 10^6	5 x 10^6	5 x 10^6	10 x 10^6	5 x 10^6
A. tamarense					
nmol STX day^{-1}	9	18	9	18	9
cell day^{-1} indiv.$^{-1}$	2.5 x 10^5	5 x 10^5	2.5 x 10^5	5 x 10^5	2.5 x 10^5
A. margaleffi					
cell day^{-1} indiv.$^{-1}$	2.5 x 10^5	5 x 10^5	2.5 x 10^5	5 x 10^5	2.5 x 10^5
Volume vessel					
Liters	2	1	0.8	2	0.5

Perna canaliculus

Mussels were placed individually in vessels containing 4 L of seawater with constant aeration. The clearance rate was measured in vessels containing 2 L of filtered seawater at the end of the pre-feeding period and 0, 1, 2, 3 and on day 4. Over the four days of the pre-feeding period, mussels were fed on 5 x 10^7 cells day^{-1} $indiv.^{-1}$ of *Tetraselmis* sp. For the next four days, the PSP group was fed on 10^6 cells day^{-1} $indiv.^{-1}$ of *A. tamarense* and the control group was fed on 10^6 cells day^{-1} $indiv.^{-1}$ of *A. margalefii*. Clearance rates were measured using an initial concentration of 10^6 cells L^{-1} of *Tetraselmis* sp. and 5 x 10^5 cells L^{-1} of the *Alexandrium* species.

Dosinia anus

This species of clam was placed individually in vessels containing 1 L of seawater with constant aeration. Clearance rates were measured before the experiment and on days 0, 2 and 4 in smaller vessels containing 0.8 L of filtered seawater. For the first four days of the experiment, clams were fed with 2.5 x 10^7 cells day^{-1} $indiv^{-1}$ of *Tetraselmis* sp. For the next four days, the PSP group was fed with 5 x 10^5 cells day^{-1} $indiv.^{-1}$ of *Alexandrium tamarense* and the control group was fed with 5 x 10^5 cells day^{-1} $indiv.^{-1}$ of *A. margalefii*. Clearance rates were measured using an initial concentration of 5 x 10^6 cells L^{-1} of *Tetraselmis* sp. and 2.5 x 10^5 cells L^{-1} of the *Alexandrium* species.

Tissue preparation

After the last clearance rate measurement, the whole tissue of the bivalves from the PSP group was individually frozen at -80°C for later quantification of PSP toxins (4 replicates = 4 individuals). Samples of tissues (n = 4 per each species of bivalve) and *A. tamarense* (n = 3 per each experiment) were quantified by HPLC with post column oxidation. The extraction and quantification of PSP toxins followed the procedure described in chapter 2 (2.3).

3.3 Results

3.3.1 Effects of *A. tamarense* on the clearance rate of bivalves

Ostrea chilensis

The clearance rates of the PSP and control oysters were highly variable over the experiment (Figure 3.1). Both groups presented similar values (0.15 L h^{-1} g^{-1}) in the pre-feeding trial when they were both fed with *Tetraselmis* sp. On day 0, when oysters were exposed for the first time to *Alexandrium* species, both groups increased their clearance rates to values close to 0.45 L h^{-1} g^{-1}. On day 2, both groups decreased their clearances rates whereas on day 4, they both increased the clearance rate. Finally, on day 6 both groups decreased the clearances rates to values close to those recorded at the start. The PSP and the control group reached their highest clearance rates on day 4. The PSP group had lowest values (0.09 L h^{-1} g^{-1}) on day 2, whereas the rate for control group was lowest (0.15 L h^{-1} g^{-1}) at the start.

There were no significant differences in clearance rate between the PSP and the control groups (repeated measures ANOVA, $F_{1,6} = 0.05$, $P = 0.82$), however, the time had a significant effect (repeated measures ANOVA, $F_{4,24} = 8.97$, $P < 0.001$) on this physiological response. Interaction between both variables was not significant (Table 3.2). On day 4, the clearance rate of the PSP group was significantly higher than the rates observed before the experiment and on days 2 and 6 (post-hoc Tukey Test).

Pecten novaezelandiae

The clearance rate of scallops fed with the toxic dinoflagellate *A. tamarense* changed over four days of exposure to PSP dinoflagellates (Figure 3.2). Both groups when fed with *Tetraselmis* sp. had similar feeding rates (0.77 L h^{-1} g^{-1}), however, when the food supply was changed (day 0), the PSP group exposed to the toxic dinoflagellates decreased its clearance rate. On the same day, the control group fed with the non toxic dinoflagellate *A.*

margalefii had a rate similar to that observed for *Tetraselmis* sp.. Low feeding rates in the PSP group were also observed on days 2 and 4 and were on average 0.20 L h^{-1} g^{-1}. The control group fed with *Tetraselmis* sp. had similar clearance rates (0.86 L h^{-1} g^{-1}) to those observed on days 0, 2 and 4 (0.91 L h^{-1} g^{-1}) when fed on *A. margalefii* .

There were significant differences in the clearance rate between the PSP and control groups (repeated measures ANOVA, $F_{1,6} = 120.78$, $P < 0.001$). The effect of time on the clearance rate of scallops exposed to different algae was also significant (repeated measures ANOVA, $F_{3,18} = 4.16$, $P = 0.02$) as well as the interaction between both variables tested (repeated measures ANOVA, $F_{3,18} = 6.63$, $P = 0.003$) (Table 3.3). On days, 0, 2 and 4, clearance rates of the PSP group were significantly lower than those rates observed in the control group and also to the clearance rates observed before the experiment when fed with *Tetraselmis* sp. (post-hoc Tukey Test).

Paphies donacina

The clearance rate of the clam *Paphies donacina* was affected by the presence of dinoflagellates in the diet (Figure 3.3). When the PSP and control groups were fed with *Tetraselmis* sp., the feeding rate was 0.13 L h^{-1} g^{-1}. Later, when the diet was changed to *Alexandrium* species, both groups decreased the feeding rate to 0.06 L h^{-1} g^{-1} and the rate did not increase for the next four days.

There were no significant differences in the clearance rates between the PSP and control groups (repeated measures ANOVA, $F_{1,6} = 0.58$, $P = 0.47$), however there was a significant effect of time (repeated measures ANOVA, $F_{3,18} = 2.35$, $P < 0.001$) on this physiological response. The interaction of both variables did not affect the clearance rate of the clams exposed to this conditions (Table 3.4). When the PSP group was fed with *Tetraselmis* sp. the clearance rate was higher than those recorded on days 0 and 4 when fed on *A. tamarense*. Also, the control group fed on *Tetraselmis* sp. had a higher rate than recorded on days 0, 2 and 4 when fed on *A. margalefii*.

Perna canaliculus

The clearance rate of mussels was unaffected by the dinoflagellate species supplied in the diet (Figure 3.4). Overall, the clearance rate of mussels fed on *Tetraselmis* sp. was 1.29 L h^{-1} g^{-1} (PSP and control groups). When the food was changed to *Alexandrium* species, there was no change in the clearance rate were The rate for the PSP group was 1.17 L h^{-1} g^{-1} and control group; 1.24 L h^{-1} g^{-1}. There were no significant differences in the clearance rates between either treatments (repeated measures ANOVA, $F_{1,6} = 1.36$, $P = 0.28$), or days of exposure (repeated measures ANOVA, $F_{1,6} = 1.65$, $P = 0.17$) to the experimental conditions. Interaction between both variables was also not significant (Table 3.5).

Dosinia anus

The clearance rate of the clam, *Dosinia anus* was unaffected by the presence of dinoflagellates species supplied in the diet (Figure 3.5). Both groups fed on *Tetraselmis* sp. had a clearance rate close to 0.22 L h^{-1} g^{-1}, which was similar to the clearance rate observed on days 0, 2 and 4 when the clams had been fed on the *Alexandrium* species (0.23 L h^{-1} g^{-1} for both groups). There were no significant differences in the clearance rates between either treatments (repeated measures ANOVA, $F_{1,6} = 0.03$, $P = 0.85$), or days of exposure (repeated measures ANOVA, $F_{3,15} = 1.95$, $P = 0.16$). Interaction between both variables was also not significant (Table 3.6).

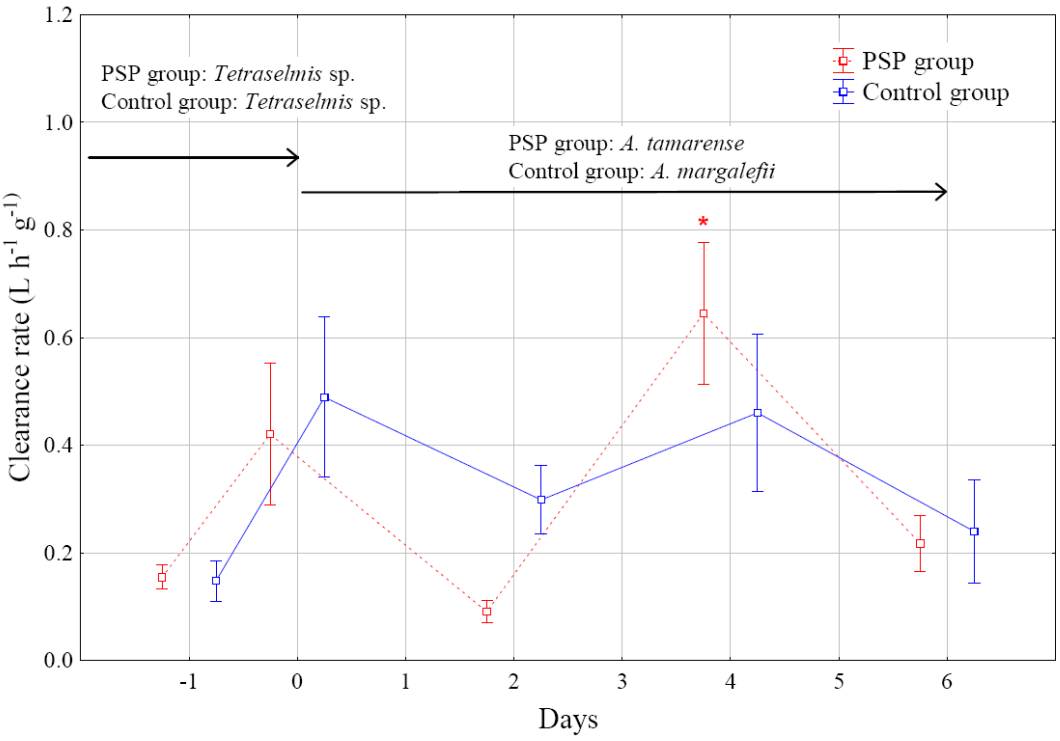


Figure 3.1: Clearance rate of *Ostrea chilensis* exposed to different microalgae. Measurements were taken on days -1, 0, 2, 4 and 6. The asterisk (*) represents significantly higher clearance rate. Values are means ± SE.

Table 3.2: Results of repeated measures ANOVA testing the effects of the algae supplied to *Ostrea chilensis* (treatment) and the days of exposure (time) on the clearance rate of oysters. Values in **bold** are of statistical significance.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.004	1	0.004	0.05	0.82
Error	0.49	6	0.08		
Days	0.99	4	0.24	**8.97**	**< 0.001**
Days*Treatments	0.16	4	0.04	1.44	0.25
Error	0.66	24	0.02		

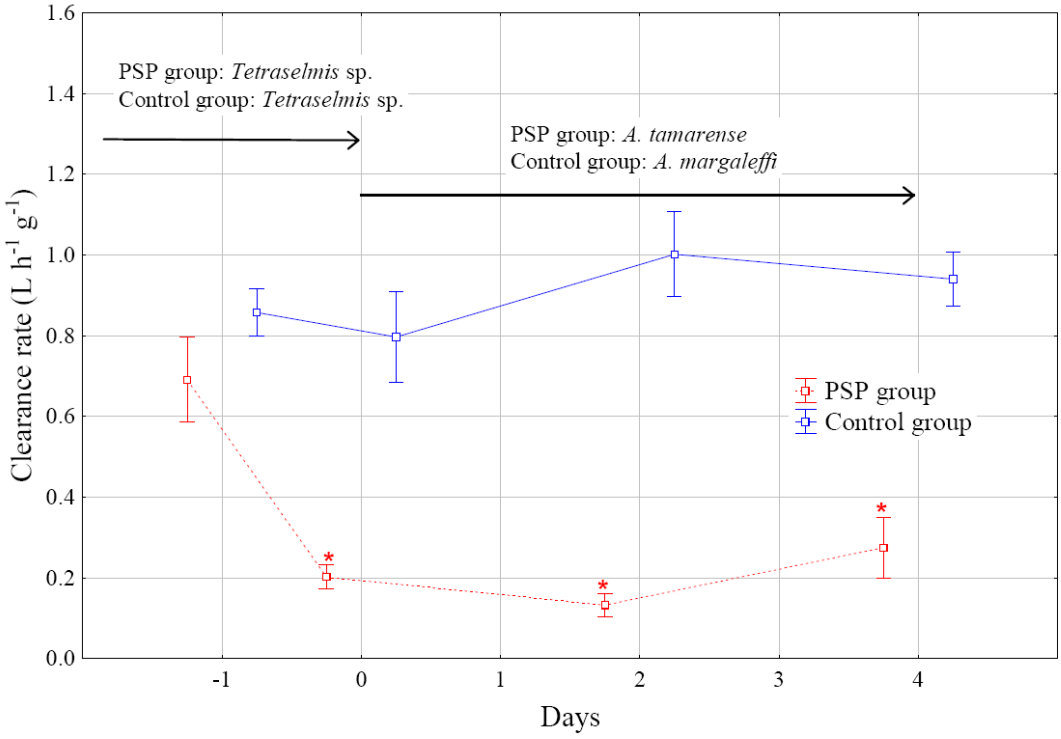


Figure 3.2: Clearance rate of *Pecten novaezelandiae* exposed to different microalgae. Measurements were taken on days -1, 0, 2 and 4. The asterisk (*) represents significantly lower clearance rate. Values are means ± SE.

Table 3.3: Results of repeated measures ANOVA testing the effects of the algae supplied to *Pecten novaezelandiae* (treatment) and the days of exposure (time) on the clearance rate of scallops. Values in **bold** are of statistical significance.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	2.63	1	2.63	**120.7**	**< 0.001**
Error	0.13	6	0.02		
Days	0.32	3	0.10	**4.16**	**0.02**
Days*Treatments	0.52	3	0.17	**6.63**	**0.03**
Error	0.47	18	0.02		

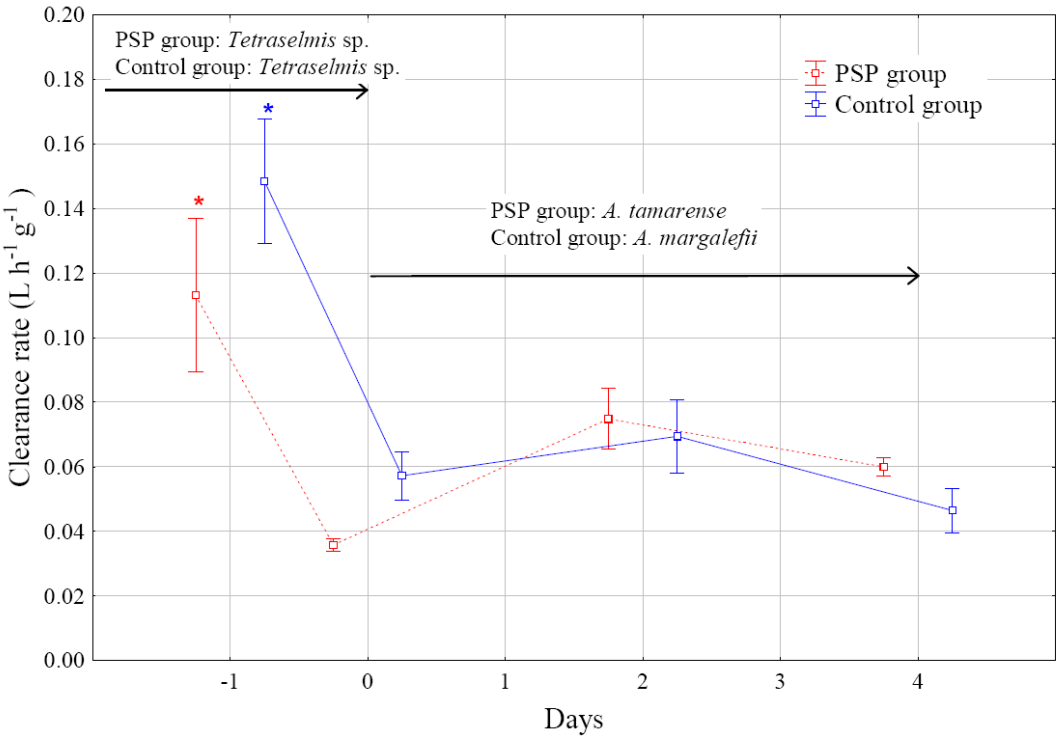


Figure 3.3: Clearance rate of *Paphies donacina* exposed to different microalgae. Measurements were taken on days -1, 0, 2 and 4. The asterisk (*) represents significantly higher clearance rate. Values are means ± SE.

Table 3.4: Results of repeated measures ANOVA testing the effects of the algae supplied to *Paphies donacina* (treatment) and the days of exposure (time) on the clearance rate of clams. Values in **bold** are of statistical significance.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.0007	1	0.0007	0.58	0.47
Error	0.007	6	0.001		
Days	0.03	3	0.011	**26.6**	**< 0.001**
Days*Treatments	0.003	3	0.001	2.35	0.1
Error	0.007	18	0.0004		

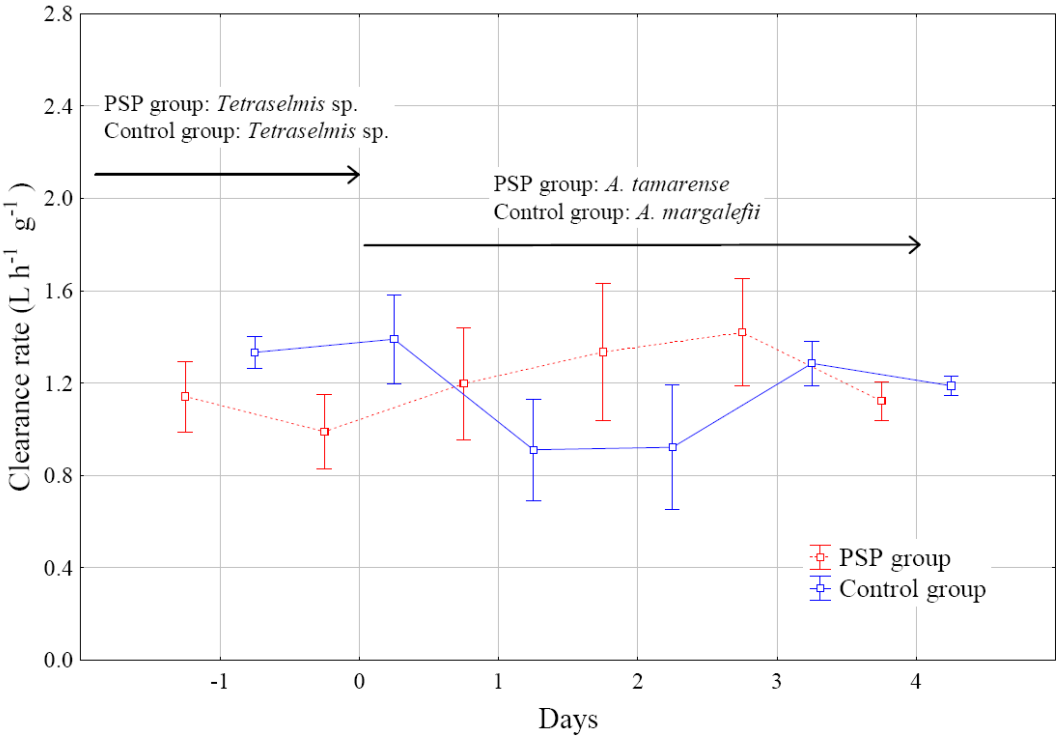


Figure 3.4: Clearance rate of *Perna canaliculus* exposed to different microalgae. Measurements were taken on days -1, 0, 1, 2, 3 and 4. Values are means ± SE.

Table 3.5: Results of repeated measures ANOVA testing the effects of the algae supplied to *Perna canaliculus* (treatment) and days of exposure (time) on the clearance rate of mussels.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.05	1	0.05	1.36	0.28
Error	0.23	6	0.03		
Days	0.38	5	0.07	1.65	0.17
Days*Treatments	0.10	5	0.02	0.43	0.82
Error	1.3	30	0.04		

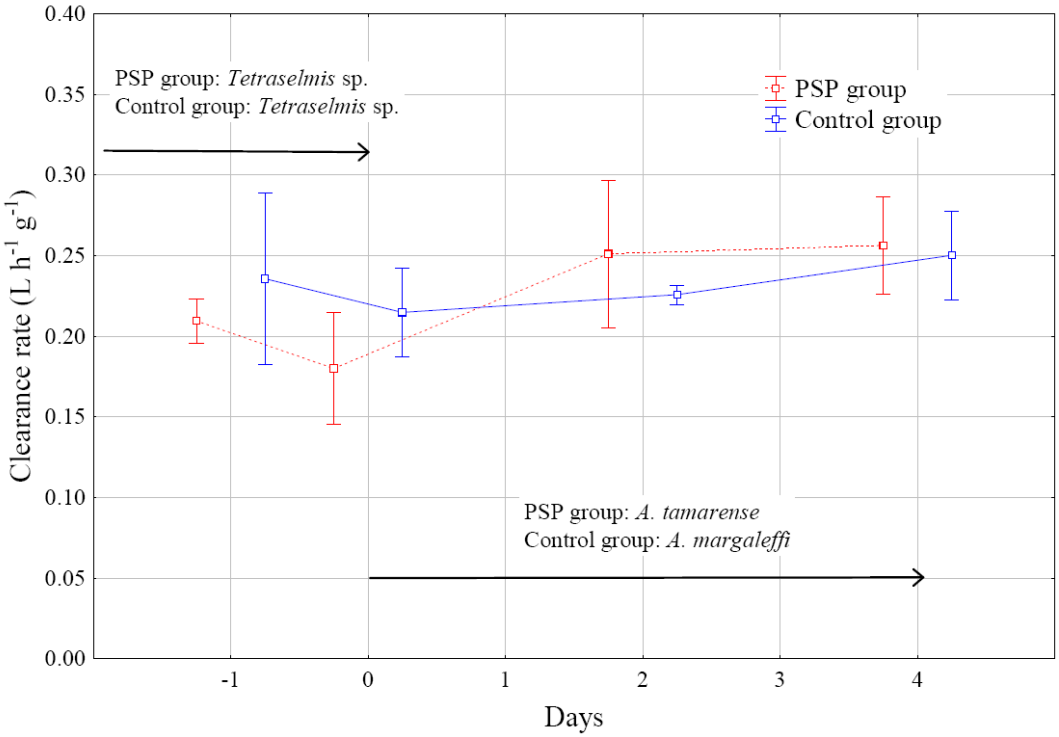


Figure 3.5: Clearance rate of *Dosinia anus* exposed to different microalgae. Measurements were taken on days -1, 0, 2 and 4. Values are means ± SE.

Table 3.6: Results of repeated measures ANOVA testing the effects of the algae supplied to *Dosinia anus* (treatment) and days of exposure (time) on the clearance rate of clams.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.0003	1	0.0003	0.03	0.85
Error	00.04	5	0.009		
Days	0.01	3	0.003	1.95	0.16
Days*Treatments	0.004	3	0.001	0.65	0.58
Error	0.02	15	0.001		

3.3.2 Toxin analyses

PSP toxin profile of *Alexandrium tamarense*

The toxin profile of *A. tamarense* was dominated by the N-sulfocarbamoyl toxin, C2 (91.5%). The other toxins identified were dcSTX (5.3%) and GTX4 (1.2%) accompanied by trace amounts of dcNeo + Neo (0.5%), STX (0.4%), dcGTX3 + GTX5 (0.4%), C1 (%0.3), GTX1 (%0.2) and GTX3 (0.1%) (Figure 3.6). Although the total toxin content was 353 ± 97 fmol cell^{-1}, the predominance of C2 resulted in a cell specific toxicity of 35 ± 9 fmol STX-diHCl equiv. cell^{-1} (Table 3.7) or 16 ± 4 pg STX-diHCl equiv. cell^{-1}.

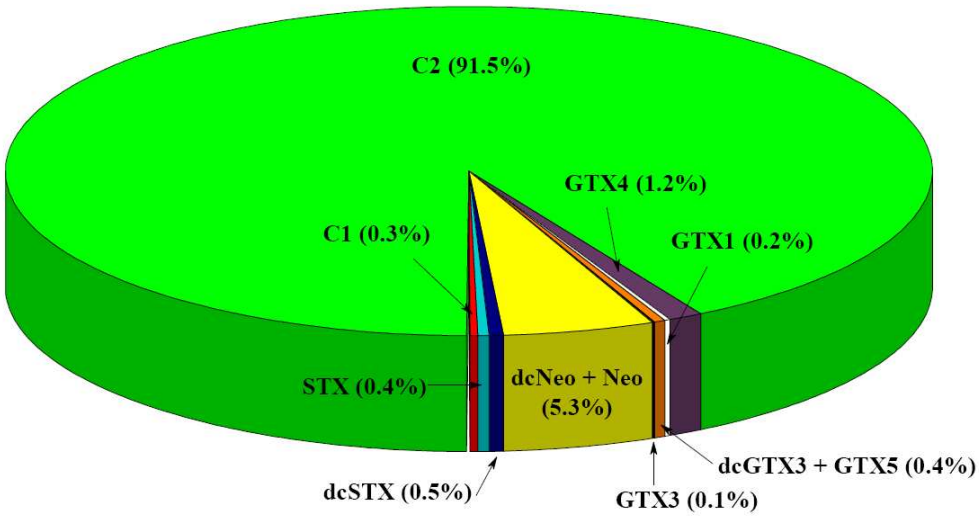


Figure 3.6: PSP toxin profile of *Alexandrium tamarense*.

Table 3.7: PSP toxin profile of *A. tamarense* expressed in fmol cell^{-1}, fmol STX-diHCl equiv. cell^{-1} and as a proportion of the total PSP content per cell (%).

	Toxic content (fmol cell^{-1})	Specific toxicity (fmol STX-diHCl equiv. cell^{-1})	Proportions of PSP toxins (%)
GTX4	0.6	0.4	1.2
GTX1	0.1	0.1	0.2
dcGTX3 + GTX5	0.4	0.1	0.4
GTX3	0.1	0.05	0.1
dcNeo + Neo	2.0	1.9	5.3
dcSTX	0.4	0.2	0.5
STX	0.2	0.2	0.4
C1	17.6	0.1	0.3
C2	332.1	32.0	91.5
Total	353	35	100

PSP toxin profile in the tissues of shellfish

Ostrea chilensis

The toxin profile of oysters was dominated by the N-sulfacarbamoyl, C2 (49%). The other major components found in the tissues were GTX1 (21%), dcNeo + Neo (11%) and GTX4 (11%). Small amounts of dcGTX2 were also detected in oysters, but were not present in the dinoflagellate. Oysters had accumulated 46 μg STX di-HCl equiv. 100 g^{-1} of tissue after six days of exposure to *A. tamarense*.

Pecten novaezelandiae

The toxin profile of scallops was dominated by the N-sulfacarbamoyl, C2 (60%), followed by the carbamoyl toxins GTX1 and GTX4 (19% and 11% respectively). The GTX2 was detected in scallops but was not observed in the dinoflagellate. Scallops had accumulated 30 μg STX di-HCl equiv. 100 g^{-1} after four days of exposure to *A. tamarense*.

Paphies donacina

The toxin profile of this clam was dominated by dcGTX3 + GTX5 (47%), while the carbamoyl toxins, GTX1 and STX were present in 13% and 10% respectively. The decarbamoyl toxin, dcGTX2 was found in the clams (10%) but was not detected in the toxin profile of the dinoflagellate. Low quantities of C2 were presented in the toxic profile of the clam. The tissues of *P. donacina* had accumulated 28 μg STX di-HCl equiv. 100 g^{-1} after four days of exposure to *A. tamarense*.

Perna canaliculus

The toxin profile of mussels was dominated by the carbamoyl toxin, GTX1 (72%) and N-sulfocarbamoyl toxin, C2 (19%). The compound GTX2, was detected in the tissues of mussels but not in the toxin profile of the dinoflagellate. Mussels had accumulated 88 μg STX di-HCl equiv. 100 g^{-1} after 4 days of feeding with *A. tamarense*.

Dosinia anus

The toxin profile of this clam was dominated by the carbamoyl toxin, GTX1 (48%) and the N-sulfocarbamoyl, C2 (37%). The GTX4 and dcNeo + Neo were also found in the tissues (6% and 4%). Small quantities of dcGTX2 were detected in the clams, but were not observed in the dinoflagellate. *Dosinia anus* had accumulated 59 μg STX di-HCl equiv. 100 g^{-1} after 4 days of exposure to *A. tamarense*.

The toxin profiles of the five species of bivalves exposure to *A. tamarense* are presented in Figure 3.7 and Table 3.8.

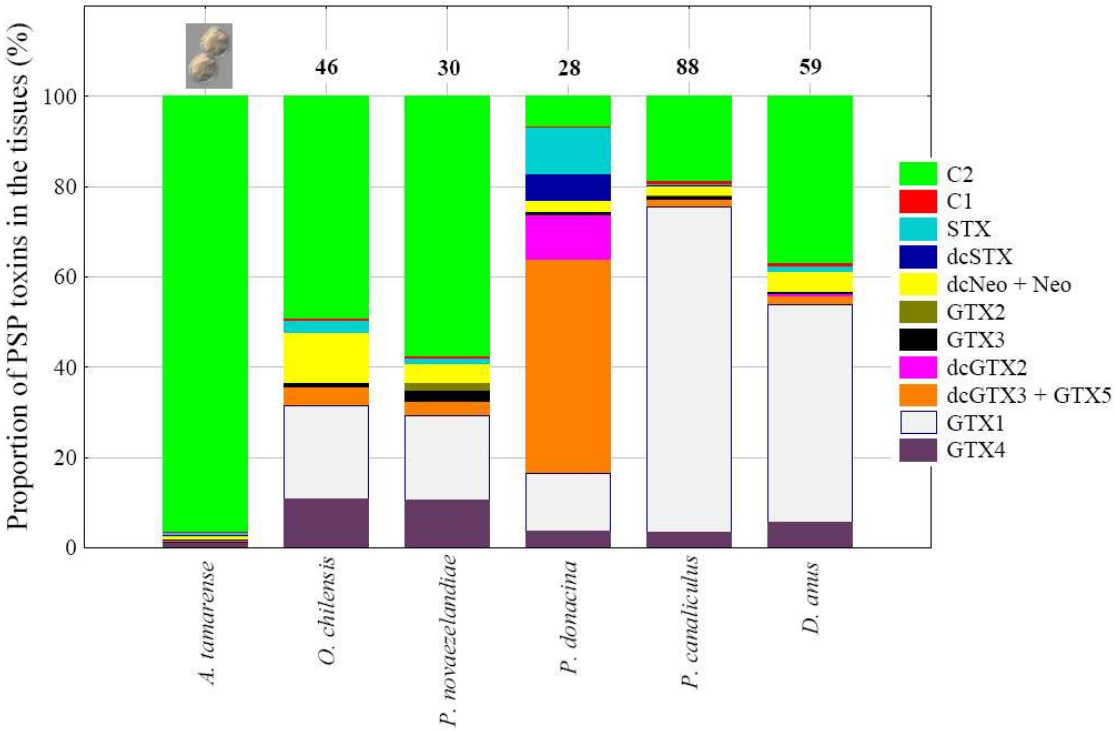


Figure 3.7: PSP toxin profiles of oysters (*O. chilensis*), scallops (*P. novaezelandiae*) clams (*P. donacina*, *D. anus*) and mussels, (*P. canaliculus*) fed with the dinoflagellate *A. tamarense*. The total content of toxins in the tissues is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}).

Table 3.8: PSP toxin profile of oysters (*O. chilensis*), scallops (*P. novaezelandiae*), clams (*P. donacina, D. anus*), and mussels (*P. canaliculus*) fed with the dinoflagellate *A. tamarense*. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in this tissue (%). The compound dominating the toxin profiles of each bivalve is shown in **bold**.

	O. chilensis		*P. novaezelandiae*		*P. donacina*		*P. canaliculus*		*D. anus*	
	T	%	T	%	T	%	T	%	T	%
GTX4	5.0	10.8	3.2	10.7	1.1	3.8	3.1	3.6	3.3	5.6
GTX1	9.6	20.7	5.5	18.6	3.5	12.7	**63.1**	**71.8**	**28.2**	**48.1**
dcGTX3 + GTX5	1.7	3.8	0.9	2.9	**13.0**	**47.3**	1.3	1.5	1.0	1.7
dcGTX2	0.1	0.2	-	-	2.7	9.8	-	-	0.4	0.6
GTX3	0.4	0.9	0.8	2.5	0.2	0.8	0.8	0.9	0.3	0.6
GTX2	-	-	0.5	1.7	-	-	0.2	0.2	-	-
dcNeo + Neo	5.1	11.0	1.3	4.2	0.7	2.4	1.6	1.8	2.6	4.4
dcSTX	-	-	-	-	1.6	5.9	0.3	0.3	-	-
STX	1.3	2.9	0.3	1.1	2.9	10.4	0.2	0.3	0.6	1.1
C1	0.2	0.5	0.2	0.6	0.1	0.2	0.5	0.6	0.5	0.9
C2	**22.7**	**49.3**	**17.2**	**57.7**	1.9	6.8	16.7	19.0	21.7	37.0
Total	46	100	30	100	28	100	88	100	59	100

3.4 Discussion

Considerable differences were observed in the feeding responses of bivalves from New Zealand exposed to the toxic dinoflagellate *Alexandrium tamarense*. The scallop, *Pecten novaezelandiae*, was the most sensitive of the species and the clearance rate was significantly reduced from the first contact with the toxic dinoflagellate. *Paphies donacina* was also sensitive to the presence of *Alexandrium* species as shown by a decrease of the clearance rate. The mussel, *Perna canaliculus* and the clam, *Dosinia anus* were insensitive to the presence of dinoflagellates in the diet and PSP toxins. Clearance rates measured on the oyster, *Ostrea chilensis* were variable throughout the experiment.

The species-specific differences in the clearance rate were also reflected in the total content of toxins in the tissues. Two species of bivalve *P. novaezelandiae* and *P. donacina* accumulated fewer toxins in their tissues than the most resistant bivalves (*P. canaliculus* and *D. anus*). Oysters accumulated more toxins than the other species, but they were exposed to two more days of feeding with *A. tamarense* therefore, they may actually have a similar intoxication response to *P. novaezalandiae* and *P. donacina*.

3.4.1 Clearance rate as indicator of sensitivity to PSP toxins

Considerable differences in clearance rates have been found between different bivalve species. Palmer (1980) compared filtration behaviour in the oyster, *Crassostrea virginica* and the scallop, *Argopecten irradians*. The scallops had relatively a constant clearance rate over 26 hours, while the oysters showed alternating periods of high and low rates. In addition, the functional state of the gill and its efficiency as a filter will affect the clearance rate. Any capacity to alter the retention efficiency of the gill in response to changes in food quantity and quality would be of considerable value to the animal (Bayne and Newell, 1983). The amount of material retained by the filtration system varies somewhat in different suspension-feeding organisms and is dependant primarily on the porosity of the gill (Foster-Smith, 1976). Other factors as temperature, particle concentration and composition (organic content, size), tidal level, body size also affect the rate of feeding in bivalves.

Filter-feeding bivalve molluscs vary widely in their capacity to ingest toxic dinoflagellates (Shumway and Cucci 1987; Lassus et al., 1989; Bricelj et al., 1991) and their feeding responses to toxic *Alexandrium* spp. as a sole food source are species-specific. Previous studies have found that the toxic dinoflagellate *Alexandrium* either inhibited (Shumway and Cucci, 1987; Bardouil et al., 1993; Lesser and Shumway, 1993;), enhanced (Shumway and Cucci, 1987; Lesser and Shumway, 1993), or did not affect (Shumway and Cucci, 1987; Bricelj et al., 1990; Marsden and Shumway, 1992) the clearance rate of bivalve molluscs. Not surprisingly the five species of bivalves from New Zealand tested in this study presented differences in clearance rate when they were exposed to dinoflagellates containing PSP toxins.

Mussels

In general, mussels (e.g. *Mytilus edulis*) are tolerant of PSP toxins and they readily feed on toxic cells. Also, a high PSP toxin level is found in their tissues (Bricelj et al., 1990). In contrast, species that attain relatively low toxicities (e.g. the oyster *Crassostrea virginica*) are highly sensitive to PSP toxins and exhibit physiological and behavioral mechanisms to avoid or reduce exposure to toxic cells such as inhibition of filtration rate (Shumway et al., 1990; Bricelj and Shumway, 1998). In this study, the New Zealand mussel, *Perna canaliculus* fed normally on toxic and non-toxic dinoflagellate cells as reported in Marsden and Shumway (1992). These authors also observed that *P. canaliculus* showed no dramatic physiological effects following short-term feeding on *A. tamarense*.

Clams

The North American soft-shell clam, *Mya arenaria,* differs in its behavioural and physiological responses to toxic *Alexandrium* cells under laboratory conditions, depending on its prior history of PSP toxin exposure in the field. *Mya arenaria*, from a population with no known history of PSP, had reduced feeding rates on toxic cells, reduced toxin accumulation rates, lower survival and greater *in vitro* nerve sensitivity to STX compared

to clams from a population that had experienced recurrent PSP outbreaks (Bricelj et al., 2005).

Bricelj et al. (1996) indicated that a significant reduction in the clearance rate of *M. arenaria* occurred only when clams were fed on the highly toxic *Alexandrium excavatum* PR18b (74.5 pg STX equiv. $cell^{-1}$), but not on other, less toxic species. In this study, the clearance rates of the New Zealand clam *Paphies donacina* fed on toxic and non-toxic *Alexandrium* species were significantly lower than the feeding rates observed when fed on *Tetraselmis* sp. Although a clear effect from the *Alexandrium* species was observed on the clearance rate of *P. donacina*, it was not possible to conclude whether the PSP toxins resulted in any physiological response.

There are three possible explanations of these results:

1. The effect of PSP toxins was masked by the effect of the *Alexandrium* cells,
2. The PSP toxins did not affect the clearance rate of *P. donacina*, or
3. The toxicity of *A. tamarense* used for feeding the bivalves (16 pg STX-diHCl $cell^{-1}$) may have been too low to cause an inhibitory effect on the feeding rate of this clam. This was observed in *M. arenaria* (Bricelj et al., 1996).

In contrast, other species of clams such as *Spisula solidissima* appear insensitive to the effects of PSP toxins and can thus attain high toxicities of PSP toxins in their tissues (Shumway et al., 1994; Silvert et al., 1998). In the present study, the clam *Dosinia anus* was insensitive to PSP toxins and to the effect of *Alexandrium* species. The results from *P. donacina* and *D. anus* from New Zealand, confirm that species that coexist in the same habitat have different responses when exposed to toxic dinoflagellates cells.

Scallops

The scallop, *Pecten magellanicus* exposed to *Protogongaulax tamarensis* fed normally, but they also showed striking behavioural responses (Shumway and Cucci, 1987). They exhibited an immediate closure of the shell valves followed by either violent swimming activity, partial sustained shell-valve closure, or a combination of the two. On addition of

clean sea water, this activity ceased. Juveniles of the giant lions-paw scallop, *Nodipecten subnodasus* showed a decreased clearance rate when exposed to *Gymnodinium catenatum* at concentrations greater than 450 cell ml^{-1} (Estrada et al., 2007).

In this study, the New Zealand scallop, *Pecten novaezelandiae* was highly affected by toxic dinoflagellates in the diet. Scallops reduced their clearance rate significantly from the first contact with *Alexandrium tamarense* cells until the end of the experiment. Control scallops fed normally on non-toxic dinoflagellate cells confirming the effects of PSP toxins on the clearance rate of the bivalve.

Oysters

In this study, as in other studies (Shumway et al., 1990; Luckenbach et al., 1993; Lassus et al., 1996), a high individual and daily variation in physiological responses in oysters was observed. Shumway and Cucci (1987) found that the clearance rate of *Crassostrea virginica* was significantly inhibited by addition of toxic cells, whereas that in *Ostrea edulis* was stimulated. Furthermore, in Maine waters *O. edulis* became toxic several days prior to any other species, including the mussel, *Mytilus edulis* (Shumway et al., 1988).

In this study, *Ostrea chilensis* did not respond clearly to any of the variables tested over the experiment, possibly due to the low number of replicates. Oysters increased and decreased clearance rates over the 6 days of exposure to *Alexandrium* species showing an erratic response over the time. The results were not conclusive and further studies are needed, including a higher number of replicates and a longer period of exposure to toxic and non-toxic dinoflagellates cells.

3.4.2 Comparison between PSP toxin profiles of bivalves fed with *A. tamarense*

PSP toxin profile of *A. tamarense*

The toxin profile of bivalves fed with *Alexandrium tamarense* showed considerable differences between each other and to the toxin profile of the dinoflagellate. *Alexandrium*

tamarense (CAWD 121) isolated in 1997 from Marsden Point, Bream Bay, has been characterised by Mackenzie et al. (2004) as having a toxin profile dominated by the N-sulfocarbamoyl toxins C3-4, with lesser amounts of C1-2 and trace amount of GTX4 and GTX1. This species had a specific toxicity of 3.2 pg STX equiv. $cell^{-1}$. The same strain was obtained from Cawthron Institute and has cultured at the University of Canterbury under the same controlled conditions of temperature and photo-period (see Chapter 2 (2.1)).

Analysis of the toxic profile of these cultures showed considerable differences to the results presented by Mackenzie et al. (2004) with *A. tamarense* cultured at University of Canterbury being dominated by the N-sulfocarbamoyl toxin, C2 (91.5%) followed by dcNeo + Neo (5.3%). Trace amounts of GTX4, dcSTX, STX, dcGTX3 + GTX5, C1, GTX1, GTX3, were also detected. The total toxin content (353 fmol $cell^{-1}$) and the specific toxicity (16 pg STX equiv $cell^{-1}$) were also higher than that registered in Mackenzie et al. (2004) (329 fmol $cell^{-1}$ and 3 pg STX equiv $cell^{-1}$ respectively).

Although conditions at University of Canterbury were set up following the same conditions as in Cawthron Institute, some differences may have occurred which modified the toxin profile of the dinoflagellate. This assumption is supported by the results obtained from three extracts of *A. tamarense* cultured at University of Canterbury and analysed in Cawthron Institute. These results (data not shown) showed similar profiles to those obtained at University of Canterbury. The total number of samples analysed were not sufficient to support further conclusions.

To clarify whether it was the controlled cultured conditions or differences in the procedures developed in both laboratories to quantify PSP toxins that were affecting the toxin profiles further studies would need to be done. These studies may have to involve analyses of the cultures from Cawthron Institute and University of Canterbury and crossed analyses of the PSP toxin profiles in both laboratories.

PSP toxin profile of bivalves

Differences between the toxin composition in ingested dinoflagellates and bivalve tissues may arise from epimerisation of the ingested toxins, chemical or enzymatic

transformations and/or selective retention of the individual toxins. These differences are greatest when dinoflagellate strains are rich in the low-potency, but highly labile N-sulfocarbamoyl toxins (Bricelj et al., 1991). These differences are notable in a few clam species that are capable of enzymatic production of decarbamoyl toxins from N-sulfocarbamoyl and, in some cases, carbamate derivatives: *Spisula solidissima* (Cembella et al., 1993; Bricelj et al., 1996), *Protothaca staminea* (Sullivan et al., 1983b), *Mactra chinensis* and *Peronidia venulosa* (Oshima, 1995a).

In this study, *Paphies donacina* presented high quantities of dcGTX3 + GTX5 and dcGTX2 in the tissues. Unfortunately, it was not possible to quantify dcGTX3 separately. However, due to the high capacity of enzymatic production of decarbamoyl toxins from N-sulfocarbamoyl observed in some other species of clams, it is likely that *P. donacina* from New Zealand has the same capacity for bioconversion. Mackenzie et al. (2004) observed that in tissues of *Paphies subtriangulata* from the Bay of Plenty, North Island of New Zealand, there were high concentrations of STX and neoSTX. This same feature was observed in this study on *P. donacina* from the South Island.

In contrast, the clam *Dosinia anus* presented a higher proportion of the carbamoyl toxin GTX1 and very low proportions of dcGTX3 + GTX5 and dcGTX2. The toxin profile of *D. anus* was similar to the one observed in the mussel *Perna canaliculus* where an active epimerisation of the β-epimers to α-epimers had taken place, leading the production of high quantities of GTX1. This reaction has been commonly observed when toxins are transferred from dinoflagellates to shellfish (Bricelj et al., 1991). β-epimers (C2, C4, GTX3, GTX4) are the favored configuration in dinoflagellates and the α-epimers (C1, C3, GTX2, GTX1) will gradually convert to the more stable a configuration in the shellfish tissues under thermodynamic equilibration conditions (Oshima, 1995a).

Fast et al. (2006) found an extreme variation between the toxin profile of the Pacific littleneck, *Protothaca staminea* and the softshell, *Mya arenaria* in their capacity for PSP toxin transformation. The littleneck clam showed a remarkable conversion of N-sulfocarbamoyl toxins to decarbamoyl derivatives whereas the soft-shell clam showed an increased concentrations in both GTX2 and GTX1 following incubation with GTX2/GTX3 epimers. Epimerisation from the β-epimers to α-epimers in the tissues of mussels has been widely reported (Bricelj et al., 1990; Oshima et al., 1990; Oshima, 1995a; Ichimi et al.,

2001) and are in agreement with the results obtained for the New Zealand mussel *Perna canaliculus* that contained high levels of GTX1.

Ostrea chilensis and *Pecten novaezelandiae* presented the lowest bioconversions of the PSP toxins among the five species of bivalves analysed in this study. The toxin profile of both species contained high quantities of the N-sulfocarbamoyl, C2 which was also dominant in the toxin profile of *Alexandrium tamarense*. GTX1 and GTX2 were also presented in the toxin profile of oysters and scallops, but in lesser proportion than in clams and mussels. Lassus et al. (2007) observed that the oyster *Crassostrea gigas* fed on *Alexandrium catenella* presented toxin profiles that were only slightly different, or the same as the toxin profile of the toxic dinoflagellate. In both toxin profiles the N-sulfocarbamoyl, C2 was the major toxin found. These results are in agreement with the toxin profile of *Ostrea chilensis* from New Zealand.

The toxin profile of the scallop, *P. novaezalendiae* also presented high quantities of C2. These results indicated slow bioconversion of toxins in scallops. Cembella et al. (1994) studied the seasonal variation of the toxin profile of the scallop *Placopecten magellanicus* exposed to *A. tamarense*. They found that the sea scallops exposed to PSP toxins contained high amounts of N-sulfocarbamoyl toxins which represented a large reservoir of potential toxicity. They also suggested that the high proportion of this toxin in the tissues of scallops indicated recent exposure to toxic dinoflagellates which is in agreement with the results observed in *P. novaezelandiae* exposed to *A. tamarense*.

The differences in the feeding responses amongst bivalves from New Zealand presented in this study and the levels of PSP toxins reached in their tissues should be a major factor for consideration when selecting species for aquaculture in areas prone to toxic algal blooms. Also, other important factor to considerate is the rate at which toxins are eliminated from bivalves after exposure to a bloom. Species that rapidly achieve elevated toxin level, such as *Perna canaliculus*, are obviously ideally suited as sentinel organisms to provide early warning of PSP outbreaks. Due to this mussels are the sentinel animal for monitoring programs worldwide. Other species more sensitive to PSP toxins, such as *Pecten novaezelandiae*, should be harvested earlier in a bloom episode in order to decrease the economic impact of this phenomenon. Sensitive species can also become more resistant when they are exposed to more re-current HABs blooms (Bricelj et al.,

2005). This suggest that constant sampling over different PSP toxin events should be carried out in order to detect possible changes in the physiology of shellfish and accumulation of PSP toxins in the tissues.

Chapter 4

Physiological effects and biotransformation of PSP toxins in *Perna canaliculus*

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4.1 Introduction

Paralytic shellfish poisoning (PSP) toxins are imidazoline derivatives which are accumulated by shellfish via the food chain and cause sporadic food poisoning in humans (Indrasena and Gill, 2000). Because of differences in charge and substituent groups within the basic saxitoxin molecule, the toxins bind with different affinities to site 1 of the sodium channel (Catterall, 1986) resulting in a range of toxicities. According to the number of charges in the molecule, PSP toxins can be classified into three main groups, namely the saxitoxin group (with two positive charges), gonyautoxin group (one positive charge) and the C toxin group (with no charge). Saxitoxin (STX) is one of the most representative toxins of the saxitoxin group and the GTX1 is known to be present as a major component in many PSP samples. The GTX4 is the 11-epimer of GTX1. The GTX3 is an 11-epimer of GTX2 which forms approximately a 7:3 equilibrium mixture of GTX2:GTX3 in solutions of neutral or higher pH. The two compounds are usually found together (Louzao et al., 1994).

Differences in PSP toxin composition between bivalve shellfish and ingested toxigenic dinoflagellates can arise via two alternatives (but not mutually exclusive) mechanisms:

1. selective retention/elimination of specific toxins, and
2. biotransformation among toxic components within tissues.

For example, some bivalves can convert PSP toxins to increase the toxic potency. A good example is the conversion of C2 toxin to GTX3. These conversions can be achieved by the reductive conversion, hydrolysis at low pH (Laycock et al., 1995) and/or enzymatic transformations (Cembella et al., 1994). Marine mussels are known to accumulate substantial amounts of some phycotoxins faster than other bivalves (Shumway, 1990). This capability, along with the periodic occurrence of toxic events, is threatening the commercialisation of the extensive cultures of this bivalve all over the world. Mussels are highly resistant to STX, and in most cases, do not exhibit adverse reactions when feeding on PSP toxin producing dinoflagellates (Bricelj et al., 1990; Marsden and Shumway, 1992; Cucci et al., 1985). This resistance, along with the generally elevated filtration rate of mussels, are probably the factors that determine their faster rate of phycotoxin accumulation, as compared to other bivalves. For example, in Maine coastal waters the toxicity of *Mytilus edulis* is detected about 12 days earlier than that the clam *Mya arenaria*, thus providing an early warning system for harvesting of the latter species. It is also well documented that *M. edulis* generally becomes 2 to 4 times more toxic than co-occurring *M. arenaria*. This clam closes its valves in the presence of toxic dinoflagellates, avoiding the ingestion of the microalgae and consequently the contamination of the tissues (White, 1982).

Mussels exhibit a remarkable degree of phenotypic plasticity in their physiological responses to environmental variables, resulting in considerable physiological differences among species, populations, and even individuals from the same population (Hawkins and Bayne, 1992). Therefore, under PSP toxin conditions it is necessary to establish the suite of physiological responses characteristic of each species, rather than relying on extrapolation of data from other species.

Although there are no authenticated records of human illness due to PSP toxins in New Zealand, nationwide phytoplankton and shellfish toxicity monitoring programs have revealed that the incidence of PSP toxins contamination and the occurrence of the toxic *Alexandrium* species are more common than previously realised (Mackenzie et al., 2004). The New Zealand mussel, *Perna canaliculus* is the major shellfish exported from New Zealand and because of its economic importance, several studies on this species have been undertaken (Marsden and Shumway, 1992; Alfaro et al., 2004; Alfaro, 2006; Petes et al., 2007). However, not many studies have focused on the effects of marine phytotoxins on the physiology of the New Zealand greenshell mussel. Marsden and Shumway (1992) demonstrated that *P. canaliculus* has the ability to survive, feed normally, and accumulate paralytic shellfish toxins from toxic dinoflagellates, but further studies measuring other physiological responses and the bioconversions of PSP toxins in this mussel have not yet been done.

The main objective of this study was to investigate the physiological effects of the toxic dinoflagellate *Alexandrium tamarense* on the New Zealand mussel, *Perna canaliculus*. Mussels were exposed to periods of intoxication and detoxification and the clearance and excretion rates measured. Toxins in the tissues of mussel exposed to the toxic dinoflagellate were also measured and compared with toxins present in the dinoflagellates. Studies were also carried out on byssus production of mussels fed with *A. tamarense* and mussel faeces were examined to test for the presence of intact cells of the toxic dinoflagellate. The presence of intact cells in the faeces of mussels could suggest the potential to regenerate a new population of dinoflagellates.

4.2 Methods

4.2.1 Collection of the mussels

The mussel *Perna canaliculus* was collected from culture ropes in Okains bay, Banks Peninsula, South Island in June, 2007 (N = 100) and August, 2008 (N = 50). Mussels were acclimated for two weeks in 60 L aquaria containing circulating seawater and constant

aeration. During this period, they were fed daily with *Tetraselmis* sp. according to the laboratory acclimation conditions (explained in Chapter 2 (2.2)).

4.2.2 Intoxication and detoxification of *P. canaliculus* exposed to toxic and non-toxic *Alexandrium* spp.

This experiment was carried out using 48 mussels collected in June, 2007 (dry weight 1.7 ± 0.5 g; shell length 67.3 ± 5.4 mm). The physiological responses (clearance rate and excretion rate) and analyses of PSP toxins in the tissues were measured over a 20 day period. Replicates in this experiment comprised independent vessels containing 6 bivalves and 10 L of sea water (salinity of 30 and 15°C) with constant aeration. *Perna canaliculus* was exposed to three different diets in order to study their responses during intoxication and detoxification conditions.

For the first two days (acclimation period) all mussels were supplied with a diet of non-toxic microalgae *Tetraselmis* sp. (5×10^7 cells^{-1} indiv^{-1}) and the non-toxic dinoflagellate *Alexandrium margalefii* (1.5×10^5 cells^{-1} indiv^{-1}). The bivalves were fed with *A. margalefii* to acclimate them to dinoflagellates cells as part of their daily diet. For the next 18 days (10 day intoxication and 8 day detoxification periods), the bivalves were divided into two groups: the PSP and control groups with four replicates in each. Over the intoxication period the PSP group was fed with a diet of *Tetraselmis* sp. (5×10^7 cells $^{-1}$ indiv^{-1}) and the toxic dinoflagellate *A. tamarense* (1.5×10^5 cells^{-1} indiv^{-1}). The control group was fed with a diet of *Tetraselmis* sp. (5×10^7 cells^{-1} indiv^{-1}) and the non-toxic dinoflagellate *A. margaleffi* (1.5×10^5 cells^{-1} indiv^{-1}). For the last 8 days (detoxification period), the PSP group was fed with the same diet as the control group (*Tetraselmis* sp. and *A. margalefii*). Details of the diets supplied to *P. canaliculus* during the different periods are given in Table 4.1.

The clearance and excretion rates were measured on days 0, 3, 6, 10, 12 and 18 of the experiment. The measurements were made on the same individuals from each replicate (repeated measures design). The clearance rate of the PSP group was measured individually

using vessels containing 2 L of aerated filtered seawater and 10^7 cells L^{-1} of *Tetraselmis* sp. and 3 x 10^4 cells L^{-1} of *A. tamarense* as the initial concentration. The control group was maintained under the same conditions, but the initial concentration for measuring the clearance rate was 10^7 cells L^{-1} of *Tetraselmis* sp. and 3 x 10^4 cells L^{-1} of *A. margalefii*. The clearance rate was measured every 30 min for 2 hours using the methodology described in Coughlan (1969) (Chapter 2 (2.3)).

Table 4.1: Composition of the diets provided to individual *P. canaliculus* over the experiment (TETR = *Tetraselmis* sp.; AT = *A. tamarense*; AM = *A. margalefii*).

Periods	Treatments	TETR (cells d^{-1})	AT (cells d^{-1})	AM (cells d^{-1})	Toxicity (nmol STX-diHCl d^{-1})
Acclimation (2 days)	PSP group	5 x 10^7	0	1.5 x 10^5	0
	Control group	5 x 10^7	0	1.5 x 10^5	0
Intoxication (10 days)	PSP group	5 x 10^7	1.5 x 10^5	0	5
	Control group	5 x 10^7	0	1.5 x 10^5	0
Detoxification (8 days)	PSP group	5 x 10^7	0	1.5 x 10^5	0
	Control group	5 x 10^7	0	1.5 x 10^5	0

After the clearance rate measurements, the mussels were placed individually in vessels containing 0.3 L of filtered seawater and the excretion rate measured over 2 hours following the methodology described in Solórzano (1969) (Chapter 2 (2.3)). On the same days, tissues samples were taken for analysis of the PSP toxins. From the PSP group, one mussel from each

replicate was dissected into: (1) digestive gland and (2) other tissues without the digestive gland (gonads + muscle + gills + mantle). Both groups of tissues were kept separately and frozen at -80°C for further processing by HPLC with post column oxidation. Also three replicates of *A. tamarense* were taken on day 10 for later toxin analyses. Details of the extraction and quantification of PSP toxins are given in Chapter 2 (2.4).

4.2.3 Byssus production in presence of toxic *A. tamarense*

This experiment was carried out using ten mussels collected in August, 2008 (dry weight 1.5 ± 0.3; length shell 81.2 ± 6.4 mm). For studying the effects of *A. tamarense* on the byssus production of *Perna canaliculus*, mussels were placed individually in 4L of aerated seawater in vessels with a concave glass bottom for better attachment (Figure 4.1).

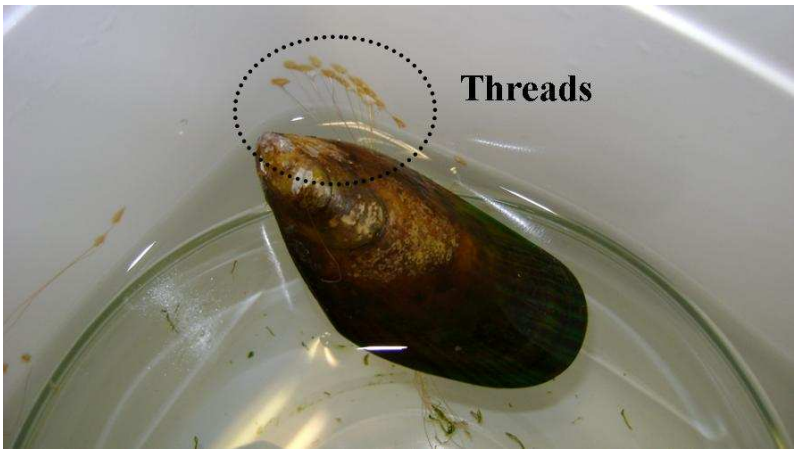


Figure 4.1: Mussel using byssus threads to attach to the wall of the vessel.

The mussels were kept without food for 2 days to void their gut contents and were then fed with *Tetraselmis* sp. for another 4 days (5 x 10^7 cells day^{-1} $mussel^{-1}$) after which byssus production was determined. Mussels were then kept without food for another 2 days. Then, for the next 15 days, five mussels (PSP group) were exposed to the toxic dinoflagellates

Alexandrium tamarense (10^6 cells day^{-1} $mussel^{-1}$) and another five mussels (control group) were fed with *Tetraselmis* sp. (5×10^7 cells day^{-1} $mussel^{-1}$). Byssus production was counted on days 1, 6, 10 and 15. On the measurement day, the water was changed and the byssus threads were cut close to the edge of the valve and the remains of old threads discarded. New byssus threads were counted the next day after 24 hours.

4.2.4 Presence of *A. tamarense* in the faeces

This experiment was carried out using five mussels collected in August, 2008 (dry weight 1.6 ± 0.3; length shell 80.2 ± 5.2 mm). The mussels were kept individually in vessels containing 4 L of filtered seawater. Food was not provided for two days to allow the mussels to void their gut contents. For the next two days mussels were fed with the toxic dinoflagellate *Alexandrium tamarense* (2×10^6 cells day^{-1} $mussel^{-1}$). After these two days, mussels were kept in filtered seawater and samples of the faeces collected over the next 24 hours were observed under fluorescence microscopy. Pictures were taken using (1) differential interference contrast and (2) fluorescence detection for visualization of chlorophyll *a*.

4.3 Results

4.3.1 Intoxication and detoxification in *P. canaliculus* exposed to toxic and non-toxic *Alexandrium* spp.

Clearance rate

The clearance rate for both the PSP and the control groups were similar during intoxication and detoxification periods (Figure 4.2). Overall, feeding rates of the PSP group were 0.96 ± 0.09 L h^{-1} g^{-1} and for the control group were 1.01 ± 0.09 L h^{-1} g^{-1}. There was no significant differences in clearance rate between the PSP and control groups (repeated measures ANOVA, $F_{1,6} = 0.12$, $P = 0.73$). The effect of time on the clearance rates was also not significant (repeated measures ANOVA, $F_{5,30} = 1.39$, $P = 0.25$), and there was no significant

interaction between treatment and time (Table 4.2).

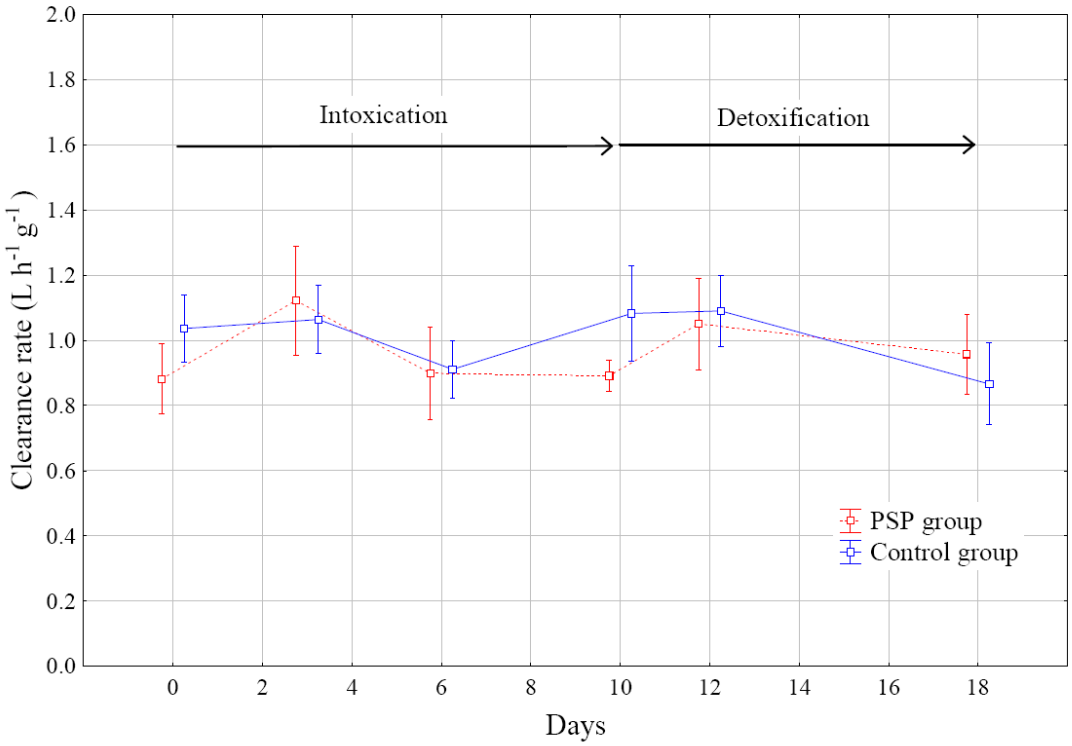


Figure 4.2: Clearance rate of *Perna canaliculus* during intoxication and detoxification periods. Measurements were taken on days 0, 3, 6, 10, 12 and 18. Values are means ± SE.

Table 4.2: Results of repeated measures ANOVA testing the effects of diet (treatment) and exposure time on the clearance rate of *Perna canaliculus.*

Source of variation	*SS*	*Df*	*MS*	*F*	*P*
Treatment	0.02	1	0.02	0.12	0.73
Error	1.03	6	0.17		
Days	0.25	5	0.05	1.39	0.25
Days*Treatments	0.12	5	0.02	0.71	0.69
Error	1.07	30	0.03		

Excretion rate

The PSP and the control groups had similar excretion rates during intoxication and detoxification periods (Figure 4.3). The PSP group excreted 29.7 ± 2.1 µg NH4-N h^{-1} g^{-1} and the control group excreted 31.4 ± 3.9 µg NH4-N h^{-1} g^{-1} during intoxication and detoxification periods. There was no significant differences in excretion rate between the PSP and the control groups (repeated measures ANOVA, $F_{1,6} = 0.12$, $P = 0.74$) and no significant effect of time (repeated measures ANOVA, $F_{5,30} = 1.19$, $P = 0.33$). Also there was no significant interaction between the two variables (Table 4.3).

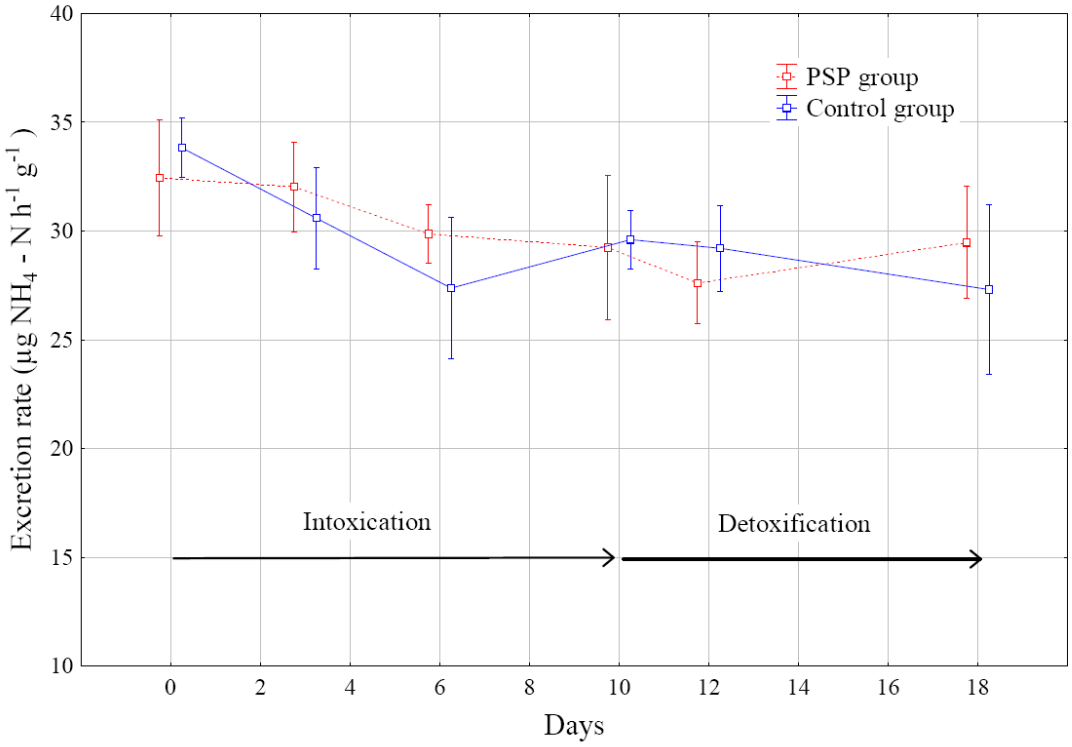


Figure 4.3: Excretion rate of *Perna canaliculus* during intoxication and detoxification periods. Measurements were taken on days 0, 3, 6, 10, 12 and 18. Values are means ± SE.

Table 4.3: Results of repeated measures ANOVA testing the effects of diet (treatment) and exposure time on the excretion rate of *Perna canaliculus.*

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	2.52	1	2.52	0.12	0.74
Error	126.2	6	21.04		
Days	150.3	5	30.07	1.19	0.33
Days*Treatments	32.4	5	6.50	0.25	0.93
Error	754.6	30	25.16		

Total toxin burden of PSP toxins in the tissues

The total toxin burden of PSP toxins in the tissues of *Perna canaliculus* was calculated by adding together the concentrations of toxins detected in the digestive gland to the toxins detected in the homogenate of the other tissues (gonads + muscle + gills + mantle). The intoxication period was characterised by an increase of the total toxin body burden in the tissues; day 3 = 28, day 6 = 188 and day 10 = 218 μg STX-diHCl equiv 100 g^{-1}. Over the detoxification period, mussels decreased the level of PSP toxins in the tissues; day 14 = 141 and day 18 = 25 μg STX-diHCl equiv 100 g^{-1} tissue (Figure 4.4).

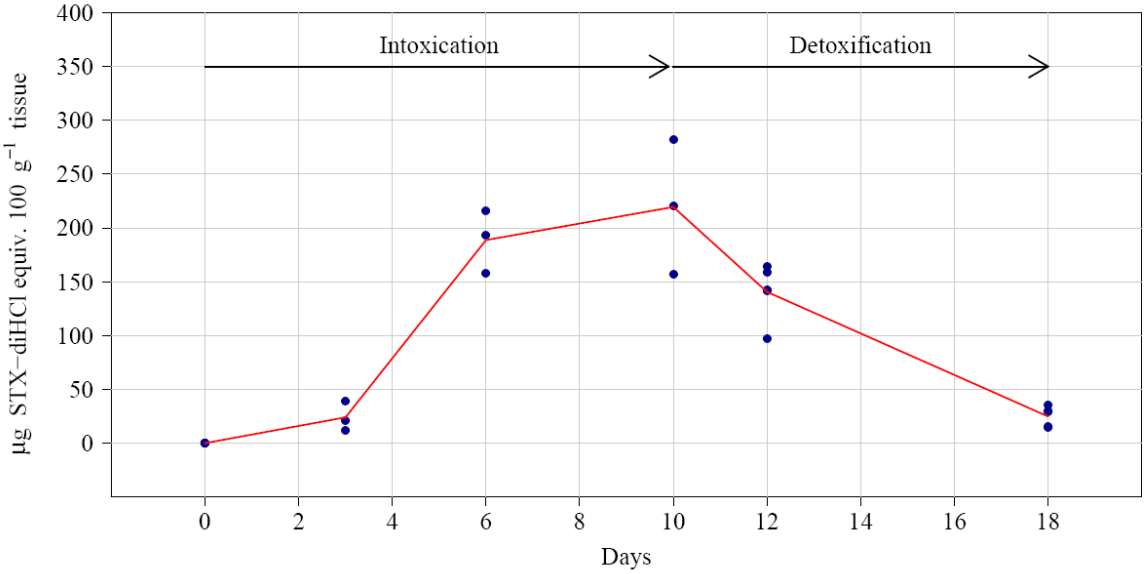


Figure 4.4: Total content of PSP toxins in the tissues of mussels during intoxication and detoxification periods. Samples were taken on days 0, 3, 6, 10, 12 and 18.

PSP toxin profile in the digestive gland

Most of the total toxin burden of *Perna canaliculus* was contained in the digestive gland over the intoxication and detoxification periods (day 3 = 57%, day 6 = 92%, day 10 = 93%, day 12 = 93%, day 18 = 64%). The PSP toxins present in the digestive gland of mussels during both periods were GTX4, GTX1, dcGTX3 + GTX5, dcGTX2, GTX3, GTX2, dcNeo + Neo, dcSTX, STX, C1 and C2 (Figure 4.5). The compounds dcGTX2 and GTX2 were detected in the digestive glands but were not present in the toxin profile of *Alexandrium tamarense*. On day 3, the N-sulfocarbamoyl toxin, C2 was present in higher quantities (41%) than the other PSP toxins observed (GTX4, dcGTX3 + GTX5, dcGTX2, GTX3, dcNeo + Neo, dcSTX, STX, C1). On this day, these other toxins were present in similar proportions, but this clearly

changed over the rest of the experiment. From day 6, GTX1 was detected and increased in concentration over the experiment, including during the detoxification period. GTX1 dominated the toxin profile at days 6, 10, 12 and 18. On the same days, GTX4 and C1 decreased in concentration with GTX4 not able to be detected on day 18. On this day, the toxin profile of the digestive gland was dominated by GTX1 (97%) and small amounts of dcGTX3 + GTX5, C1 and C2 (Table 4.4).

Figure 4.5: PSP toxin profile of the digestive gland from mussels during intoxication and detoxification periods. Values are expressed as a percentage (%) of the total content of toxins in this tissue. The total content of toxins in this tissue is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}) with the corresponding proportion of the total toxin burden of the mussels.

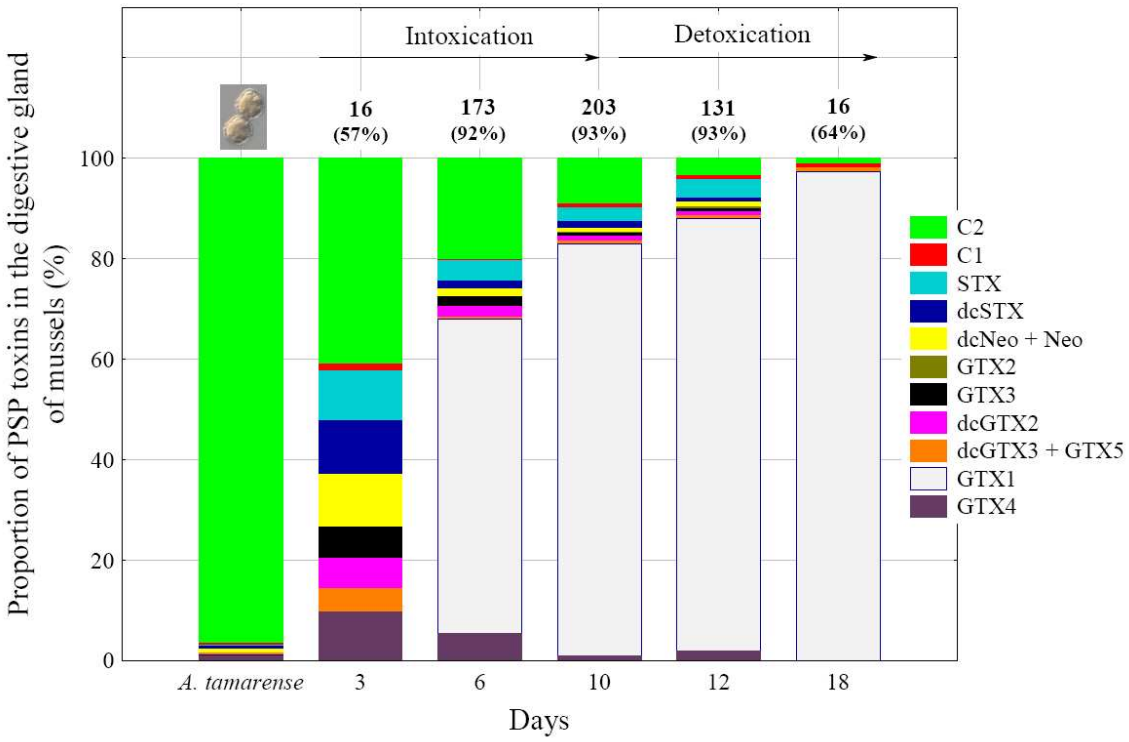


Table 4.4: PSP toxin profile of the digestive gland from mussels exposed to intoxication and detoxification periods. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in this tissue (%). On each day, the compound dominating the toxin profiles is shown in **bold**.

	Day 3		Day 6		Day 10		Day 14		Day 18	
	T	%	T	%	T	%	T	%	T	%
GTX4	1.5	9.8	9.2	5.3	2.0	1.0	2.4	1.9	-	-
GTX1	-	-	**108.1**	**62.6**	**166.4**	**82.0**	**112.7**	**86.2**	**15.6**	**97.4**
dcGT3 + GTX3	0.7	4.6	0.8	0.5	1.4	0.7	0.7	0.5	0.1	0.7
dcGTX2	0.9	6.0	3.8	2.2	1.6	0.8	0.9	0.7	-	-
GTX3	1.0	6.3	3.3	1.9	1.4	0.7	0.8	0.6	-	-
GTX2	-	-	-	-	0.3	0.2	0.5	0.4	-	-
dcNeo + Neo	1.6	10.4	2.6	1.5	1.7	0.8	1.3	1.0	-	-
dcSTX	1.7	10.7	2.6	1.5	2.5	1.2	1.2	0.9	-	-
STX	1.6	10.0	7.2	4.2	5.8	2.9	4.8	3.7	-	-
C1	0.2	1.3	0.4	0.2	1.4	0.7	0.9	0.7	0.1	0.8
C2	**6.4**	**41.0**	34.7	20.1	18.5	9.1	4.5	3.4	0.2	1.2
Total	16	100	173	100	203	100	131	100	16	100

PSP toxin profile in other tissues

Overall, the homogenate of the other tissues (gonads + muscle + gills + mantle) contained low quantities of the PSP toxins (day 3= 43%, day 6 = 8%, day 10 = 7%, day 12 =7%, day 18 =36%). The PSP toxins contained in these tissues were GTX4, GTX1, dcGTX3 + GTX5, dcGTX2, GTX3, dcNeo + Neo, dcSTX, C1 and C2 (Figure 4.6). The dcGTX2 was not present in the toxic dinoflagellate *Alexandrium tamarense*. Over the intoxication and detoxification periods, GTX4 dominated the toxin profile of these tissues but its proportion decreased with time. In contrast, GTX1 was detected from day 6 and the relative proportion increased over time (with the exemption of day 12). The C2 was presented in high proportion over the intoxication period, but decreased during detoxification. The toxins dcNeo + Neo were present from days 3 to 12 but were not detected on day 18 (Table 4.5).

Figure 4.6: PSP toxin profile of the other tissues from mussels during intoxication and detoxification periods. Values are expressed as a percentage (%) of the total content of toxins in these tissues. The total content of toxins in these tissues is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}) with the corresponding proportion of the total toxin burden of the mussels.

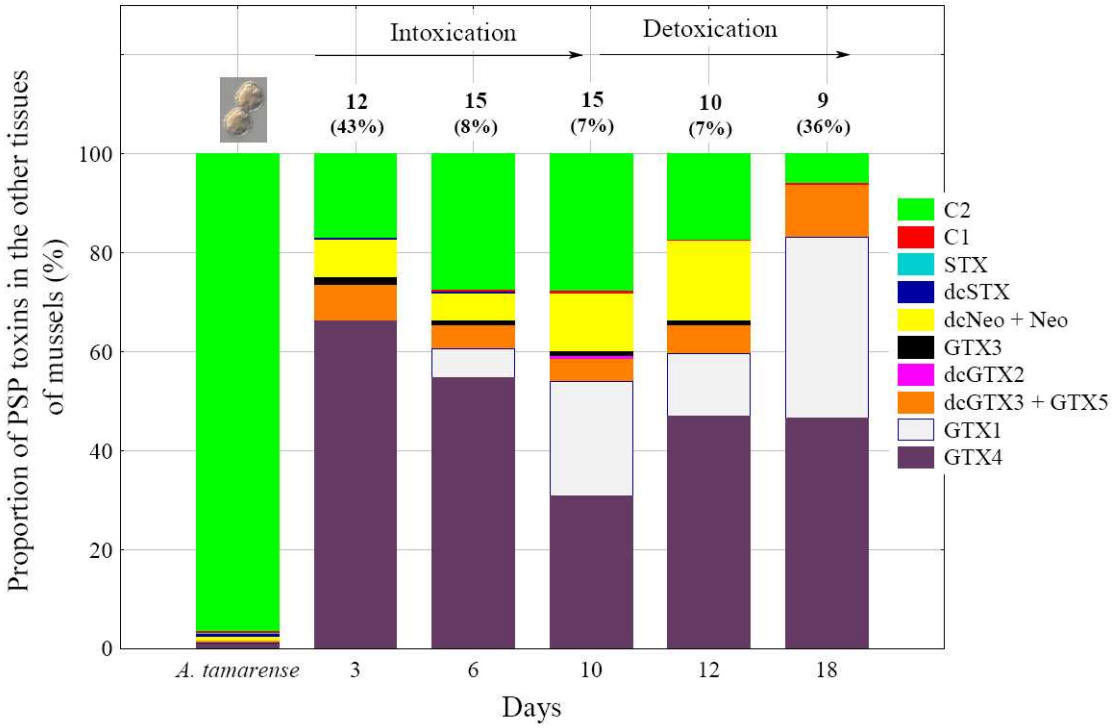


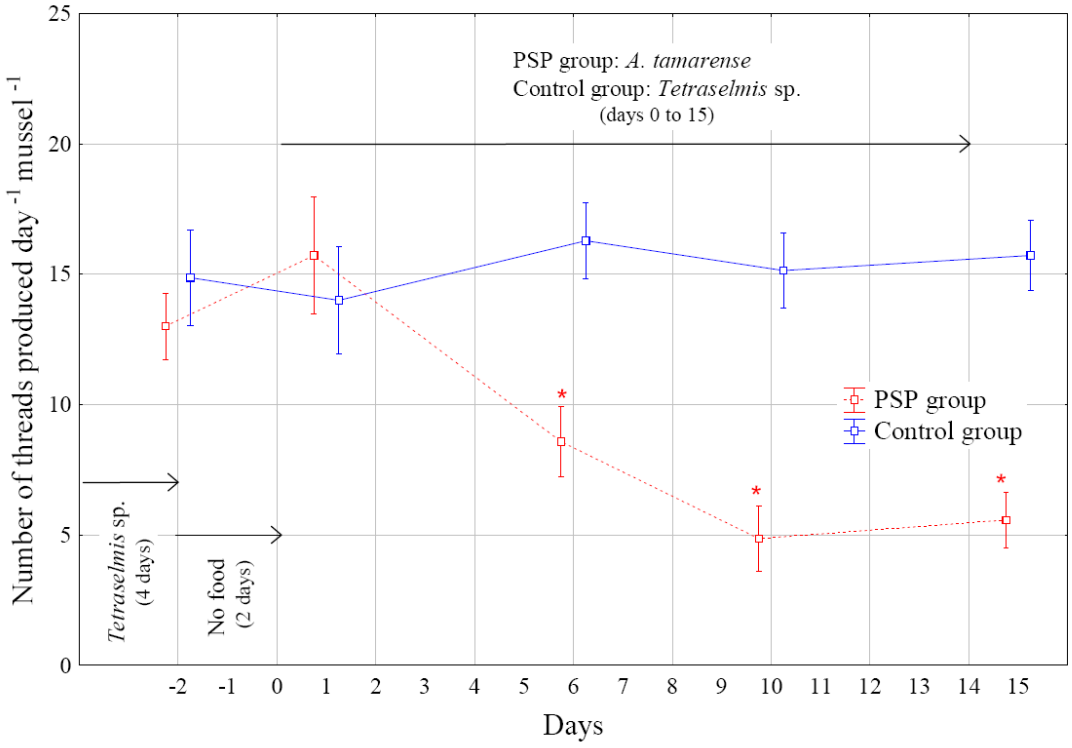
Table 4.5: PSP toxin profile of the other tissues from mussels exposed to intoxication and detoxification periods. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in this tissue (%). On each day, the compound dominating the toxin profiles is shown in **bold**.

	Day 3		Day 6		Day 10		Day 14		Day 18	
	T	%	T	%	T	%	T	%	T	%
GTX4	**8.1**	**66.2**	**8.0**	**54.8**	**4.5**	**30.8**	**4.6**	**47.0**	**4.1**	**46.5**
GTX1	-	-	0.8	5.7	3.4	23.2	1.2	12.7	3.2	36.6
dcGT3 + GTX3	0.9	7.2	0.7	4.7	0.7	4.5	0.5	5.5	0.9	10.5
dcGTX2	-	-	-	-	0.1	0.4	-	-	-	-
GTX3	0.2	1.5	0.2	1.1	0.1	1.0	0.1	1.1	-	-
dcNeo + Neo	0.9	7.7	0.8	5.4	1.7	11.7	1.6	16.0	-	-
dcSTX	0.04	0.3	0.04	0.3	-	-	-	-	-	-
C1	-	-	0.1	0.5	0.1	0.5	0.02	0.3	0.03	0.3
C2	2.1	17.0	4.0	27.4	4.0	27.8	1.7	17.5	0.5	6.0
Total	12	100	15	100	15	100	10	100	16	100

4.3.2 Byssus production in presence of toxic *A. tamarense*

The byssus production of *Perna canaliculus* was affected by the presence of the toxic dinoflagellates (Figure 4.7). Overall, the byssus production of mussels fed on *Tetraselmis* sp. was 15 ± 4 threads day^{-1} mussel^{-1}.

Figure 4.7: Byssus production of *Perna canaliculus* exposed to *Alexandrium tamarense* and *Tetraselmis* sp. Measurements were taken on 2 days before exposure to the toxic algae and on days, 1, 6, 10 and 15. The asterisk (*) represents significant lower production of byssus. Values are means ± SE.



At the start of the experiment, the byssus production of the PSP group fed on *Tetraselmis* sp. and the control group fed on *Alexandrium tamarense* was similar. Later in the

experiment (day 6), the number of threads produced by mussel in the PSP group decreased to values of 9 ± 4 threads day^{-1} mussel^{-1}. On days 10 and 15, the PSP group still produced fewer threads than the control group (5 ± 3 and 6 ± 3 threads day^{-1} mussel^{-1} respectively).

There were significant differences in the byssus production between the PSP and the control group (repeated measures ANOVA, $F_{1,12}$ = 44.60, P < 0.001). The effect of time (repeated measures ANOVA, $F_{4,48}$ = 3.25, P < 0.05) and the interaction between both variables (repeated measures ANOVA, $F_{4,48}$ = 5.42, P < 0.001) on the byssus production of mussels were also significant (Table 4.6). On days 6, 10 and 15, the PSP group produced significantly lower number of threads than the control group (post-hoc Tukey analysis).

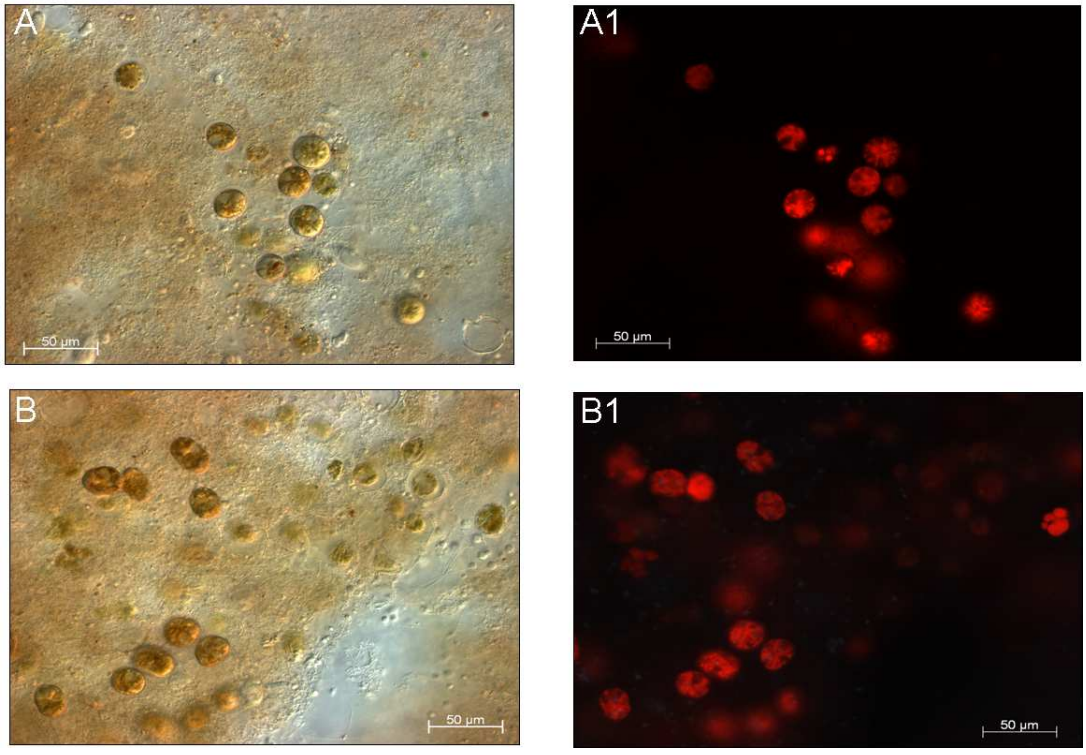
Table 4.6: Results of repeated measures ANOVA testing the effects of toxic and non-toxic diets (treatment) and days of exposure on the byssus production of *Perna canaliculus*. Values in bold are of statistical significance.

Source of variation	SS	df	MS	F	P
Treatment	560.1	1	560.1	**44.6**	**< 0.001**
Error	150.7	12	12.56		
Days	241.1	4	60.26	**3.25**	**< 0.05**
Days*Treatments	400.9	4	100.2	**5.42**	**< 0.001**
Error	887.6	48	18.5		

4.3.3 Presence of *A. tamarense* in the faeces of mussels

Observations using differential interface contrast microscopy showed intact cells of the toxic dinoflagellate *A. tamarense* in the faeces of the five mussels tested. Observations using fluorescence detection microscopy showed that some of these cells contained still chlorophyll *a* which was observed as a red colour (Figure 4.8).

Figure 4.8: Faeces from mussels fed with *A. tamarense*. Pictures A and B were taken using differential interface contrast microscopy. Pictures A1 and B1 are the same samples and pictures were taken using fluorescence detection microscopy. Chlorophyll *a* contained inside of the cells is showed in red colour.



4.4 Discussion

The results of this study showed that the New Zealand mussel, *Perna canaliculus* was able to ingest the dinoflagellate *Alexandrium tamarense* and over ten days, accumulated 218 µg STX di-HCl equiv. 100 g^{-1} in the tissues. Following a detoxification period of 8 days, mussels decreased the PSP toxins in the tissues to a safe total toxin burden of 25 µg STX di-HCl equiv.

100 g^{-1} in the tissues. The PSP toxins did not affect the clearance and the excretion rate of mussels during intoxication and detoxification periods. The byssus production of mussels exposed to *A. tamarense* was significantly lower than the mussels fed on *Tetraselmis* sp. Intact cells of A. tamarense were able to pass through the digestive system of mussels, and the presence of chlorophyll *a* in the intact cells suggested that these cells might have the potential to regenerate a new population of toxic dinoflagellate.

4.4.1 Intoxication and detoxification processes of *P. canaliculus* exposed to *A. tamarense*

Clearance and excretion rates

The clearance rate of *Perna canaliclus* fed with the toxic dinoflagellate *Alexandrium tamarense* was not affected by the PSP toxins and the mussels fed actively on the three species of algae used during intoxication and detoxification periods. Marsden and Shumway (1992) observed that the same species of mussel from the Marlborough Sounds presented similar responses to those observed in this study. These authors demonstrated that *P. canaliculus* had the ability to survive, feed normally, and accumulate PSP toxins from the dinoflagellate *A. tamarense* (GT429).

Shumway and Cucci (1987) described differences in the response of mussels to toxic dinoflagellates of the genus *Alexandrium* that were dependent on the bivalve's prior exposure history. *Mytilus edulis* from areas free of PSP toxins exhibited 75% mortality, copious production of white mucus, shell valve closure and mantle retraction when exposed to *Alexandrium* (formerly *Protogonyaulax tamarensis* clone GT429), whereas mussels from Maine, which experience recurrent algal blooms, were not visibly affected by PSP toxins. The New Zealand mussel *Perna canaliculus* tested in this study came from a bay free of PSP toxins and the population had never been exposed to a PSP toxic algal bloom. Therefore, the resistance of this mussel to the PSP toxin due to previous exposure to toxic algal blooms has been discounted.

Navarro et al. (2008) studied the effect of different diets containing the toxic dinoflagellate *Alexandrium catenella* (10, 30, 50 and 100% of toxic cells) on the clearance rate of the Chilean mussel *Mytilus chilensis*. They found that the clearance rate of mussels decreased with an increase of *A. catenella* in the diet. This effect was observed over 24 h of exposure to diets containing 30 and 50% of the toxic dinoflagellate and over 72 h of exposure to the diet containing 100% of the toxic cells. After exposure, *M. chilensis* recovered producing clearance rates similar to the control mussels. These results suggest that the New Zealand mussel *P. canaliculus* may have been exposed to a concentration of toxic algae too low to produce an effect on their clearance rates. Moreover, if a short-term effect had occurred, under the conditions of this experiment, the frequency of the sampling may not have allowed detection.

Excretion rates of *Perna canaliculus* were unaffected during intoxication and detoxification periods. Navarro and Contreras (2010) found that *Mytilus chilensis* exposed to *Alexandrium catenella* increased the excretion rate over time which was significantly correlated with the accumulation of PSP toxins in the tissues. They suggested that the degradation of the PSP toxins, a rich source of nitrogen, produced high concentrations of ammonia which had to be eliminated via the excretory organs in order to maintain equilibrium in the bivalve. In contrast, excretion rates of *P. canaliculus* fed on *Alexandrium* species were similar over both periods and did not increase over the time. These results could be explained by the resistance of mussels to PSP toxins or due to the concentration of toxic algae used that may have been to low to produce an effect. Further studies using higher concentrations or different toxicities of toxic algae and more frequent sampling (e.g. hourly) would answer whether *P. canaliculus* is completely resistant to PSP toxins or has the ability to respond differently to other PSP-conditions.

PSP toxins in the tissues of mussels

Following six days exposure to PSP dinoflagellates , the levels of toxin in the tissue of *Perna canaliculus* reached 188 μg STX di-HCl equiv. 100 g^{-1} tissue which is over the safety limit level for human consumption (80 μg STX di-HCl equiv. 100 g^{-1} tissue). This rapid capacity to accumulate PSP toxins has been widely reported in mussels which are used as a sentinel shellfish in monitoring programs (Bricelj and Shumway, 1998).

There were considerable differences in the toxin profile between the dinoflagellate *A. tamarense* and the mussel *P. canaliculus*. Mussel tissue contained a higher proportion of carbamoyl toxins whereas the dinoflagellate was dominated by the N-sulfocarbamoyl derivative, C2. These results agree with other studies (Oshima et al., 1990; Bricelj et al., 1991; Cembella et al., 1993; Choi et al., 2003) that have shown transformation of PSP toxins between the dinoflagellate and the shellfish. This transformation means that bivalves usually have lower proportions of N-sulfocarbomyl toxins and higher proportions of carbamoyl toxins than the toxigenic dinoflagellates that were ingested.

The amount of PSP toxins in the digestive gland was higher than in the other tissues as has been reported in other studies (Bricelj et al., 1990; Choi et al., 2003; Kwong et al., 2006). Toxins were more rapidly accumulated and eliminated from the digestive gland than from the other tissues. In fact, several studies have shown that the digestive gland had the most rapid elimination rates of PSP toxins relative to other tissues (Bricelj et al., 1990, 1991). From day 6, the toxin profile of the digestive gland *in P. canaliculus* was dominated by GTX1, whereas the contribution of GTX4 decreased over the time. This might be explained by epimerisation from less stable β-epimers (C2, GTX4, GTX3) to the more stable α-epimers (C1, GTX1, GTX2) which is what has been most commonly observed in bivalves (Oshima, 1995a). This process occurs by epimerisation of the C-11 hydroxysulfate moiety at neutral pH and can be accelerated at higher pH and higher temperatures. Oshima (1995a) also found that this process was faster with N-sulfocarbamoyl derivatives (C1 and C2) than with carbamoyl derivatives (GTX2 and GTX3). In shellfish toxins to, epimerisation proceeds gradually, until it reaches equilibrium at a β: α ratio close to 1:3. Thus, the relative ratio of epimers provides information

on how long the toxins have been retained by shellfish. Epimerisation in the other tissues was slower than in the digestive gland and the proportion of GTX4 found was higher than in the digestive gland. Consequently, the proportion of GTX1 in the other tissues was lower than in the digestive gland demonstrating the slower epimerisation.

4.4.2 Byssus production of mussels affected by *A. tamarense*

The ability of *Perna canaliculus* to produce byssus was affected by the presence of *A. tamarense* in the diet. One of the most important aspects of mussel life styles is the ability to secrete strong byssal threads and attach to a variety of substrates (Alfaro, 2006). This is crucial to the survival of the species and it is an informative index of activity in the mussels. Shumway and Cucci (1987) observed that the byssus production in *Mytilus edulis* was inhibited in the presence of *Protogonyaulax tamarensis*, but in *Modiolus modiolus* it was not inhibited. The decreased byssus production in the New Zealand mussel registered in this study agreed with a previous study on the same species of mussel (Marsden and Shumway, 1992).

In the present study we observed that *Perna canaliculus* exposed to *A. tamarense* had significantly decreased byssus production . This could be critical in aquaculture systems where threads play an integral role in the attachment of mussels to culture ropes. Mussels exposed to an algal bloom could become contaminated, but also their ability to attach to the culture ropes may be diminished. Extensive losses of cultured mussels due to this phenomenon has not been described in the literature, nevertheless, further studies need to be done, especially in another reproductive stages where the byssus production is crucial in the life cycle of bivalve species (e.g. juvenile).

4.4.3 Implications of the presence of toxic cells in the faeces of bivalves

Observations made on the faeces of *Perna canaliculus* fed with the toxic dinoflagellate *A. tamarense* confirm that not all ingested cells are digested and that some of them passed intact through the digestive system to be ejected in the faeces. This phenomenon has been also reported for other bivalve species. Bardouil et al. (1993) and Laabir and Gentien (1999) found intact cells of *Alexandrium minutum* and *Alexandrium tamarense* in the faeces of the oyster *Crassostrea gigas*. Undigested cells may result from the resistance of the algal cell walls to shellfish gastric juices. Oysters ingesting naked dinoflagellate species (without theca) usually produce faeces made up of split cells, whereas oysters consuming dinoflagellates with thick thecae produce faeces containing intact or encysted cells in proportions ranging from 50 to 90% (Laabir and Gentien, 1999).

Several problems may arise from the presence of toxic phytoplankton cells in the faeces: a possible secondary contamination of other bivalves via re-suspension in the water column (Kikuchi et al., 1996), and, in the event of shellfish transfer, the contamination of another area, previously free of toxic cells (Guéguen et al., 2008). Hégarèt et al. (2008) also found intact, non-digested cells of *Alexandrium fundyense* in the faeces of *Mytilus edulis* and on culture, these cells were able to produce a new population of dinoflagellates. This suggests that transplanted bivalve molluscs can potentially serve as vectors for introduction of harmful algae to other areas. The results presented in this study are important in the management, restoration and aquaculture activities involving shellfish transportation activities in New Zealand. It is strongly recommended that aquaculturists avoid transportation of contaminated bivalves to free PSP areas. This consideration should be applied within New Zealand coast and other fisheries areas around the world to avoid the increasing expansion of harmful algae blooms.

In conclusion, the results of the present study the New Zealand mussel, *P. canaliculus* exposed to the toxic dinoflagellate *A. tamarense,* confirm that mussels have the capacity to become intoxicated and to detoxify rapidly. Like many other mussel species around the world, *P. canaliculus* is suitable as a sentinel species for early detection of toxic algal blooms. The

effects of toxic algae on bivalves depends on multiple factors, such as the concentration and toxicity of the algae in the diet and the history of bivalve exposure to toxic bloom (Bricelj et al., 2005). Studies on *P. canaliculus* involving other variables such as different toxic algae and different populations of mussels will provide more information about the plasticity of *P. canaliculus* for coping with different PSP toxic condition. Modelling these responses under different PSP toxic conditions will help to understand the implications of future toxic algal blooms in New Zealand involving commercial shellfish species as *P. canaliculus*.

Chapter 5

Physiological effects and biotransformation of PSP toxins in *Paphies donacina*

5.1 Introduction

Surf-clams are bivalves found in the surf zone of exposed sandy beaches throughout New Zealand. The group comprises seven species of the families Mesodesmatidae (*Paphies donacina*), Mactridae (*Spisula aequilatera*, *Mactra murchisoni*, and *M. discors*) and Veneridae (*Dosinia anus*, *D. subrosea*, and *Bassina yatei*). Surf-clams are distributed in discrete depth zones, which allow individual species to be targeted by fishing. The species mix and biomass of clams change over time as a result of variable recruitment, heavy mortality from erosion during storms, high temperatures and low oxygen levels during calm summer periods, blooms of toxic algae and excessive freshwater outflow (Cranfield and Michael, 2001).

The clam *Paphies donacina* (tuatua) occurs mainly around the South Island, but also on the north coast of Stewart Island, and on the east and west coast of the North Island. The adults are found from low tide to about 4 m, and the juveniles may extend to the mid-tide mark. The greatest numbers of spat are found near the high tide mark, and recruitment between years is highly variable (Cranfield et al., 1993). Tuatua are an important handpicked resource of the Maori, especially in Pegasus Bay, Canterbury.

During mid-summer (December to January) 1992–1993, the first documented case of paralytic shellfish poisoning (PSP) contamination of shellfish within New Zealand occurred. The PSP toxins were detected by mouse bioassay in a variety of shellfish species from the Bay of Plenty in the North Island. The source of this contamination was a bloom of the toxic dinoflagellate *Alexandrium minutum* apparently associated with a local upwelling event (Chang et al., 1996). After the cessation of the event, the PSP toxicity of most shellfish species within the Bay of Plenty declined rapidly. However, in *Paphies subtriangulata*, the PSP toxicity declined gradually and it was not until 22 months after the initial contamination event that the toxicity of this shellfish could not be detected by mouse bioassay (Mackenzie et al., 1996a). The magnitude and nature of the PSP toxin retention by shellfish varies depending on the shellfish species and it has been found that surf-clams retain PSP toxins for considerable periods of time (Shumway et al., 1994).

Physiological processes of shellfish (e.g. filtration rate, oxygen consumption, excretion rate) can be affected when bivalves feed on toxic algae (Gainey and Shumway, 1988). Shumway and Cucci (1987) found that the effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on seven species of bivalve from three geographical localities were species-specific and varied with the collection locality. The responses included the closure of the shell-valve and siphon retraction (*Mya arenaria*), increased rates of particle clearance (*Ostrea edulis*), production of mucus (*Placopecten magellanicus*), or no effect (*Spisula solidissima*). Li et al. (2002) observed that the clearance rate of the clam *Ruditapes philippinarum* was reduced with an increase of the PSP toxin level in the tissues and they suggested the intoxication of the ciliary system. Moreover, the scope for growth was significantly reduced (negative values) when clams reached just 17 µg STX equiv. 100 g^{-1} in the tissues. Many laboratory experiments have shown that observations of the inhalant and exhalant openings of bivalves are relatively simple and they can provide information about the feeding behaviour (Thorin et al., 1998). It has been demonstrated that the degree of opening of the inhalant and exhalant siphons can control a bivalve's pumping activity (Foster-Smith, 1975; Newell and Bayne, 1980; Eaton, 1983). Exhalant flow can be strongly reduced by rapid constrictions of inhalant and mostly exhalant apertures (Foster-Smith, 1976). .

Burrowing in bivalves is accomplished by repeated probing-anchoring sequences, in which the longitudinal and radial muscles of the foot alternately contract (Trueman et

al., 1966). The contractions of the radial muscles dilate the distal tip of the foot which acts as an anchor for the contraction of the longitudinal muscles which drag the shell into the sediment. Bricelj et al. (1996) compared the toxin body burden of the soft-shell clam *Mya arenaria* and the surf-clam *Spisula solidissima* and found that the surf-clam accumulated 20 times more toxins in the tissues than the soft-shell clam. After exposure to the PSP toxins, the clearance rate of *M. arenaria* decreased significantly and the ability to reburrow in sediments was impaired, whereas *S. solidissima* was unaffected under the same PSP conditions. These results correlated well with the high level of toxins detected in the surf-clam after 11 days of exposure to *Alexandrium* spp. (2759 µg STX equiv. 100 g^{-1}). These authors suggested that the burrowing incapacitation index, conducted under standardised conditions, provides a simple and rapid index to characterise the individual resistance to PSP toxins of infaunal bivalves.

Because bivalve species differ in the ability to accumulate PSP toxins, it is necessary to carry out studies on different New Zealand bivalves species. The objective of this chapter was to investigate the physiological responses of *Paphies donacina* to the toxic dinoflagellate *Alexandrium tamarense*. The clams were exposed to intoxication and detoxification periods and the clearance and excretion rates were measured. During these conditions, tissues were taken to study the distribution of the PSP toxins in the clams. Complementary experiments investigated the siphon activity and the ability to burrow in the presence of *A. tamarense*.

5.2 Methods

5.2.1 Collection of the clams

Paphies donacina was collected by hand from South New Brighton beach in May, 2008 (N = 15) and August, 2009 (N = 60). Bivalves were acclimated for two weeks in either 60 L aquaria containing 15 cm of sand (behavioural studies) or 60 L aquaria without sand (physiological studies), circulating seawater and constant aeration. During this period, they were fed daily with *Tetraselmis* sp. according to the laboratory acclimation conditions (explained in Chapter 2 (2.2)).

5.2.2 Intoxication and detoxification of *P. donacina* exposed to toxic and non-toxic *Alexandrium spp*. – physiological studies

This experiment was carried out using 48 clams collected in August, 2009 (dry weight 2.8 ± 0.7 g; shell length 84.5 ± 6.4 mm). The physiological responses (clearance and excretion rates) and the analysis of PSP toxins in the tissues were measured over a 20 day period. The replicates in this experiment comprised independent vessels containing 6 clams, 6 L of seawater (salinity 30 ppt and 15°C) and constant aeration. The clams were divided into the PSP and the control group (4 replicates each) and fed with mixtures of algae: *Tetraselmis* sp. (non-toxic), *Alexandrium tamarense* (toxic dinoflagellate), and *A. margalefii* (non-toxic dinoflagellate). Both groups were exposed to 2 days of acclimation to the non toxic dinoflagellate *Alexandrium margalefii* before the PSP group was exposed to *A. tamarense* for 10 days (intoxication), while the control group was fed with a non-toxic diet (*A. margalefii*). Finally, the PSP group was exposed to a detoxification period of 8 days. Details of the diets supplied to *P. donacina* during the different periods are given in Table 5.1.

The clearance and excretion rates were measured on days 0, 3, 6, 10, 14 and 18 on the same individual from each replicate (repeated measures design). The clearance rate of the PSP group was measured individually using vessels containing 0.8 L of filtered seawater and a mixture of 5 x 10^6 cells L^{-1} of *Tetraselmis* sp. and 2.5 x 10^5 cells L^{-1} of *A. tamarense*, as the initial concentration. The control group was maintained under the same conditions, but fed 5 x 10^6 cells L^{-1} of *Tetraselmis* sp. and 2.5 x 10^5 cells L^{-1} of *A. margalefii*. The measurements were taken over 2 hours and the decrease of the cells in the seawater was monitored every 30 min. No pseudofaeces were produced under these conditions.

After the clearance rate measurements, the clams were transferred to vessels containing 0.5 L of filtered seawater and the excretion rate quantified after two hours. The clearance and excretion rates were measured following the methodologies described in Coughlan (1969) and Solórzano (1969) respectively (Chapter 2 (2.3)). On the same days, the tissue samples were taken for analysis of PSP toxins. From the PSP group, one clam from each replicate was dissected into: (1) digestive gland and (2) other tissues without the

digestive gland (gonads + muscle + gills + mantle). The two groups of tissues were kept separately and frozen at -80ºC for further processing. On day 10, three replicates of *Alexandrium tamarense* were taken for later toxin analyses. Details of the extraction and quantification of the PSP toxins are given in Chapter 2 (2.4).

Table 5.1: Composition of the diets provided to individual *P. donacina* in the physiological experiment (TETR = *Tetraselmis* sp.; AT = *A. tamarense*; AM = *A. margalefii*).

Periods	Treatments	TETR (cells d^{-1})	AT (cells d^{-1})	AM (cells d^{-1})	Toxicity (nmol STX-diHCl d^{-1})
Acclimation (2 days)	PSP group	2.5×10^7	0	5×10^5	0
	Control group	2.5×10^7	0	5×10^5	0
Intoxication (10 days)	PSP group	2.5×10^7	5×10^5	0	17
	Control group	2.5×10^7	0	5×10^5	0
Detoxification (8 days)	PSP group	2.5×10^7	0	5×10^5	0
	Control group	2.5×10^7	0	5×10^5	0

5.2.3 Behavioural studies

Burrowing and siphon activity

This experiment was carried out using eight clams collected in May, 2008 (dry weight 2.9 ± 1.2 g; shell length 80.6 ± 9.8 mm). Prior to the experiment, the clams were acclimated for two weeks in 60 L aquaria containing circulating seawater and 15 cm of sand in the

bottom of the aquaria. The sand was taken from the place the clams had been collected from. The bivalves were transferred to 20 L experimental aquaria containing 15 cm of sand in the bottom, 15 cm of seawater on the top and constant aeration. In these experimental aquaria, *Paphies donacina* was fed for 2 days on a diet of 2.5 x 10^7 cells day^{-1} $clam^{-1}$ of *Tetraselmis* sp. (pre-trial). Over the next three days, the clams were divided into the PSP group (four replicates) which fed 10^6 cells day^{-1} $clam^{-1}$ of *A. tamarense* and the control group (four replicates) which fed 2.5 x 10^7 cells day^{-1} of *Tetraselmis* sp.

In every sampling day, two different behaviours were measured: the activity of the siphons, measured in the morning after the clams had been fed and the depth of the burrowing, measured in the afternoon (Figure 5.1). To measure the activity of the siphons, the clams were observed for one minute during feeding activity and observations of both inhalant and exhalant siphons were done. Behaviours were classified into:

1. both siphons opened (OO)
2. both siphons closed (CC)
3. inhalant siphon opened (IO)
4. exhalant siphon closed (EO)
5. erratic contractions of the inhalant siphon (IE)
6. erratic contractions of the exhalant siphon (EE)

To determinate the burrowing depth, a piece of string (40 cm) was attached to the umbo of each clam which was placed on the top of the sand and allowed to burrow. After 30 min, and using the string attached, the burrowing depth of the clam was measured. At the end of the experiment, the length of the shell was measured using a vernier caliper.

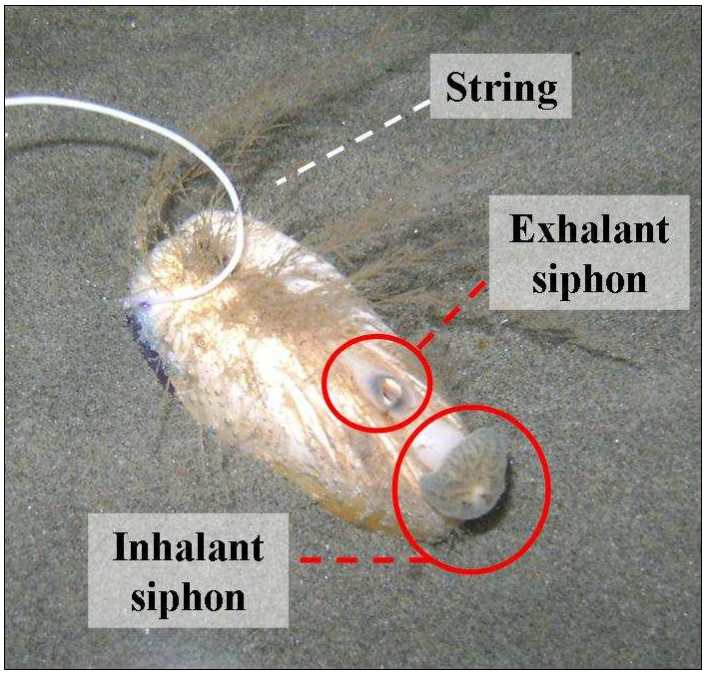


Figure 5.1: Inhalant and exhalant siphons of *Paphies donacina* studied in the behavioural experiments. The string attached to the shell of the clam was used for measuring the burrowing depth.

5.3 Results

5.3.1 Intoxication and detoxification of *Paphies donacina* exposed to toxic and non-toxic *Alexandrium* spp.

Clearance rate

The clearance rates for the PSP and the control groups were similar over the intoxication period. Overall, the rate for clams exposed to *Alexandrium tamarense* was 0.12 ± 0.05 L h^{-1} g^{-1} and the rate for the control clams exposed to *Alexandrium margalefii* was 0.11 ± 0.06 L h^{-1} g^{-1} (Figure 5.2). On day 14 (detoxification period), both groups had similar rates to those measured in the intoxication period. At the end of the detoxification period (day 18), the PSP and the control groups had increased the clearance rates to the highest values measured in the experiment (PSP group: 0.29 ± 0.07; control group: 0.21 ± 0.06 L h^{-1} g^{-1}).

There were no significant differences in the clearance rate between the PSP and the control group (repeated measures ANOVA, $F_{1,6} = 1.93$, $P = 0.21$). In contrast, there was a

significant effect of time on the clearance rate (repeated measures ANOVA, $F_{5,30} = 13.84$, $P < 0.001$). The interaction between both variables tested was not significant (Table 5.2). On day 18, the PSP and the control group had significantly higher clearance rates than in the rest of the experiment (post-hoc Tukey Test).

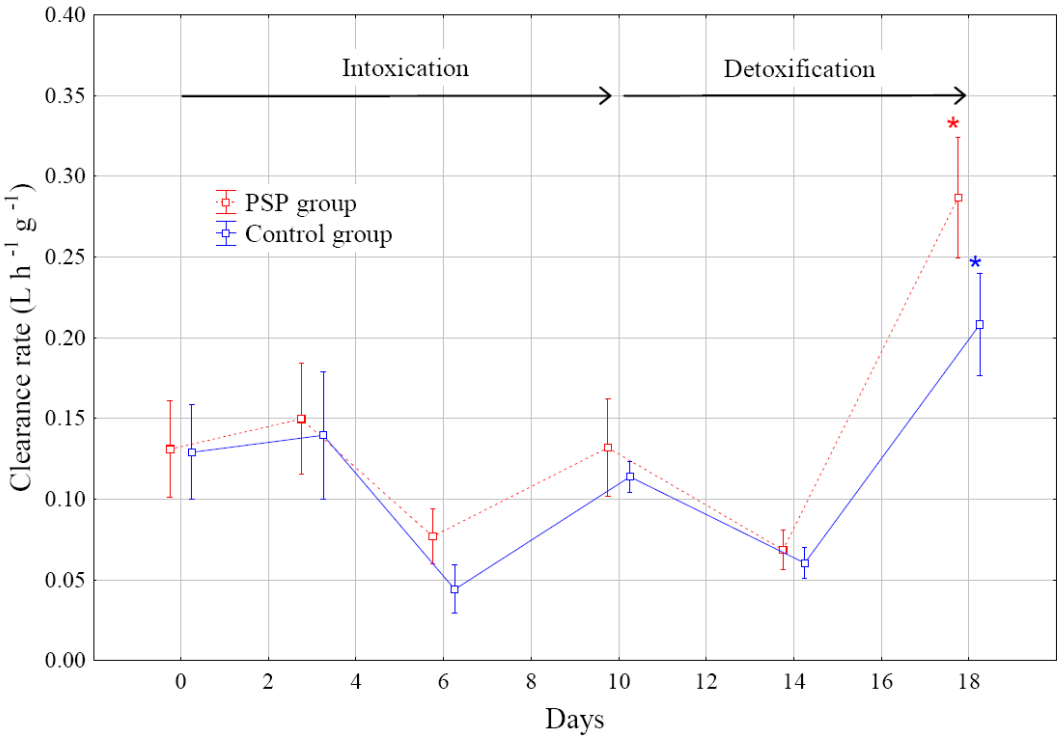


Figure 5.2: Clearance rate of *Paphies donacina* during intoxication and detoxification periods. Measurements were taken on days 0, 3, 6, 14 and 18. The asterisk (*) represents significant higher clearance rate. Values are means ± SE.

Table 5.2: Results of repeated measures ANOVA testing the effects of diet (treatment) and exposure time on the clearance rate of *Paphies donacina.*Values in **bold** are of statistical significance.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.007	1	0.007	1.9	0.21
Error	0.003	6	0.003		
Days	0.03	5	0.03	**13.8**	**< 0.001**
Days*Treatments	0.001	5	0.001	0.6	0.69
Error	0.002	30	0.002		

Excretion rate

The PSP and the control groups had similar excretion rates over the experiment (Figure 5.3). Overall, the PSP group excreted 19.0 ± 5.3 µg NH4-N h^{-1} g^{-1} over the intoxication period and 21.3 ± 5.3 µg NH4-N h^{-1} g^{-1} over the detoxification period. Similar values were found in the control clams and the excretion rate was 21.2 ± 6.1 µg NH4-N h^{-1} g^{-1} during both experimental periods (intoxication and detoxification).

There were no significant differences in the excretion rate of the PSP and the control groups (repeated measures ANOVA, $F_{1,6}$ = 0.001, P = 0.97). Neither the effect of time (repeated measures ANOVA, $F_{5,30}$ = 2.19, P = 0.08) nor the interaction between both variables tested were significant (Table 5.3).

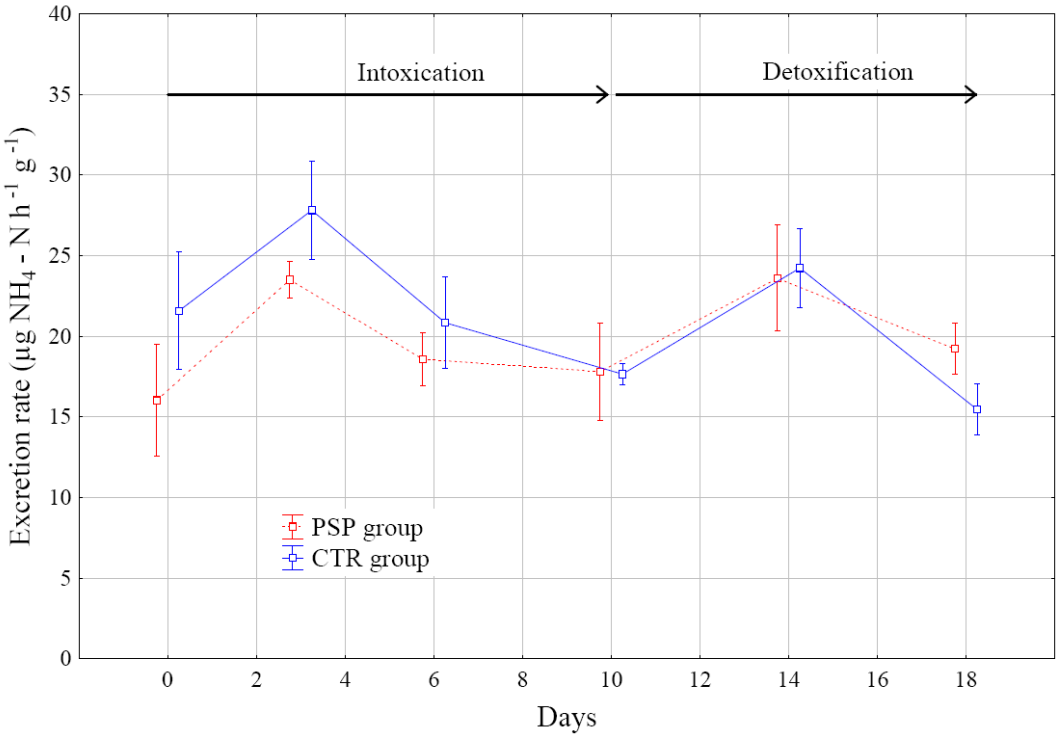


Figure 5.3: Excretion rate of *Paphies donacina* during intoxication and detoxification periods. Measurements were taken on days 0, 3, 6, 10, 14 and 18. Values are mean ± SE.

Table 5.3: Results of repeated measures ANOVA testing the effects of diet (treatment) and exposure time on the clearance rate of *Paphies donacina.*

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.04	1	0.04	0.001	0.97
Error	162.4	6	27.1		
Days	433.9	5	86.8	2.19	0.08
Days*Treatments	244.8	5	49.0	1.23	0.31
Error	1188.4	30	39.6		

Total toxin burden of PSP toxins in the tissues

The total toxin burden of PSP toxins in the tissues of *Paphies donacina* was calculated by adding together the concentrations of toxins detected in the digestive gland to the toxins detected in the homogenate of the other tissues (gonads + muscle + gills + mantle). The intoxication period was characterised by an increase of the total toxin body burden in the tissues; day 3 = 138, day 6 = 329 and day 10 = 532 µg STX-diHCl equiv 100 g^{-1}. Over the detoxification period, clams had not decreased the total toxin burden and they remained toxic for human consumption (more than 80 µg STX-diHCl equiv. 100 g^{-1} tissue); day 14 = 489 and day 18 = 635 µg STX-diHCl equiv 100 g^{-1} tissue (Figure 5.4).

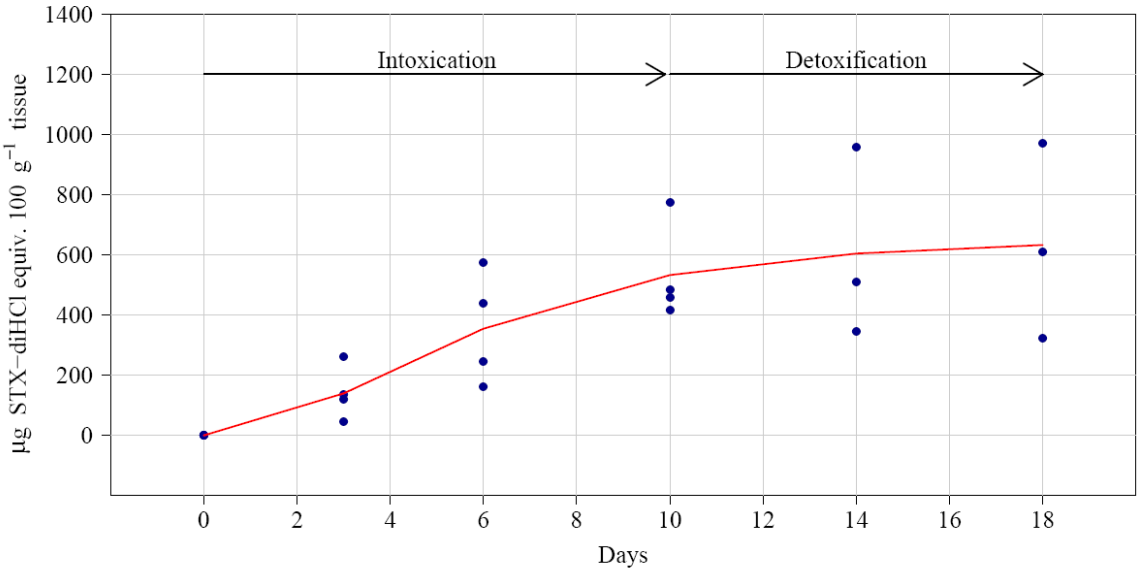


Figure 5.4: Total content of PSP toxins in the tissues of clams during intoxication and detoxification periods. Samples were taken on days 0, 3, 6, 10, 12 and 18.

PSP toxin profile in the digestive gland

Throughout the experiment, most of the total toxin burden of *Paphies donacina* was contained in the digestive gland (day 3 = 98%, day 6 = 98%, day 10 = 98%, day 12 = 97%, day 18 = 97%). The PSP toxins present in the digestive gland during both periods were GTX4, GTX1, dcGTX3 + GTX5, dcGTX2, GTX3, GTX2, dcNeo + Neo, dcSTX, STX and C2 (Figure 5.5). The toxins dcGTX2 and GTX2 were detected in the digestive gland, but were not present in the toxin profile of *A. tamarense*. The toxin profile of the digestive gland was mainly dominated by GTX1 followed by dcGTX3 + GTX5, dcNeo + Neo, STX and C2. The toxin C2 was mainly detected in the intoxication period. The proportion of GTX1 increased over the experiment whereas GTX4 and dcGTX3 + GTX5 decreased. In the detoxification period both dcSTX and STX increased in relative proportions. The toxins dcNeo + Neo were present in similar proportions over the experiment (Table 5.4).

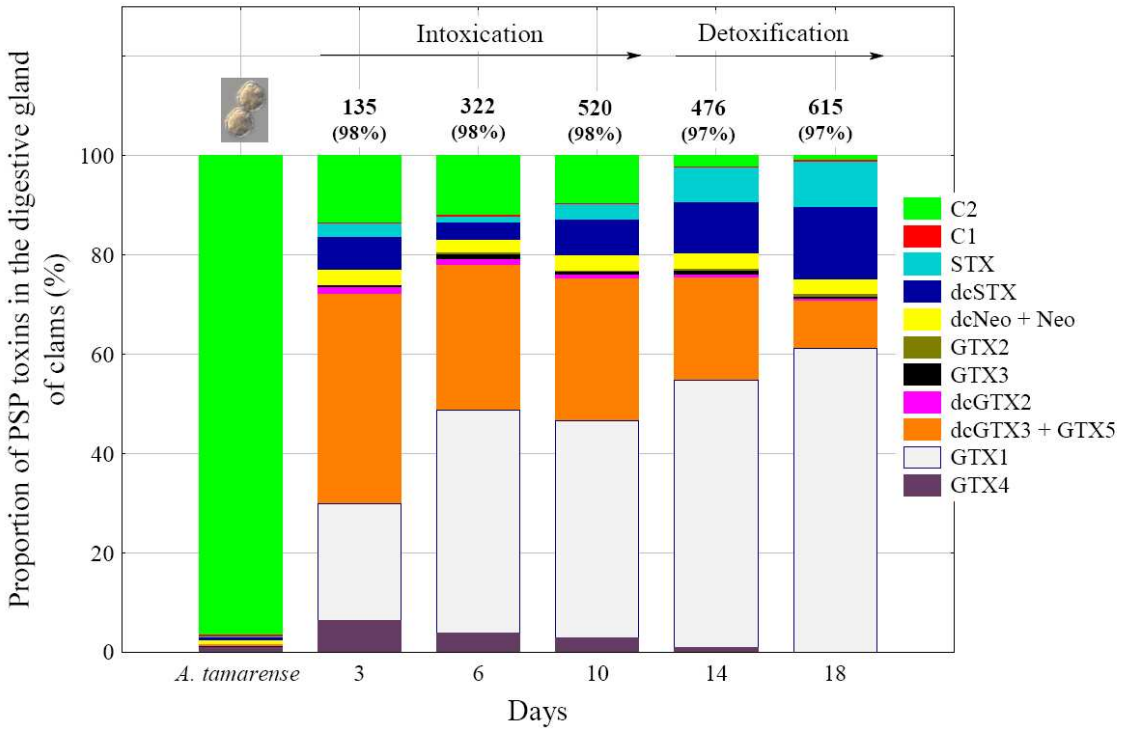


Figure 5.5: PSP toxin profile of the digestive gland from clams during intoxication and detoxification periods. Values are expressed as a percentage (%) of the total content of toxins in this tissue. The total content of toxins in this tissue is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}) with the corresponding proportion of the total toxin burden of the clams.

Table 5.4: PSP toxin profile of the digestive gland from clams exposed to intoxication and detoxification periods. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in this tissue (%). On each day, the compound dominating the toxin profiles is shown in **bold**.

	Day 3		Day 6		Day 10		Day 14		Day 18	
	T	%	T	%	T	%	T	%	T	%
GTX4	86	6.5	12.5	3.9	15.2	2..9	4.6	1.0	-	-
GTX1	31.4	23.5	**144.6**	**44.9**	**227.3**	**43.7**	**256.3**	**53.8**	**362.5**	**59.0**
dcGT3 + GTX3	**56.4**	**42.2**	93.5	29.0	148.5	28.5	97.9	20.6	74.8	12.2
dcGTX2	1.7	1.3	3.6	1.1	3.7	0.7	3.2	0.7	3.6	0.6
GTX3	0.6	0.5	3.8	1.2	3.7	0.7	3.1	0.7	3.6	0.6
GTX2	-	-	1.0	0.3	0.5	0.1	1.7	0.4	4.9	0.8
dcNeo + Neo	4.1	3.1	8.4	2.6	16.8	3.2	15.2	3.2	17.4	2.8
dcSTX	8.9	6.6	10.9	3.4	37.1	7.1	48.8	10.2	85.3	13.9
STX	3.8	2.8	4.2	1.3	16.0	3.1	32.9	6.9	54.9	8.9
C1	0.2	0.2	0.7	0.2	1.3	0.3	1.5	0.3	1.8	0.3
C2	18.1	13.5	38.8	12.1	50.1	9.6	10.8	2.3	5.9	1.0
Total	135	100	322	100	520	100	476	100	615	100

PSP toxin profile in the other tissues

The homogenate of the other tissues (gonads + muscle + gills + mantle) contained low quantities of the PSP toxins (day 3 = 2%, day 6 = 2%, day 10 = 2%, day 12 = 3%, day 18 = 3%). The PSP toxins presented in these tissues were GTX4, GTX1, dcGTX3 + GTX5, dcGTX2, GTX3, dcNeo + Neo, dcSTX, STX, and C2 (Figure 5.6). The toxin dcGTX2 was not present in the toxic profile of *A. tamarense*. The toxin profile of the other tissues was comprised mainly by dcGTX3 + GTX5, dcGTX2 and dcSTX. The presence of dcNeo + Neo was detected from day 6 onwards and in constant proportions. For most of the experiment, dcGTX3 + GTX5 dominated the toxic profile of these tissues, but this proportion decreased over time. In contrast, dcSTX and dcGTX2 increased over this time period. The carbamoyl toxin, GTX1 was found only on day 18 (Table 5.5).

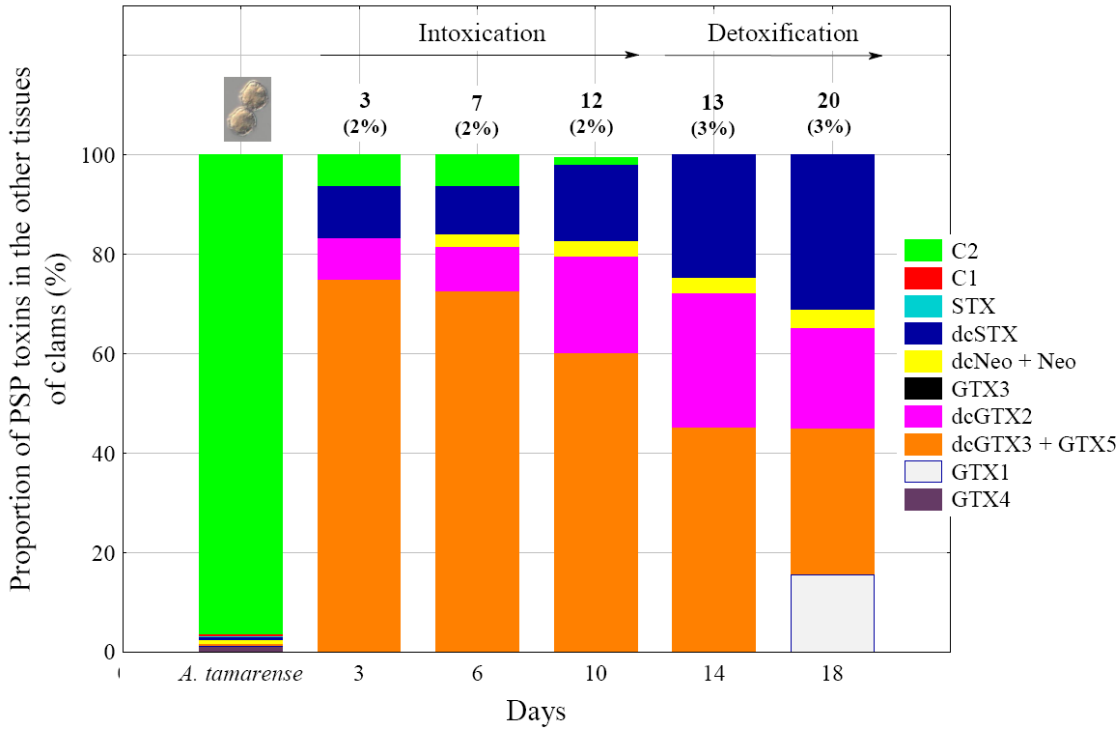


Figure 5.6: PSP toxin profile of the other tissues from clams during intoxication and detoxification periods. Values are expressed as a percentage (%) of the total content of toxins in this tissue. The total content of toxins in this tissue is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}) with the corresponding proportion of the total toxin burden of the clams.

Table 5.5: PSP toxin profile of the other tissues from clams exposed to intoxication and detoxification periods. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in this tissue (%). On each day, the compound dominating the toxin profiles is shown in **bold**.

	Day 3		Day 6		Day 10		Day 14		Day 18	
	T	%	T	%	T	%	T	%	T	%
GTX1	-	-	-	-	-	-	-	-	3.2	15.5
dcGT3 + GTX3	**2.2**	**74.8**	**5.2**	**72.5**	**7.5**	**60.0**	**5.8**	**45.0**	**6.0**	**29.3**
dcGTX2	0.2	8.3	0.6	8.9	2.4	19.5	3.5	27.1	4.1	20.2
dcNeo + Neo	-	-	0.2	2.6	0.4	3.1	0.4	3.0	0.8	3.7
dcSTX	0.3	10.5	0.7	9.8	1.9	15.4	3.2	24.8	6.4	31.2
C1	0.2	6.4	-	-	0.1	0.5	-	-	-	-
C2	-	-	0.5	6.3	0.2	1.5	-	-	-	-
Total	3	100	7	100	12	100	13	100	20	100

5.3.2 Behavioural studies

Siphon activity

The inhalant siphon of *Paphies donacina* exposed to *Tetraselmis* sp. and *Alexandrium tamarense* did not show any effect to the different algae supplied. In contrast, the exhalant siphon showed different behaviour over the experiment (Table 5.6). Of the eight clams used in this experiment, six had normal siphon activity on the first day of the pre-trial (75%) and two clams had both siphons closed. The next days seven clams had normal siphon activity (88%) and one clam had both siphons closed.

On day 0, of the four clams fed on *A. tamarense*, three presented erratic contractions of the exhalant siphon (75%) whereas the inhalant siphon remained opened (IO/EE). On day 1, all clams in the PSP group presented the same behaviour observed on day 0 (IO/EE). On day 2, two clams presented the behaviour IO/EE (50%) whereas one clam had both siphons closed and one clam had both siphons opened. On days 0 and 2, all control clams fed *Tetraselmis* sp. had both siphons opened. On day 1, one control clam had both siphons closed. No erratic contractions of the exhalant siphon was observed in control clams.

Burrowing depth

The burrowing depth of *Paphies donacina* fed with *Tetraselmis* sp. and *A. tamarense* was variable over the experiment. One clam in the PSP group and one clam in the control group did not burrow at any time. The rest of the clams burrowed between 2 and 6.5 cm. The PSP group burrowed to the same depth when fed either *Tetraselmis* sp. or the toxic dinoflagellate *A. tamarense*. The burrowing depth of the control clams fed *Tetraselmis* sp. was also similar. There were no significant differences in the burrowing depth between the PSP and the control group (repeated measures ANOVA, $F_{1,6} = 0.01$, $P = 0.89$). Neither the time nor the interactions between both variables tested were significant (Table 5.8).

Table 5.6: Summary of siphon activity of *Paphies donacina* exposed to the non-toxic algae, *Tetraselmis* sp. and the toxic dinoflagellate, *A. tamarense*. The behaviours were classified as: both siphons opened (OO), both siphons closed (CC), inhalant siphon opened (IO), exhalant siphon closed (EO), erratic contractions of the inhalant siphon (IE) and erratic contractions of the exhalant siphon (EE).

Treatment	Clam	Pre-trial		Day 0	Day 1	Day 2
		Tetraselmis spp.		*A. tamarense*		
PSP	1 (6.9 cm)	OO	OO	CC	IO/EE	IO/EE
group	2 (7.6 cm)	OO	OO	IO/EE	IO/EE	IO/EE
	3 (8.1 cm)	OO	OO	IO/EE	IO/EE	CC
	4 (8.3 cm)	CC	OO	IO/EE	IO/EE	OO
		Tetraselmis spp.		*A. tamarense*		
Control	5 (6.7 cm)	OO	OO	OO	OO	OO
group	6 (8.4 cm)	OO	OO	OO	OO	OO
	7 (9.3 cm)	CC	OO	OO	CC	OO
	8 (9.3 cm)	OO	CC	OO	OO	OO

Table 5.7: Summary of the burrowing depth (cm) of *Paphies donacina* exposed to the non-toxic algae *Tetraselmis* sp. and the toxic dinoflagellate *Alexandrium tamarense*.

Treatment	Clam	Pre-trial		Day 0	Day 1	Day 2	Average ± SD
		Tetraselmis spp.				*A. tamarense*	
PSP	1 (6.9 cm)	2	2	3	2	3	2.4 ± 0.5
group	2 (7.6 cm)	5	5	4	4	3.5	4.3 ± 0.7
	3 (8.1 cm)	6.5	4	6	5	5	5.3 ± 1.0
	4 (8.3 cm)	0	0	0	0	0	-
		Tetraselmis spp.				*A. tamarense*	
Control	5 (6.7 cm)	6	5	4	5	4	5.0 ± 0.7
group	6 (8.4 cm)	3.5	2	4	4	2	3.1 ± 1.0
	7 (9.3 cm)	0	0	0	0	0	-
	8 (9.3 cm)	5	5	4.5	5	5	4.9 ± 0.2

Table 5.8: Results of repeated measures ANOVA testing the effect of *Tetraselmis* sp. and *Alexandrium tamarense* (treatment) and days of exposure on the burrowing depth of *Paphies donacina*.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.04	1	0.04	0.001	0.97
Error	162.4	6	27.1		
Days	433.9	5	86.8	2.19	0.08
Days*Treatments	244.8	5	49.0	1.23	0.31
Error	1188.4	30	39.6		

5.4 Discussion

The results of this study showed that the clam, *Paphies donacina* was able to ingest the toxic dinoflagellate *Alexandrium tamarense*, which led to accumulation of PSP toxins in the tissues. The physiological and behavioural responses to the toxic algae were variable and demonstrated that *P. donacina* responded differently to the species of algae supplied during the experiment. Nevertheless, it was not possible to conclude whether clams were affected by the dinoflagellate cells (e.g. size, shape) as well as the PSP toxins.

5.4.1 Intoxication and detoxification of *P. donacina* exposed to *A. tamarense*

Clearance and excretion rates

The clam, *Paphies donacina* fed *Alexandrium* species (toxic and non-toxic) presented low clearance rates. Moreover, during the first 14 days, the clams had lower clearance rates (~ 0.12 L h^{-1} g^{-1}) than those reported in Marsden (1999). This author had measured the rates using the same species of bivalve fed *Isochrysis galbana* and had observed clearance rates between 0.25 and 0.5 L h^{-1} g^{-1}. *Paphies donacina* exposed to dinoflagellates reached similar values to those reported in Marsden (1999) on day 18. These results suggest that *P. donacina* was maybe affected by the dinoflagellates cells (e.g size, shape) and a period of time of 18 days was necessary for acclimation to the dinoflagellate species.

Bricelj et al. (1991) studied the responses of the clam, *Mercenaria mercenaria* to PSP conditions and observed that clams showed no evidence of decreasing ingestion of toxic cells with increasing body burden of toxins, but they presented different clearance rates according to the toxicity of the strain used to feed them. This suggested that *M. mercenaria* had some toxin-recognition mechanism. In the present study on *Paphies donacina*, there was no significant effect of the PSP toxins on the clearance rate.

Two possible hypothesis may arise from these results:

1. the PSP toxins did not affect the clearance rate of *P. donacina*, or
2. the effect of PSP toxins was too small to be identified and was masked by the effect of the *Alexandrium* species (e.g. size, shape).

Both hypothesis could be tested using other species of dinoflagellates with higher toxicities. Lower clearance rates in the PSP group than in the control group would indicate an effect due to the toxins.

The excretion rate of *Paphies donacina* was not affected by the algae supplied during the experiment. MacDonald et al. (1998) measured excretion rates in the clam, *Mya arenaria* exposed to different concentrations (1 to 14 mg L^{-1}) and qualities (25 to 80% of organic matter) of seston and observed a range of excretion rates between 6 to 30 µg NH4-N h^{-1} g^{-1}. These authors concluded that *M. arenaria* exposed to different conditions was able to maintain relatively constant ammonia excretion rates. Overall, the excretion rate of *P. donacina* was 20.5 ± 5.7 µg NH4-N h^{-1} g^{-1} which was within the range of *M. arenaria* observed by MacDonald et al. (1998).

PSP toxins in the tissues of clams

Despite the low rates of feeding, *Paphies donacina* was able to accumulate high levels of PSP toxins in the tissues over the 10 days of exposure to *Alexandrium tamarense* (532 µg STX di-HCl equiv. 100 g^{-1}). Over the intoxication period, the total content of the PSP toxins in the tissues increased, however, over the detoxification period, the total content of the PSP toxins in the tissues did not decrease. These findings are in agreement with the study of Mackenzie et al. (1996a), where an slow and erratic decline in toxicity within *Paphies subtriangulata* was observed after disappearance of *A. minutum* from the plankton. The toxicity of the clam did not fall below the official action level of 80 µg STX di-HCl equiv. 100 g^{-1} until 10 months after the initial contamination event.

Other species of bivalves have been found to retain high toxin levels (months to years); the Alaska butter clam, *Saxidomus giganteus*, the sea scallop *Placopecten magellanicus*, and the surfclam *Spisula solidissima* (Quayle, 1969; Shumway et al., 1988; Shumway, 1990, Beitler and Liston, 1990; Shumway and Cembella, 1993; Cembella et al., 1993, 1994). *Paphies donacina* tissues did not detoxify during 8 days of non-toxic conditions, therefore further studies involving a longer detoxification period are necessary for a better understanding of detoxification processes in this species of bivalve.

The digestive gland of *P. donacina* accumulated more than 97% of the total PSP toxin content of the body, both during the intoxication and detoxification periods. The epimerisation of the hydroxysulfate group at C11 took place in this tissue leading to GTX1 at an increasing rate over the experiment. A change of β-epimers (e.g. C2, C4, GTX3, GTX4) to α- epimers (e.g. C1, C3, GTX2, GTX1) is commonly observed when toxins are transferred from dinoflagellates to shellfish (Bricelj et al., 1991). β-epimers are the favoured configuration in dinoflagellates and they gradually convert to the more stable α configuration in the shellfish tissues under thermodynamic equilibrium conditions (Oshima, 1995a).

The total content of toxins in the other tissues was much lower (less than 3%) than in the digestive gland and was dominated by dcGTX3 + GTX5, dcGTX3 and dcSTX. It has been reported that in the conversion of C toxins to the corresponding decarbamoyl derivatives, enzymes may be involved, and this has been demonstrated in several Pacific clam species including *Protothaca staminea* (Sullivan et al., 1983a). Oshima, 1995a studying Japanese clams, *Mactra chinensis* and *Peronidia venulosa* demonstrated that they contained an enzyme that catalysed hydrolysis of N-sulfacarbamoyl groups which converted C1 and C2 to dcGTX2 and dcGTX3. He also, observed that C2 was hydrolysed much faster than C1 indicating that the orientation of the 11-hydroxysulfate in the substrate toxins greatly affected the rate of turn over of the enzyme.

The digestive gland of *P. donacina* also contained higher levels of STX and dc-STX than in the other tissues. These toxins are known for their high relative toxicity. In the other tissues the presence of GTX1 was detected only on day 18 and the tissues did not contain GTX2 over the experimental periods. These results indicate that the epimerisation in the other tissues occurred at a slower rate than in the digestive gland.

5.4.2 Effect of *A. tamarense* on the siphon activity and burrowing of *P. donacina*

The clams fed with *A. tamarense* presented erratic contractions of the exhalant siphon. This sensitivity may be related with the low clearance rates measured in clams. Thorin et al. (1998) studied the siphon activity of *Mya arenaria* and they reported that the exhalant siphon was more active than the inhalant siphon. This sensitivity in the exhalant siphon of *P. donacina* did not affect the burrowing capacity under the same experimental conditions.

Bricelj et al. (1996) studied the ability of *Mya arenaria* and *Spisula solidissima* to reburrow in sediment following exposure to toxic *Alexandrium* cells and this provided a useful index of in vivo sensitivity to PSP toxins. They found that the ability of *M. arenaria* to burrow was markedly impaired in the presence of toxic *Alexandrium* species whereas *S. solidissima* remained unaffected. The same responses were observed in the clearance rates, but the authors did not carry out control experiments using non-toxic species of *Alexandrium.*

The main finding of this study on *P. donacina*, was the capacity of clams to retain high toxic compounds as STX, dcSTX, and GTX1 during the detoxification period. This phenomenon is a critical for public health management as it only takes trace level contamination to raise the toxicity above the 80 µg STX di-HCl equiv. 100 g^{-1}. It is therefore advisable that during a toxic algal bloom event in New Zealand, appropriate information about the risk involved in the consumption of bivalves that only slowly detoxify be transmitted promptly to the population. Bivalves such as *Paphies subtriangulata* and *P. donacina* could retain high level of toxins for long periods of time even though the toxic bloom has disappeared. After a toxic algal bloom, consumers need to be aware that while some bivalves (e.g. *Perna canaliculus*) are safe for human consumption others could still remain toxic for a long period of time.

Chapter 6

Physiological effects and biotransformation of PSP toxins in *Pecten novaezelandiae*

6.1 Introduction

There are some 400 known living species in the bivalve family Pectinidae, commonly known as scallops. They occur in all seas from the polar regions to the tropics. Most of the commercially important species occur in the inshore waters of the continental shelves, but scallops are found in waters of all depths from the intertidal zone down to some 7,000 m. They are extremely attractive and prominent animals with many species of considerable commercial importance as the flesh is considered a luxury food in many countries (Brand, 2006).

The presence of toxic algae and the potential for blooms have clear, negative effects on the development of aquaculture. Not only do these outbreaks pose a threat to public health (numerous deaths have been attributed to paralytic shellfish poisoning (PSP) over the years), but they are also responsible for mass mortalities of shellfish and they can result in great economic hardship to the coastal fishing industries and aquaculture facilities (Shumway, 1990). Slowly, progress is being made towards understanding the nature and

causes of toxicity in shellfish, but the toxins pose serious problems to harvesters, seafood processors, consumers and regulatory agencies.

Like other suspension-feeding bivalve molluscs, the scallops accumulate toxins associated with harmful algal species and they become a serious threat to public health. It is noteworthy that many species bind the toxins in various tissues for extended periods of time and the bivalves become unsuitable for human consumption even when blooms are not evident (Shumway and Cembella, 1993; Bricelj and Shumway, 1998). In addition to becoming vectors for these algal toxins, many species of scallops are themselves adversely affected by the toxins.

The effects of the PSP toxins on the larvae and juvenile stages of shellfish could also cause severe economic losses in the aquaculture industry, due to the decreased production of adults in the next harvest season. Yan et al. (2003) studied the effects of *Alexandrium tamarense* on early life stages of the scallop, *Argopecten irradians concentricus* and found that the toxic algae had adverse effects on scallop activity, including mobility of the D-shape larvae, attachment, climbing ability of juveniles and possible chronic inhibitory effects on growth.

It is well known that the accumulation of PSP toxins in bivalves is species-specific. For example, in the South China Sea, the scallop, *Chlamys nobilis* has a much higher PSP burden than the clam, *Ruditapes philippinarum* (Li et al., 2001). These authors concluded that these observations may be partly explained by the differences in feeding and absorption behaviour. Bricelj and Shumway (1998) suggest that other processes, especially the biotransformation of the PSP toxins, may also play a significant role in defining the inter-species differences in PSP body burden among marine bivalves.

Cembella et al. (1994) described seasonal variation in the PSP toxicity of *Placopecten magellanicus* from the Gulf of Maine. This was most likely due to persistence and magnitude of toxic blooms, physiological responses, seasonal changes in water temperature and perhaps genotypic differences among the scallop populations. These authors found that selective retention and in vivo biotransformation of specific PSP toxin analogues were the major determinant of toxicity in the tissues of the scallops. The toxin bioconversion was differentiated into two general classes; firstly epimerisation occurring

during toxin uptake and early in detoxification and secondarily reductive transformation affected by removal of sulfate and N-1 hydroxyl groups, which become evident later in detoxification. They also observed that the formation of decarbamoyl derivatives in the tissues of the scallops was less active than in other bivalves such as the littleneck clam, *Protothaca staminea*. The dcSTX an dcGTX derivatives were often dominant in the toxin profile of this clam (Sullivan et al., 1983a).

The study of the detoxification kinetics may help to estimate the time required to reduce the PSP toxicity in shellfish tissues to a level making them safe for human consumption. In this context, bivalves fall into two general categories in terms of detoxification capacities. Rapid to moderate detoxifiers, such as mussels (e.g. *Mytilus edulis),* take only a few weeks (10.6% day^{-1}) while slow detoxifiers such as some clams (e.g. *Spisula solidissima*) and scallops (e.g. *Placopecten magellanicus*) exhibit average detoxification rates of just 0.7 to 0.5% day^{-1} (Bricelj and Shumway, 1998).

At least 18 different species of Pectinidae have been recorded from New Zealand waters (Powell, 1979). Of these, only two, *Pecten novaezelandiae* and *Chlamys delicatula* are large enough or occur at sufficient density to have encouraged any commercial exploitation. *Pecten novaezelandiae* occurs sporadically around the entire coastline including Stewart Island and the Chatham Island. It is found on a wide variety of substrates in semi-estuarine and coastal waters, from low tide to at least 90 m depth (Bull, 1991).

A fishery for *P. novaezelandiae* has existed for over 40 years (Marsden and Bull, 2006). Scallops are fished commercially in various locations, but the main beds are in the north end of South Island (Golden Bay, Tasman Bay and the Marlborough Sound) and the north of the North Island (Bream Bay, Spirits Bay, the Bay of Plenty, Hauraki Gulf) (Bull, 1991). Since 1989, a rotational fishing regime, coupled with extensive stock enhancement, has been operated with great success in Tasman Bay and Golden Bay to stabilise the fishery and achieve environmental and commercial sustainability (Arbuckle and Metzger, 2000).

Because of the potential threats of increasing toxic algal blooms in New Zealand waters to the scallops industry (Marsden and Bull, 2006), it is necessary to investigate the effect of the PSP toxins on the physiology of *P. novaezelandiae.* In this study scallops

were exposed to intoxication and detoxification periods and fed on different algae, including the toxic dinoflagellate *Alexandrium tamarense*. Over these periods, the clearance and excretion rates were measured and the level of PSP toxins accumulated in the tissues was also determined. In an independent experiment, the scallops were exposed to a higher concentration of *A. tamarense* and the oxygen consumption was measured.

6.2 Methods

6.2.1 Collection of the scallops

Pecten novaezelandiae (N = 125) was collected by dredging at Ketu Bay, Pelorous Sound, Malborough in February, 2009. To avoid major disturbances of the seabed (20 m depth) a small recreational dredge (Foil Dredge) was used for the collection, at a boat speed of 4 knots. The scallops were placed in a purpose-built holding tank with flowing fresh seawater for the journey to Havelock. In previous collections (2008), this bivalve species was found to be easily stressed and high mortality rates occurred under laboratory conditions (100% of the bivalves had died after 1 month). Commercially-caught scallops and dry packing specimens for transportation resulted in poor survival. Because of these experiences, a different transportation system was designed.

At Havelock, the scallops were transferred to heavy-duty plastic bags containing seawater sourced from the collection location. Each bag, containing 15 scallops and 25 L of seawater, was placed inside a chilli bin (400 x 560 x 350 mm) and icepacks positioned around the bags to keep the temperature of the water cool during transfer. A battery-powered aeration system was set up and 2 bubbler stones were placed in the seawater of each bag to provide constant aeration. The transportation of the scallops from Ketu Bay to Christchurch took 10 hours with several stops to check the temperature of the seawater, the aeration systems and the condition of the bivalves. By the half way point, the scallops had produced significant amounts of foam, so the seawater was changed at the University of Canterbury's Edward Percival Marine Station in Kaikoura. As well as changing the seawater, the bivalves were placed in aquaria with continuous flowing seawater, one hour prior to the start of the second part of the journey.

The scallops arrived in good conditions at the aquaria facilities at the University of Canterbury with only minor production of foam. They were placed in aquaria with 800 L of circulating seawater and constant aeration. *Tetraselmis* sp. was supplied daily before the start of the experiments (2 weeks) and ammonia level in the water was also checked daily. The water was immediately changed when the level of ammonia started to reach high concentrations and only minor mortality only occurred under these conditions.

6.2.2 Intoxication and detoxification of *P. novaezelandiae* exposed to toxic and non-toxic *Alexandrium* spp.

This experiment was carried out using 48 scallops (dry weight 3.7 ± 0.7 g; shell length 94.3 ± 7.0 mm). The physiological responses (clearance and excretion rates) and analysis of the PSP toxins in the tissues were measured over a 20 day period. Replicates comprised independent aquaria containing six scallops and 50 L of seawater (salinity 30 ppt and 15ºC) with constant aeration. The PSP group contained four replicates as well as the control group. The shellfish were exposed to three periods of different diets in order to study the responses during intoxication and detoxification conditions.

Over the first two days (acclimation period) both groups were fed *Tetraselmis* sp. and the non-toxic dinoflagellate *Alexandrium margalefii*. For the next 10 days, the PSP group was fed *Tetraselmis* sp. and the toxic dinoflagellate *A. tamarense* (intoxication period). Over the last 8 days (detoxification period), the PSP group and the control group were fed *Tetraselmis* sp. and *A. margalefii*. The cell concentrations fed to the scallops over the experiment are listed in Table 6.1.

The clearance and excretion rates were measured following the methodologies described in Coughlan (1969) and Solórzano (1969) respectively (Chapter 2 (2.3)). These physiological responses were measured on the same individual from each replicate (repeated measures design) on days 0, 3, 6, 10, 14 and 18. The clearance rates for the PSP group were measured using vessels containing 2 L of filtered seawater. As an initial concentration scallops were fed 5 x 10^6 cells L^{-1} of *Tetraselmis* sp. And 2.5 x 10^5 cells L^{-1} of *A. tamarense*. The control group was maintained under the same conditions, but fed 5 x

10^6 cells L^{-1} of *Tetraselmis* sp. and 2.5 x 10^5 cells L^{-1} of *A. margalefii*. Measurements were taken over 2 h and the decrease of cells in the seawater was monitored every 30 min. No pseudofaeces were produced under these conditions.

Table 6.1: Composition of the diets provided to individual *P. novaezelandiae* over the experiment (TETR = *Tetraselmis* sp.; AT = *A. tamarense*; AM = *A. margalefii*).

Periods	Treatments	TETR (cells d^{-1})	AT (cells d^{-1})	AM (cells d^{-1})	Toxicity (nmol STX-diHCl d^{-1})
Acclimation (2 days)	PSP group	2.5 x 10^7	0	5 x 10^5	0
	Control group	2.5 x 10^7	0	5 x 10^5	0
Intoxication (10 days)	PSP group	2.5 x 10^7	5 x 10^5	0	17
	Control group	2.5 x 10^7	0	5 x 10^5	0
Detoxification (8 days)	PSP group	2.5 x 10^7	0	5 x 10^5	0
	Control group	2.5 x 10^7	0	5 x 10^5	0

After measuring the clearance rate, scallops were transferred to vessels with 0.5 L of filtered seawater and the excretion rate quantified after 1 h. For the analysis of the PSP toxins in the tissues, samples were taken on the same days and one bivalve from each replicate was dissected into: (1) digestive gland, (2) adductor muscle, and (3) other tissues without both the digestive gland and the adductor muscle (gonads + gills + mantle). The three groups of tissues were kept separately and frozen at –80°C for further processing. Also three replicates of *A. tamarense* were taken for toxin analysis. Details of the extraction and quantification of PSP toxins are given in Chapter 2 (2.4).

6.2.3 Effects of PSP toxins in the oxygen consumption of *P. novaezelandiae*

In this experiment *Pecten novaezelandiae* was exposed to a higher concentration of *A. tamarense* cells than was used in the previous experiment, and the oxygen consumption was measured. Eight scallops (dry weight 3.4 ± 0.4 g; shell length 90.8 ± 2.8 mm) were placed individually in vessels containing 5 L of seawater (salinity 30 ppt, 15°C) and constant aeration. The PSP group (four replicates) was fed 106 $cell^{-1}$ $day^{-1}$$scallop^{-1}$ of the toxic dinoflagellate *A. tamarense* for a 10 day period. The toxicity of the diet supplied to the PSP group was 35 nmol STX-diHCl d^{-1} $scallop^{-1}$. The control group (four replicates) was fed 106 $cell^{-1}$ $day^{-1}$$scallop^{-1}$ of the non-toxic dinoflagellate *A. margalefii* over the same period. On days 0, 3, 6 and 10, the oxygen consumption was measured on the same bivalve from each replicate using a repeated measures design.

Oxygen consumption was measured in a closed respirometer chamber (Figure 6.1) where the scallop was isolated in a fixed volume (0.5 L) of stirred water Bivalves were settled in the chambers for one hour and 100% oxygenated filtered seawater was passed through the system. The temperature was controlled using a double chamber system where the outside part of the chamber contained recirculating fresh water at 15°C.

At the start, each chamber was closed and a 1mL water sample was taken and injected into the oxygen unicell electrode. The same procedure was repeated every 20 min for two hours. The oxygen consumption of the scallop (MO2) was calculated from the decrease in the oxygen partial pressure of the water (PO2) in the chamber. A chamber without animals was used as control for record the decreasing of oxygen over the same time interval.

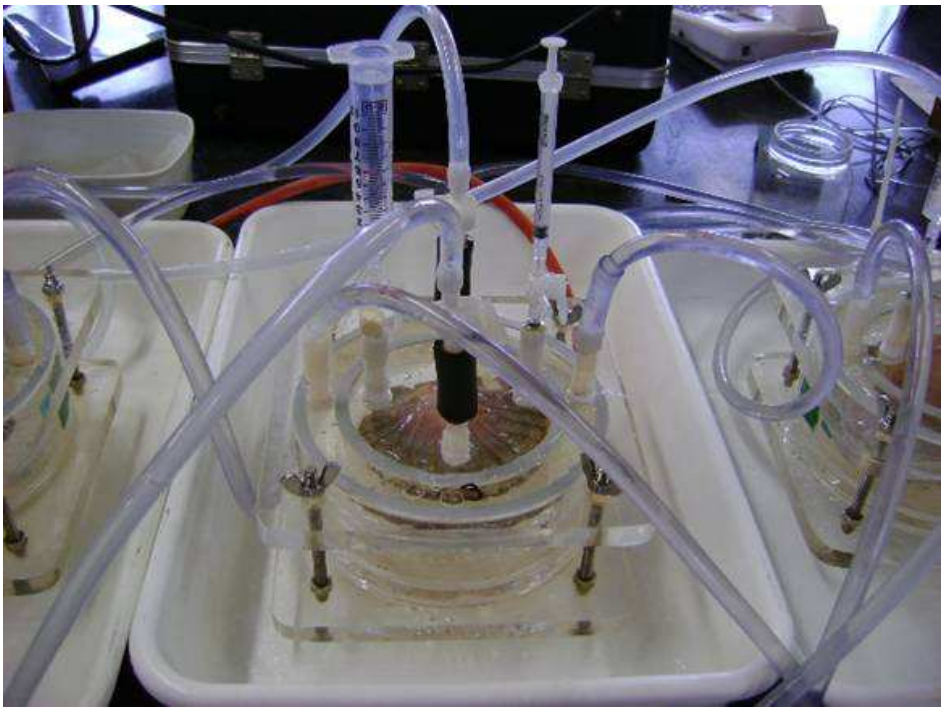


Figure 6.1: A respirometer chamber closed hermetically containing a scallop.

$$MO_2 = \frac{\left(\Delta PO_{2\,(sample)} - \Delta PO_{2\,(control)}\right) a \cdot V \cdot 60}{T \cdot W}$$

Where

MO_2 = µmol g^{-1} h^{-1}

a = solubility of oxygen in seawater at 15°C (µmol L^{-1} $Torr^{-1}$)

V = Volume of the experimental chambers (L)

T = experimental temperature (°K)

W = weight of the animal (g)

Finally, the results were transformed to *mL* O2 using the following equivalence:

1 *mol* O2 = 22.4 *L* O_2

6.3 Results

6.3.1 Intoxication and detoxication of *Pecten novaezelandiae* exposed to toxic and non-toxic *Alexandrium* spp.

Clearance rate

During both experimental periods, the clearance rate of the PSP group was lower than that found in the control group (Figure 6.2). On days 0, 3 and 6, the PSP group had their lowest average values of 0.15 ± 0.11 L h^{-1} g^{-1}. On days 10, 14 and 18, the PSP group had increased the average clearance rate to 0.44 ± 0.11 L h^{-1} g^{-1} . Overall, the clearance rate of the control group was similar from day 0 to 18 and was 0.79 ± 0.22 L h^{-1} g^{-1}. There were significant differences in the clearance rate between the PSP and the control group (repeated measures ANOVA, $F_{1,6}$ = 28.79, P = 0.001). The effect of time was also significant on the clearance rate (repeated measures ANOVA, $F_{5,30}$ = 3.18, P = 0.02). The interaction between both variables tested was not significant (Table 6.2). On days 0, 3 and 6, the PSP group had significantly lower clearance rates than those measured for the control group. They were no significant differences between both treatments on days 10, 14 and 18 (post-hoc Tukey analysis).

Excretion rate

The scallops exposed to a toxic and to a non-toxic dinoflagellate diet had similar excretion rates during the intoxication and detoxification periods. In both treatments, the highest values were measured on day 14 and the lowest on day 0. Overall, the excretion rate of the PSP group was 70.2 ± 12.4 µg NH4-N h^{-1} g^{-1} and the control group was 69.7 ± 15.0 µg NH4-N h^{-1} g^{-1}, during intoxication and detoxification periods. There were no significant differences in the excretion rate between PSP and control groups (repeated measures ANOVA, $F_{1,6}$ = 0.12, P = 0.73). In contrast, there was a significant effect of time on the excretion rate (repeated measures ANOVA, $F_{5,30}$ = 11.3, P < 0.001). The interaction between both variables was not significant (Table 6.3). Both groups presented higher excretion rates on day 14 than those measured on day 0 (post-hoc Tukey analysis).

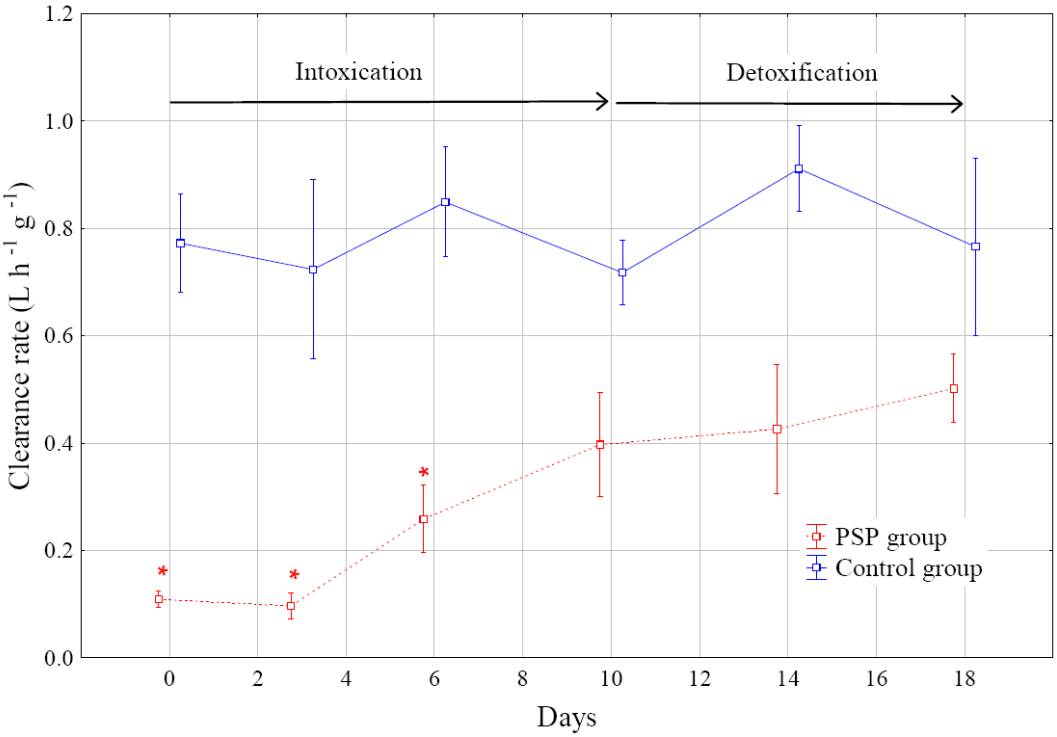


Figure 6.2: Clearance rate of *Pecten novaezelandiae* during intoxication and detoxification periods. The asterisk (*) represents significant lower clearance rate. Measurements were taken on days 0, 3, 6, 10, 14 and 18. Values are means ± SE.

Table 6.2: Results of repeated measures ANOVA testing the effects of toxic and non toxic diets (treatment) and days of exposure on the clearance rate of *Pecten novaezelandiae.* Values in **bold** are of statistical significance.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	2.90	1	2.90	**28.8**	**0.01**
Error	0.60	6	0.10		
Days	0.41	5	0.08	**3.18**	**< 0.05**
Days*Treatments	0.27	5	0.05	2.10	0.09
Error	0.79	30	0.02		

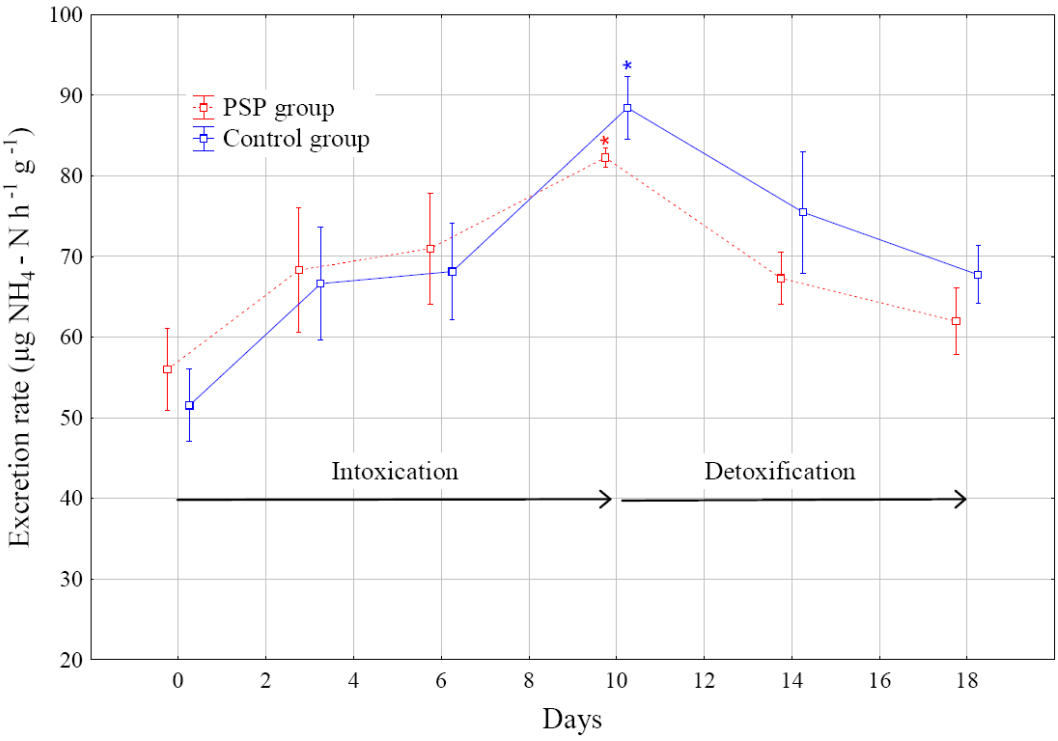


Figure 6.3: Excretion rate of *Pecten novaezelandiae* during intoxication and detoxification periods. The asterisk (*) represents significant higher excretion rate. Measurements were taken on days 0, 3, 6, 10, 14 and 18. Values are means ± SE.

Table 6.3: Results of repeated measures ANOVA testing the effects of toxic and non toxic diets (treatment) and days of exposure on the excretion rate of *Pecten novaezelandiae*. Values in **bold** are of statistical significance.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	41.3	1	41.3	0.12	0.74
Error	2018.1	6	336.4		
Days	4193.2	5	838.6	**11.3**	**< 0.001**
Days*Treatments	297.0	5	59.4	0.80	0.55
Error	2210.1	30	73.7		

Total toxin burden of PSP toxins in the tissues

The total toxin burden of PSP toxins in the tissues of *Pecten novaezelandiae* was calculated by adding together the concentration of toxins detected in the digestive gland, adductor muscle and in the homogenate of the other tissues (gonads + gills + mantle). After conversion to STX di-HCl equivalents the scallops fed *Alexandrium tamarense* for 10 days had accumulated 298 µg STX di-HCl eq. 100 g^{-1} tissues (Figure 6.4).

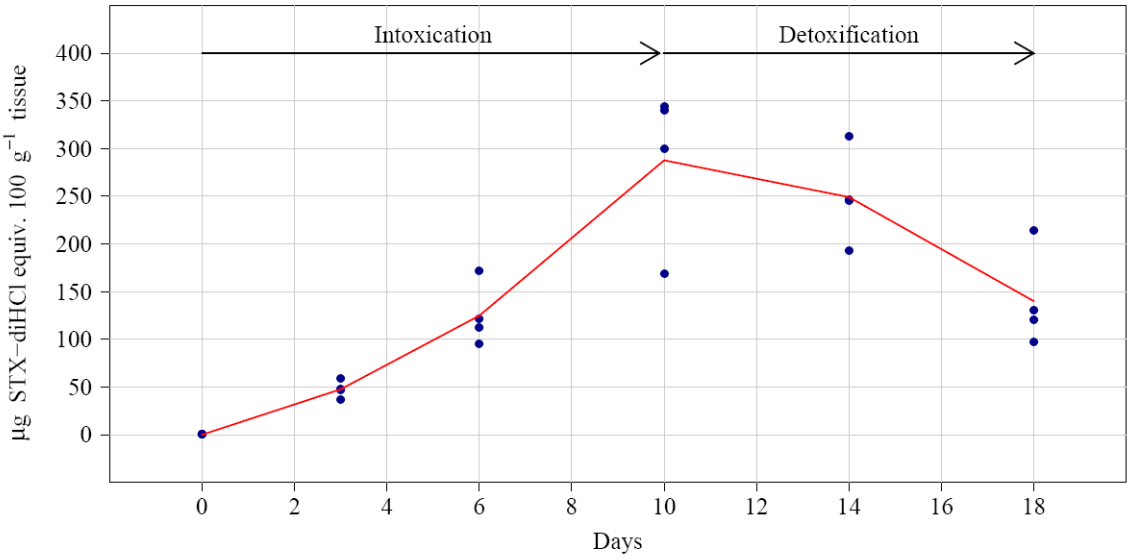


Figure 6.4: Total toxin burden of PSP toxins µg STX di-HCl equiv. 100 g^{-1} in the tissues of scallops exposed to intoxication and detoxification periods.

PSP toxin profile in the digestive gland

Most of the total toxin burden of *Pecten novaezelandiae* was contained in the digestive gland (day 3 = 47%, day 6 = 54%, day 10 = 79%, day 14 = 98%, day 18 = 97%). The highest concentration of PSP toxins accumulated in this tissue was measured in the detoxification period. Over both periods, the PSP toxins present in the digestive gland were GTX1, dcGTX3 + GTX5, dcGTX2, GTX3, GTX2, dcNeo + Neo, dcSTX, STX, C1 and C2. The toxins dcGTX2 and GTX2 were detected in the digestive gland, but were not present in the toxin profile of *A. tamarense*. On day 3, the toxic profile of the digestive gland contained high quantities of the N-sulfacarbamoyl, C2 (91%) as in the toxin profile of the toxic dinoflagellate *A. tamarense*. By day 6, this toxin profile changed and GTX1 dominated over the rest of the experiment, followed by C2 (Figure 6.5 and Table 6.4).

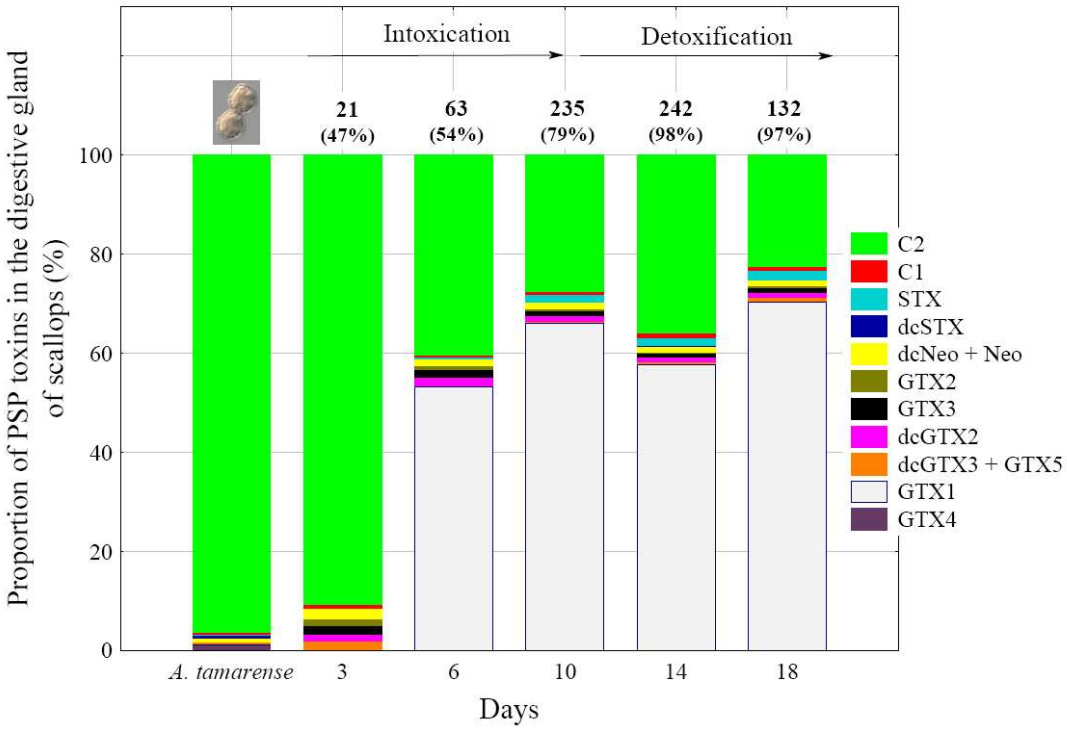


Figure 6.5: PSP toxin profile of the digestive gland from scallops during intoxication and detoxification periods. Values are expressed as a percentage (%) of the total content of toxins in this tissue. The total content of toxins in this tissue is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}) with the corresponding proportion of the total toxin burden of the scallops.

Table 6.4: PSP toxin profile of the digestive gland from scallops exposed to intoxication and detoxification periods. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in this tissue (%). On each day, the compound dominating the toxin profiles is shown in **bold**.

	Day 3		Day 6		Day 10		Day 14		Day 18	
	T	%	T	%	T	%	T	%	T	%
GTX1	-	-	**33.4**	**53.3**	**154.8**	**66.0**	**139.8**	**57.7**	**93.0**	**70.4**
dcGTX3 + GTX5	0.4	1.8	-	-	0.7	0.3	1.0	0.4	0.8	0.6
dcGTX2	0.3	1.4	1.0	1.6	2.4	1.0	2.0	0.8	1.3	1.0
GTX3	0.4	1.7	1.0	1.6	2.4	1.0	2.0	0.8	1.3	1.0
GTX2	0.3	1.3	0.5	0.9	1.0	0.4	0.6	0.3	0.4	0.3
dcNeo + Neo	0.5	2.2	0.8	1.3	3.1	1.3	2.9	1.2	1.6	1.2
dcSTX	-	-	-	-	0.2	0.1	0.3	0.1	0.2	0.1
STX	-	-	0.2	0.3	3.6	1.5	3.9	1.6	2.4	1.8
C1	0.2	0.8	0.3	0.5	1.5	0.6	2.1	0.9	1.1	0.8
C2	**19**	**90.8**	25.3	40.4	65.0	27.7	87.5	36.1	30.0	22.7
Total	21	100	63	100	235	100	242	100	132	100

PSP toxin profile in the adductor muscle

Overall, low concentrations of PSP toxins were measured in the adductor muscle of scallops (day 3 = 16%, day 6 = 31%, day 10 = 13%, day 14 = 1%, day 18 = 2%). Over both periods, the PSP toxins present in the adductor muscle were GTX4, GTX1, GTX3, GTX2, C1 and C2. The presence of dcGTX3 + GTX5 was detected only in the detoxification period. The toxin GTX2 was detected in the adductor muscle, but was not present in the toxin profile of *A. tamarense*. Over the intoxication period, the toxin profile of this tissue was dominated (93%) by GTX4. Over the detoxification period, the N-sulfacarbamoyl, C2 was dominant (52%) in the toxic profile of this tissue followed by GTX4, C1, GTX3 and dcGTX3 + GTX5 (Figure 6.6 and Table 6.5).

Figure 6.6: PSP toxin profile of the adductor muscle from scallops during intoxication and detoxification periods. Values are expressed as a percentage (%) of the total content of toxins in this tissue. The total content of toxins in this tissue is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}) with the corresponding proportion of the total toxin burden of the scallops.

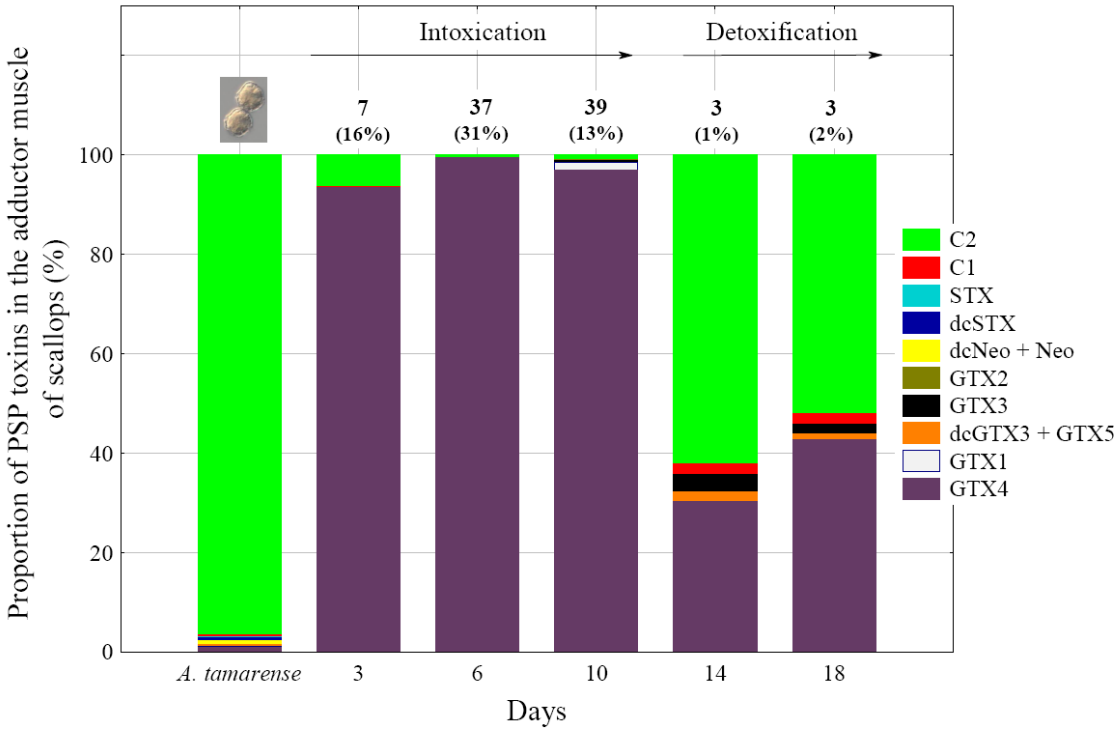


Table 6.5: PSP toxin profile of the adductor muscles from scallops exposed to intoxication and detoxification periods. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in this tissue (%). On each day, the compound dominating the toxin profiles is shown in **bold**.

	Day 3		Day 6		Day 10		Day 14		Day 18	
	T	%	T	%	T	%	T	%	T	%
GTX4	**6.5**	**93.4**	**36.4**	**99.5**	**37.4**	**96.9**	1.0	30.2	1.3	42.8
GTX1	-	-	-	-	0.6	1.6	-	-	-	-
dcGTX3 + GTX5	-	-	-	-	-	-	0.1	2.1	0.04	1.2
GTX3	-	-	0.01	0.04	0.2	0.4	0.1	3.5	0.1	1.9
GTX2	-	-	-	-	0.03	0.1	-	-	-	-
C1	0.01	0.2	-	-	0.02	0.04	0.1	2.1	0.1	2.2
C2	0.4	6.4	0.2	0.5	0.4	1.0	**2.0**	**62.1**	**1.5**	**51.9**
Total	7	100	37	100	39	100	3	100	3	100

PSP toxin profile in the other tissues

Overall, low amounts of PSP toxins were measured in the other tissues (day 3 = 37%, day 6 = 15%, day 10 = 8%, day 14 = 1%, day 18 = 1%). Over both periods, the PSP toxins present in this tissue were GTX4, dcGTX3 + GTX5, GTX3, C1 and C2. The toxins GTX2 and dcNeo + Neo were measured only on day 3 of the intoxication period. The toxin GTX2 was detected in the other tissues, but was not present in the toxin profile of *A. tamarense*. Over both periods, the main toxin detected was GTX4, but higher proportions were measured in the intoxication. More C2 was measured in the detoxification period and also C1, GTX3 and dcGTX3 + GTX5 (Figure 6.7 and Table 6.6).

Figure 6.7: PSP toxin profile of the other tissues from scallops during intoxication and detoxification periods. Values are expressed as a percentage (%) of the total content of toxins in these tissues. The total content of toxins in these tissues is placed above the bars (µg STX di-HCl equiv. 100 g^{-1}) with the corresponding proportion of the total toxin burden of the scallops.

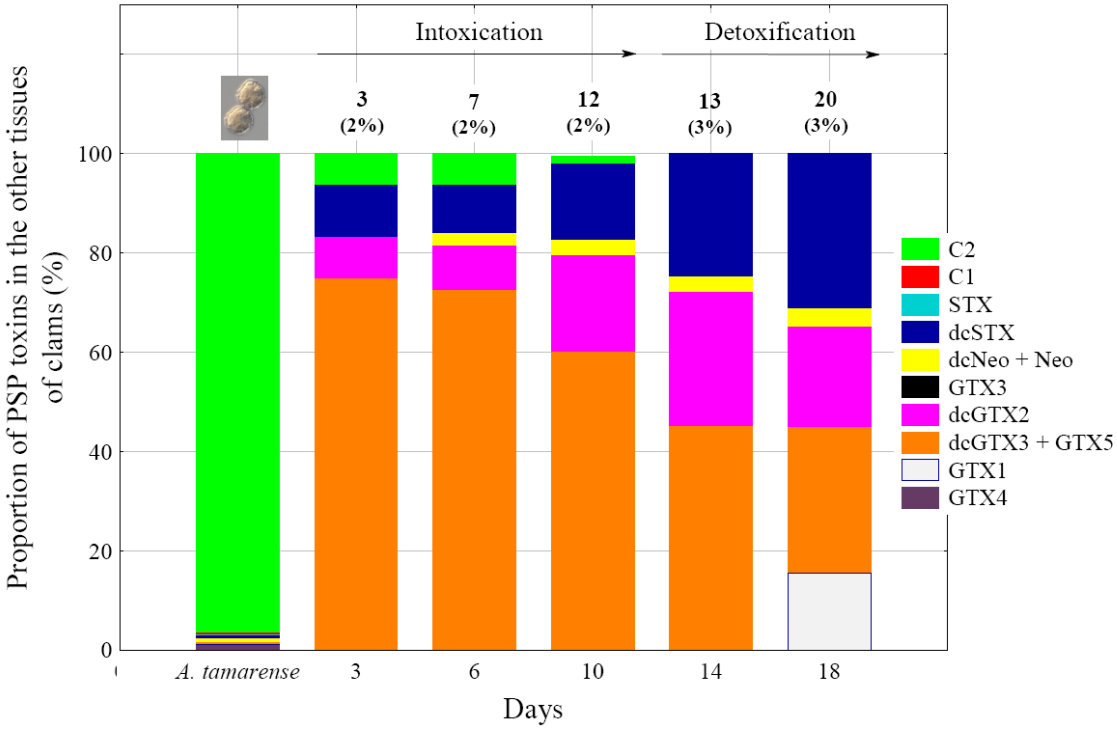


Table 6.6: PSP toxin profile of the other tissues from scallops exposed to intoxication and detoxification periods. Values are expressed in µg STX di-HCl equiv. 100 g^{-1} (T) and as a proportion of the total content of toxins in these tissues (%). On each day, the compound dominating the toxin profiles is shown in **bold**.

	Day 3		Day 6		Day 10		Day 14		Day 18	
	T	%	T	%	T	%	T	%	T	%
GTX4	**15.3**	**91.8**	**16.6**	**95.4**	**20.7**	**87.8**	**1.1**	**66.5**	**0.8**	**65.0**
dcGTX3 + GTX5	0.01	0.1	0.02	0.1	0.1	0.4	0.01	0.7	0.02	1.6
GTX3	0.03	0.2	0.1	0.5	0.2	0.8	0.1	3.3	0.1	7.1
GTX2	0.1	0.6	-	-	-	-	-	-	-	-
dcNeo + Neo	0.04	0.2	-	-	-	-	-	-	-	-
C1	0.03	0.2	-	-	0.03	0.1	0.02	1.5	0.03	2.5
C2	1.2	7.0	0.7	4.0	2.6	10.8	0.5	28.0	0.3	23.8
Total	17	100	17	100	24	100	2	100	1	100

6.3.2 Effect of PSP toxins on the oxygen consumption of *P. novaezelandiae*

The oxygen consumption of scallops fed with the toxic dinoflagellate *Alexandrium tamarense* was constant over the experiment and the average was 0.21 ± 0.03 ml h^{-1} g^{-1}. The control group fed with the dinoflagellate Alexandrium margalefii also had constant values of oxygen consumption and the average was 0.19 ± 0.03 ml h^{-1} g^{-1} (Figure 6.8).

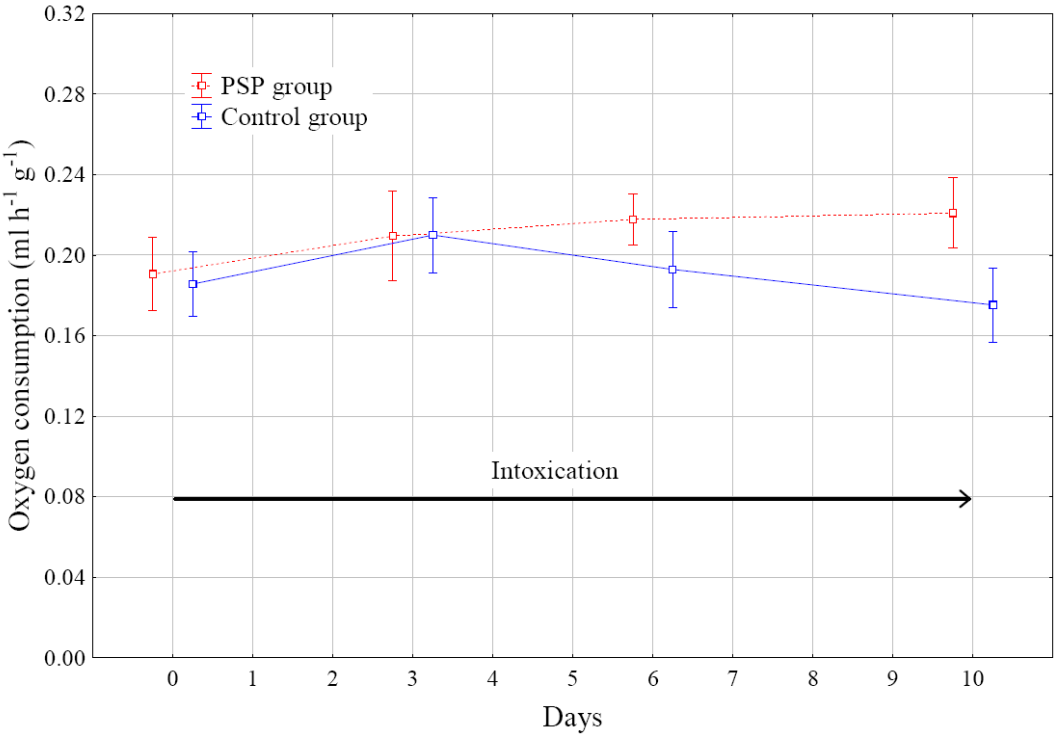


Figure 6.8: Oxygen consumption of *Pecten novaezelandiae* during 10 days of exposure to *Alexandrium* spp. Measurements were taken on days 0, 3, 6 and 10. Values are means ± SE.

There were no significant differences in the oxygen consumption between the PSP and the control group (repeated measures ANOVA, $F_{1,6}$ = 1.07, P = 0.33). Neither the effect of time (repeated measures ANOVA, $F_{3,18}$ = 0.83, P = 0.49), nor the interaction between both variables tested (repeated measures ANOVA, $F_{3,18}$ = 1.03, P = 0.4) were significant (Table 6.7).

Table 6.7: Results of repeated measures ANOVA testing the effects of toxic and non-toxic diets (treatment) and days of exposure on the oxygen consumption of *Pecten novaezelandiae*.

Source of variation	*SS*	*df*	*MS*	*F*	*P*
Treatment	0.002	1	0.0028	1.07	0.33
Error	0.01	6	0.025		
Days	0.002	3	0.0007	0.83	0.49
Days*Treatments	0.002	3	0.0008	1.03	0.40
Error	0.015	18	0.0008		

6.4 Discussion

The main findings of this study were that *Pecten novaezelandiae* was sensitive to PSP toxins with significantly lower clearance rates during the intoxication period. However, when these scallops were fed on a non-toxic diet, clearance rate increased to similar values found in the control scallops. Responses to PSP toxins were not found in either the excretion rate or oxygen consumption which suggest that *P. novaezelandiae* may have the ability to regulate its physiology in adverse conditions such as exposure to PSP toxins.

Although the scallops had a decreased clearance rate when exposed to *Alexandrium tamarense*, they were able to ingest the toxic cells over a period of 10 days and accumulated 298 µg STX di-HCl equiv. 100 g^{-1} tissues. The digestive gland slowly accumulated most of the PSP toxins present in the tissues, and epimerisation of GTX4 to GTX1 had taken place actively in this tissue. The adductor muscle and the other tissues contained less PSP toxins and relatively high proportions of GTX4 and C2. This demonstrated that epimerisation did not take place in these tissues or occurred in low rates that were not detected in this experiment.

6.4.1 Physiological responses of *P. novaezelandiae* exposed to *A. tamarense*

Clearance and excretion rates

The experiment that investigated the physiological effects of the PSP toxins on *P. novaezelandiae* over a 20 day period (intoxication and detoxification conditions), showed that scallops exposed to 17 nmol STX-diHCl d^{-1} $scallop^{-1}$ had significantly lower clearance rates than those measured for the control group. The clearance rate of the PSP group increased when fed on a non-toxic diet during a detoxification period. Both groups had lower feeding rates than those found in the scallop *Argopecten purpuratus* collected from an aquaculture site in the north of Chile (Navarro et al., 2000). These authors measured a clearance rate of 1.5 L h^{-1} g^{-1} for *A. purpuratus*, which was was twice the rate (0.79 L h^{-1} g^{-1}) measured in the present study for control scallops *Pecten novaezelandiae*).

Navarro et al. (2000) also observed that *A. purpuratus* could actively regulate the clearance rate and did not simply switch on and off their feeding activity. They suggested that chemical receptors on the gills and/or labial palps were able to detect specific nutritive compounds present in the diet. The clearance rate of *Pecten novaezelandiae* fed with the non-toxic dinoflagellate *A. margaleffi* increased during the detoxification period which suggested regulation of this response when scallops were not exposed to PSP toxins. Receptors may therefore be involved in the detection of PSP toxins in the diet. The effects of PSP toxins on the clearance rate of *P. novaezelandiae* in this study is in agreement with other studies that have shown negative impacts of toxic dinoflagellate on scallops. For example, in complementary studies (Lesser and Shumway, 1993; Smolowitz and Shumway, 1997), the juveniles *Argopecten irradians* and *Placopecten magellanicus* were exposed to 105 cells L_1 of *Gyrodinium aureolum* for a week. These studies clearly demonstrated a species-specific impact of these algae on the scallops. Mortality was 100% in *A. irradians* and 0% for *P. magellanicus*. Reduced clearance rates were noted in *A. irradians* and production of copious amounts of mucus noted in *P. magellanicus*. No mortalities were registered in either species when exposed to *Alexandrium tamarense*. Estrada et al. (2007)

Physiological effects and biotransformation of PSP toxins have been found in other scallops. Juveniles of the giant lions-paw scallop, *Nodipecten subnodasus* also decreased the clearance rate when fed with *Gymnodinium catenatum* at concentrations greater than 450 cell ml^{-1}. Shumway and Cucci (1987) found n that *P. magellanicus* exposed to toxic dinoflagellates may elicit vigorous swimming and clapping activity accompanied by the copious production of mucus and pseudofaeces, albeit without apparent significant reduction in clearance rate. Li et al. (2001) found that *Chlamys nobilis* ingested *Alexandrium tamarense* as a food source and the feeding rates were twice the rate recorded for the clam, *Ruditapes philippinarum* fed on the same toxic algae.

Although *P. novaezelandiae* had an increased clearance rate when exposed to the non-toxic diet, further studies that included longer period of exposure to toxic cells would determinate whether this scallop was able to manage PSP toxins for a longer period of time. Algal blooms can disappear in weeks or months and the effect of the PSP toxins on the clearance rate of *P. novaezelandiae* may be more dramatic when exposed to longer PSP exposures.

The PSP toxins did not affect the excretion rate of *P. novaezelandiae* (70 µg NH4-N h^{-1} g^{-1}) which was higher than those measured for the scallop, *Placopecten magellanicus* (MacDonald et al., 1998). That scallop had excretion rates between 18 and 35 µg NH4-N h^{-1} g^{-1} which was similar to the 38 µg NH4-N h^{-1} g^{-1} measured on the scallop, *Argopecten purpuratus* from Chile (Navarro et al., 2000). Because there have been no other measurents of the excretion rate of *P. novaezealandiae* it was not possible to conclude from this study whether the rates were normal or higher than expected. Further studies that investigate the excretion rate of this species of scallop when exposed to different conditions (quality and quantity of seston) including toxic algae species, would better help the understanding of these results.

Oxygen consumption

Oxygen consumption was also determined for *P. novaezelandiae* exposed to a higher number of cells of *A. tamarense* (35 nmol STX-diHCl d^{-1} $scallop^{-1}$) than was used in the previous experiment (17 nmol STX-diHCl d^{-1} $scallop^{-1}$). Over 10 days, the oxygen

consumption of the scallops was similar in the PSP and the control group. Overall, the oxygen consumption measured in both treatments (0.20 ml^{-1} h^{-1} g^{-1}) was similar to those measured on the scallop, *P. magellanicus* (0.22 to 0.33 ml^{-1} h^{-1} g^{-1}) (MacDonald et al., 1998) and slightly lower than those measured in *A. purpuratus* (0.5 ml^{-1} h^{-1} g^{-1}) (Navarro et al., 2000).

The oxygen uptake experiment on *P. novaezelandiae* did not include measurements of the clearance rate, but it is likely that this response may have been also affected due to the higher number of toxic cells used. Assuming that the clearance rate of scallops was affected, the oxygen consumption rates of the PSP group (similar to the control group) would reflect the capacity of this species to regulate its metabolism in order to cope more efficiently in adverse conditions such as the presence of high levels of PSP toxins.

6.4.2 PSP toxins in the tissues of the scallops

Pecten novaezelandiae exposed to *A. tamarense* slowly accumulated PSP toxins in the tissues. The rate of intoxication may be related with the low clearance rate measured during the intoxication period. Scallop tissues also detoxified slowly and it was estimated that over a period of 21 days they would have reached the safety limit level for human consumption.

Bivalve species differ markedly in the capacity to eliminate PSP toxins. They fall into two general categories in terms of their detoxification capacity. Rapid to moderate detoxifiers such as the blue mussel, *Mytilus edulis*, take only a few weeks. Slow detoxifiers, most notably *Saxidomus giganteus*, *Spisula solidissima*, *Placopecten magellanicus* and *Patinopecten yessoensis* typically take several months to years. The level of PSP toxins measured in *P. novaezelandiae* over the detoxification would classify this species as a slow detoxifier due to the high levels of PSP toxins which still remained in the tissues (136 µg STX di-HCl eq. 100 g^{-1}) after 8 days of feeding on a non-toxic diet.

Changes in the toxic profile of shellfish tissues may arise from selective retention or elimination of individual toxins, epimerisation, or from a variety of biotransformation processes: reductive conversion, hydrolysis at low pH, or enzymatic conversion (Oshima,

1995b). Strong evidence supporting the metabolic bioconversion of toxins rather than selective kinetics is provided by the appearance of toxins in bivalve tissues that were not detected in the dinoflagellate cells. After 3 days of exposure to PSP toxins, the toxic profile of the digestive gland of *Pecten novaezelandiae* was similar to the toxin profile found in *Alexandrium tamarense*. The proportions of the PSP toxins in the digestive gland changed over the experiment indicating that epimerisation had taken placed. This is characterised by the increase in GTX1 and the decrease of GTX4. This bioconversion occurred slowly and was detected from day 6. The slower epimerisation of GTX4 may be related to the slow accumulation of PSP toxins in this tissue which reached its highest level during the detoxification period.

Following exposure to PSP toxins the adductor muscle of the New Zealand scallop contained higher concentrations (up to 39%) of the PSP toxins than the 3% of the total toxin burden observed in the same tissue for *Chlamis nobilis* (Choi et al., 2003) and *Placopecten magellanicus* (Shimizu and Yoshioka, 1981; Shumway et al., 1988). In most scallops the adductor muscle is one tissue that accumulates low concentrations of PSP toxins, the current results on *P. novaezelandiae* are not fully understood. Therefore, further studies are necessary on the New Zealand scallop to investigate the capacity of the adductor muscle to accumulate toxins before it can be concluded that this species has different mechanisms to scallops from other parts of the world.

The toxin profile of the adductor muscle and the other tissues were similar in toxin composition. All of the tissues contained high proportions of GTX4 and epimerisation from GTX4 to GTX1 did not take place. Over the detoxification period, the proportion of GTX4 decreased but the absence of GTX1 indicated that scallops eliminated GTX4 from this tissues rather than convert it to GTX1 as was clearly observed in the digestive gland. It seems that these tissues accumulated and eliminated GTX4 more readily than C2 (the proportions of C2 were lower over the intoxication period and higher over the detoxification period).

6.4.3 Implications for the aquaculture sector

The results of this study indicate that the New Zealand scallop, *P. novaezelandiae* has a slow rate of detoxification of PSP toxins which may have negative consequences for the aquaculture sector. When scallops become too toxic for human consumption and areas are closed, the period of time before re-opening the market for this species may be longer than for other bivalves such as the New Zealand mussel *Perna canaliculus*. One way to minimize this negative impact on the scallop aquaculture sector would be an early warning system for the extraction of scallops before they become toxic for human consumption. Because scallops live subtidally, the toxic algae may be less abundant and the onset of toxicity may occur later than in other bivalves (e.g. mussels) that live closer to the sea surface. For this reason regular testing of potential sentinel species such as *P. canaliculus* co-existing with the scallops in areas such as Malborough Sound, could alert the scallop aquaculture sector to the potential threat of a toxic event. To substantiate this hypothesis it is a necessity that samples of mussels and scallops tissue be taken *in situ* from the same area during a toxic algal bloom in order to investigate the intoxication and detoxification in both species under natural conditions.

From this current research it is expected that, in the presence of a toxic PSP algal bloom, *P. novaezelandiae* would become toxic later than other bivalve species because of their to sensitivity to the PSP toxins and the depth where they live. If it could be established that the scallops become toxic later than the mussels then the extraction of scallops could be maintained for a longer period of time with a concomitant decrease in the negative impact on the aquaculture sector. It is essential that further studies be carried out to confirm this hypothesis. This information could then be included in the shellfish monitoring system for New Zealand.

Chapter 7

General discussion

Numerous literature studies have supported the wide range of responses of bivalves to toxic algal blooms and the species-specific effects of these blooms on the physiology, behaviour and rates of accumulation of PSP toxins in the tissues of the bivalves. For shellfish management purposes it is essential to have specific information for individual species, especially those that are used in aquaculture. In this context, this thesis investigated the physiological effects and bioconversion of the PSP toxins in marine bivalves from New Zealand using controlled laboratory experiments.

The species chosen for this study were:

1. the mussel, *Perna canaliculus*, from Banks Peninsula,
2. the clams, *Paphies donacina* and *Dosinia anus* from New Brighton,
3. the scallop, *Pecten novaezelandiae*, from Malborough Sound, and
4. the oyster, *Ostrea chilensis*, from Lyttelton Harbour.

Several physiological and behavioural responses of shellfish to dinoflagellates species were investigated in this thesis. Measurements of clearance rate were used to test the sensitivity of the bivalves to the PSP toxins. Other studies that involved intoxication and detoxification periods were carried out on three species of bivalves (*P. canaliculus, P. donacina, P. novaezelandiae*), using physiological responses (clearance and excretion rate) and analysis of PSP toxins in the tissues over these periods. Other responses in bivalves fed with the toxic cells were also carried out. These included byssus production, recording the

presence of toxic cells in the faeces of mussels, the siphon activity and burrowing depth in clams and the oxygen consumption in scallops.

Overall, the results showed a species-specific effect of the PSP toxins on the physiology of bivalves from New Zealand, which was related to the level of toxins in the tissues. This chapter compares the responses of bivalves exposed to PSP toxins, highlighting the main findings for each species and comparing their sensitivity to PSP toxins. The bioconversion of PSP toxins in the tissues also indicated species-specific differences among the bivalves.

7.1 Effects of PSP toxins on the physiology of bivalves

Various behavioural, physiological and cellular responses of bivalves to harmful algae have been described. Several studies have assessed the responses of bivalve species to toxic dinoflagellates, and these include changes in valves closure, filtration rate, feeding rate, byssus production, oxygen consumption and cardiac activity or neurophysiological effects (Shumway et al., 1985; Shumway and Cucci, 1987; Gainey and Shumway, 1988; Lesser and Shumway, 1993). Responses vary according to the toxin strain present, the bivalve species and the geographical environment, as well as among individuals in a given locality.

A summary of the different responses observed in the five species of bivalves from New Zealand exposed to *Alexandrium* species is presented below and in Table 7.1.

Clearance rate

In the present study, the effect of the PSP toxins was tested using two species of dinoflagellates that differed in the capacity to produce PSP toxins. These were the PSP toxic producing species, *Alexandrium tamarense* and a PSP non-producer, *A. margaleffi*.

The New Zealand mussel, *Perna canaliculus* fed actively on toxic and non-toxic dinoflagellates species and no effect resulting from intoxication of the PSP toxins was observed over the period of the study. Marsden and Shumway (1992) also observed that the same species of mussel from the Marlborough Sounds had similar responses to those observed in this study. Studies on other species of mussels have reported that these bivalves are highly resistant to STX, and in most cases, did not exhibit adverse reactions when feeding on PSP toxins from dinoflagellates (Cucci et al., 1985; Bricelj et al., 1990; Marsden and Shumway, 1992). This resistance, along with their generally elevated filtration rate most likely explain their faster rate of phycotoxin accumulation, as compared to other bivalves.

The clearance rates of the clam, *Paphies donacina* fed with toxic and non-toxic *Alexandrium* species were significantly lower than the feeding rates observed when fed with *Tetraselmis* sp. Although a clear effect of the *Alexandrium* species was observed on the clearance rate of *P. donacina*, it was not possible to conclude whether or not the PSP toxins were also affecting this physiological response. Bricelj et al. (1996) found a significant reduction in the clearance rate of *M. arenaria* only when clams were fed the highly toxic *Alexandrium excavatum* PR18b (74.5 pg STX equiv. $cell^{-1}$), but this was not observed with other, less toxic species. In the case of *P. donacina*, further studies need to be carried out using other species of microalga with higher toxicity to test the potential effect of PSP toxins in this bivalve.

In contrast, the clam, *Dosinia anus* was unaffected by the PSP toxins and to the effects of *Alexandrium* species. The two species of clams from New Zealand (*P. donacina* and *D. anus*), which inhabit the same substrate had different feeding responses when exposed to dinoflagellate cells. Studies on other species of clams, such as *Spisula solidissima* show that clams can be also insensitive to the effects of PSP toxins and can thus attain high toxicities (Silvert et al., 1998).

The clearance rate of the scallop, *P. novaezelandiae* was clearly affected by the PSP toxins which decreased the clearance rates. These results agree with other studies that have shown negative impacts of toxin dinoflagellates on scallops. For example, in complementary studies (Lesser and Shumway, 1993; Smolowitz and Shumway, 1997), when juvenile *Argopecten irradians* and *Placopecten magellanicus* were exposed to 10^5

cells L^{-1} of *Gyrodinium aureolum* for a week there was a species-specific impact of this algae on the scallops. Mortality was 100% in *A. irradians* and 0% in *P. magellanicus*. Reduced clearance rates were noted in *A. irradians* and production of copious amounts of mucus noted in *P. magellanicus*.

The oyster, *Ostrea chilensis* exposed to PSP toxins did not respond clearly to any of the variables tested over the experiment (treatments and time of exposure). Oysters increased and decreased clearance rates over the 6 days of exposure to *Alexandrium* species with an erratic response over that time. The results were not conclusive and further studies need to be carried out using a greater number of replicates. Luckenbach et al. (1993) and Lassus et al. (1996) also reported a high individual and daily variation in physiological responses in oysters. Shumway and Cucci (1987) found a species-specific effect of PSP toxins on oysters and reported that the clearance rate of *Crassostrea virginica* was significantly inhibited by addition of toxic cells, whereas that in *Ostrea edulis* was stimulated.

Excretion rate

Excretion rates were measured for *Perna canaliculus*, *Paphies donacina* and *Pecten novaezelandiae*. These species of bivalve did not show any affect of the PSP toxins on the excretion rate when exposed to toxic algae and also over the detoxification period. In contrast, Navarro and Contreras (2010) observed that *Mytilus chilensis* exposed to *Alexandrium catenella* had an increased excretion rate over time, which significantly correlated with the accumulation of PSP toxins in the tissues. They suggested that the degradation of the PSP toxins, a rich source of nitrogen, produced high concentrations of ammonia which needed to be eliminated via the excretory organs in order to maintain equilibrium within the bivalve. The same effect was not observed in the three species of bivalves studied, but further studies using higher numbers of toxic cells are needed to test any potential effect of the PSP toxins on this physiological response.

Byssus production of mussels

The mussel, *Perna canaliculus*, exposed to PSP toxins had a decreased capacity to produced byssus over a 15 day period. The decreased byssus production in this study was in agreement with a previous study on the same species of mussel, reported by Marsden and Shumway (1992).

One of the most important aspects of mussel life styles is the ability to secrete strong byssal threads and for attachment to a variety of substrates (Alfaro, 2006). This ability is crucial to the survival of the species and it is an informative index of activity in the mussels. Shumway and Cucci (1987) observed that byssus production in *Mytilus edulis* was inhibited in the presence of *Protogonyaulax tamarensis*, but production was not inhibited in *Modiolus modiolus*.

The effect of PSP toxins on the byssus production in *P. canaliculus* is of importance for aquaculture systems, because of the significant role that the threads play in the attachment of mussels to culture ropes. During a toxic algal bloom, mussels will become contaminated, but their ability to attach to the culture ropes may also be diminished. Extensive losses of cultured mussels due to this phenomenon have not been described in the literature, nevertheless, further studies should be carried out especially in other reproductive stages where byssus production is crucial in the life cycle of bivalve species (e.g. juvenile).

Presence of toxic cells in the faeces of mussels

Observations on the faeces of *Perna canaliculus* fed with the toxic dinoflagellate *A. tamarense* confirmed that not all ingested cells were digested in the mussel and that a proportion of the dinoflagellate was able to pass intact through the digestive system and be ejected in the faeces. Hégarèt (2008) also found intact, non-digested cells of *Alexandrium fundyense* in the faeces of *Mytilus edulis* and on culture these cells were able to produce a new population of dinoflagellates. This demonstrated that transplanted bivalve molluscs could potentially serve as vectors for the introduction of harmful algae.

The results presented in this study are important in the management, restoration and aquaculture activities involving shellfish transportation activities in New Zealand. It is strongly recommended that aquaculturists avoid transportation of contaminated bivalves to other areas. This consideration should be applied within New Zealand coast and other fisheries areas around the world to avoid the increasing expansion of harmful algae blooms.

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Siphon activity and burrowing depth of clams

The clam, *Paphies donacina* presented with *A. tamarense* showed erratic contractions of the exhalant siphon. This sensitivity of the exhalant siphon of *P. donacina* did not affect the burrowing capacity under the same experimental conditions. Bricelj et al. (1996) studied the ability of *Mya arenaria* and *Spisula solidissima* to reburrow in sediment following exposure to toxic *Alexandrium* cells and this provided a useful index of in vivo sensitivity to PSP toxins. These authors found that clearance rates and the ability of *M. arenaria* to burrow was markedly impaired in the presence of toxic *Alexandrium* species, whereas *S. solidissima* remained unaffected. However, as in the present study the authors did not carry out experiments using non-toxic species of *Alexandrium* to confirm the specific PSP effect. Nevertheless these preliminary behavioural studies plus the physiological results indicate that some level of sensitivity to dinoflagellate cells and/or PSP toxins is present in *P. donacina*.

Oxygen consumption

The oxygen consumption of the scallop, *Pecten novaezelandiae* was unaffected by the PSP toxins and similar rates of oxygen consumption were measured in the PSP and the control group. Li et al. (2002) found the same response in *Perna viridis* following 6 days of exposure to different proportions of *A. tamarense* (20, 50 and 100%). Marsden and Shumway (1993) concluded that oxygen consumption was unaffected in five species of juvenile filter feeding bivalves after 1 hour of exposure to *A. tamarense*. Nevertheless, they also suggested that a longer exposure to toxic algae may have resulted in greater activity or valve closure, but this was not observed in the present study of *P. novaezelandiae* over a period of 10 days.

Although the responses presented here for New Zealand shellfish species agree with previous literature collected from other parts of the world, it is important to undertake further studies with a higher number of replicates to obtain more robust data. The main problem in controlled laboratory studies is the amount of microalgae needed to feed the bivalves. If specialist culture facilities could be accessed, then the number of replicates could be increased and it would also be possible to provide conditions more similar to a bloom events, which then could be tested. This approach would be interesting to explore in future research in New Zealand.

7.2 Bioconversion of PSP toxins in the tissues of bivalves

The relative proportions of different PSP toxins in the tissues of bivalves are frequently different from those found in the producer dinoflagellate. This can be explained by toxin-specific uptake, elimination or by transformation between toxin types. Differential uptake has not been found (Bricelj and Shumway, 1998) and only one previous experiment with *Spisula solidissima* suggested a differential elimination (Silvert et al., 1998).

The results in this thesis also confirm biotransformation of toxins in the tissues of New Zealand shellfish. The toxic profile of the dinoflagellate *Alexandrium tamarense* was dominated by C toxins whereas the toxin profile of the shellfish was dominated by carbamoyl or decarbamoyl toxins (higher relative toxicity). Also, the toxin profile of the tissues of the shellfish differed between species and over time. Therefore, our results confirmed that bioconversions of PSP toxins had taken placed in the tissues establishing species-specific differences among bivalves.

For a better understanding of the bioconversion processes observed in this thesis, it is necessary to distinguish the differences between PSP analogues and their chemical structure (Table 7.2 and Figure 7.1). First, a large proportion of the PSP toxins produced by dinoflagellates occur as C toxins which have a sulphate groups and a sulfocarbamoyl group. These sulfate groups can be selectively removed from the C toxins to make other toxins that were not abundant in the algal source. Thus, the range of toxins that may be derived from one source can be markedly increased by chemical inter-conversions

(Laycock et al., 1995). These bioconversions produce finally other toxic PSP analogues as a result of chemical reactions, such as reduction, epimerisation, hydrolysis and decarbamoylation, where the molecule changes its original structure.

Table 7.1: Structure of the PSP derivatives (Cembella et al., 1993).

Carbamoyl		N-sulfacarbamoyl		Decarbamoyl				
R4:OCONH2		R4: OCONHSO3		R4:OH				
Toxin	Relatively Toxicity	Toxin	Relatively toxicity	Toxin	Relatively toxicity	R1	R2	R3
STX 1	1	(B1) GTX5	0.06	dcSTX	0.51	H	H	H
Neo	0.92	(B2) GTX6	0.06	dcNeo	-	OH	H	H
GTX1	0.99	C3	0.01	dcGTX1	-	OH	H	OSO_3^-
GTX2	0.36	C1	0.01	dcGTX2	0.15	H	H	OSO_3^-
GTX3	0.64	C2	0.01	dcGTX3	0.38	H	OSO_3^-	H
GTX4	0.73	C4	0.06	dcGTX4	-	OH	OSO_3^-	H

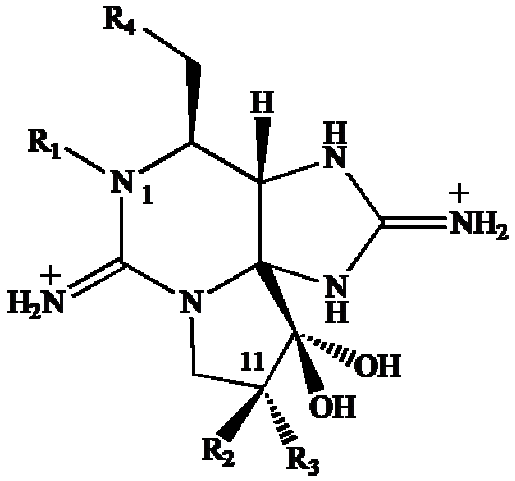

Figure 7.1: The general structure of PSP toxins (Cembella et al., 1993)

Four main bioconversion have been reported for PSP toxins (Elke et al., 2007) which were also observed in the tissues of New Zealand bivalves:

1. **Reductive transformation in shellfish:** Reductive toxin transformations are characterised by a decrease in the N-OH toxin group (GTX1, GTX4, Neo) which is accompanied by an increase in the N-H toxin group (GTX2, GTX3, STX) as well as by the loss of the sulphate group at C11 (GTX1, and GTX4 to give Neo, GTX2 and GTX3 to give STX). Both of these reactions can be induced by natural reductants (cystein, glutathione), or by the enzymatic activity of bacteria commonly found in molluscs (Kotaki et al., 1985; Smith et al., 2001).

2. **Hydrolytic cleavages:** Toxins bearing a N-sulfocabamoyl moiety (C1, C2, C3, C4, GTX5, GTX6) can be non-enzymatically converted to their carbamate counterparts (GTX2, GTX3, GTX1, GTX4, STX, Neo) at pH values < 7 (Hall and Reichardt, 1984; Oshima, 1995a). This hydrolytic cleavage was not effective in the ten species of shellfish analysed by Elke et al. (2007), but has been used extensively in analyses of PSP toxins where the carbamate standards derivatives were not available (Laycock et al., 1995).

1. **Epimerisation:** PSP toxins with a C11 hydroxysulfate undergo epimerisation through a keto-enol equilibrium. The β-epimers (C2, C4, GTX3, GTX4) primarily produced by dinoflagellates, are usually transformed to the thermodynamically more stable α-epimers (C1, C3, GTX2, GTX1) after uptake by shellfish (Oshima, 1995a; Bricelj and Shumway, 1998).

2. **Enzymatic decarbamoylation:** Enzymatic hydrolysis of the N-sulfocarbamoyl and the carbamoyl toxins to decarbamoyl is probably restricted to a few bivalve species (*Mactra chinensis, Peronidia venulosa, Protothaca staminea*) (Sullivan et al., 1983a; Oshima, 1995a). Oshima demonstrated that *M. chinensis* and *P. venulosa* contained an enzyme that catalysed hydrolysis of N-sulfacarbamoyl groups which converted the C1 and C2 toxins to dcGTX2 and dcGTX3. Also, Oshima observed that C2 was hydrolysed much faster than C1 indicating that the orientation of the

11-hydroxysulfate in the substrate toxins greatly affected the rate of turn over of the enzyme.

The most important finding regarding to PSP biotransformations is the clear difference between the toxic profile of *A. tamarense*, dominated by C toxins, and the toxic profile of the five species of shellfish tested in this study. Moreover, the increase of the amount of certain toxins in the tissues of the bivalves (e.g. GTX1) and the decreased of other toxins (e.g. GTX4) re-confirmed these bioconversion and helped to identified specific reactions (e.g. epimerisation) taking place in the tissues. As have been discussed in the previous chapter, these reactions were specie-specific and tissue-specific.

Overall, epimerisation was the reaction that took place in the tissues of all the bivalves studied and this was characterised by in increase of GTX1 and a decrease of GTX1. Decarbamoylation, which has a final product decarbamoyl analogue, was observed in the tissues of the clam, *P. donacina*. In the detoxification period, reductive reactions had taken place in the scallop, *P. novaezelandiae* and was characterised by an increase of GTX3 and a decrease of GTX4. A summary of these bioconversions with the corresponding final products is presented in Table 7.3.

7.3 Future directions for the aquaculture sector and ongoing research

7.3.1 Comparative sensitivity of New Zealand bivalves to PSP toxins

The results of this research showed that the bivalve species in this study differ greatly in the kinetics, anatomical distribution and capacity for biotransformation of the PSP toxins. The different sensitivity of bivalve species to PSP toxins can be determined on the basis of their physiological and behavioural responses. By integrating these responses an overall classification scheme emerges on the potential for toxin accumulation in the commercial and non-commercial species in New Zealand.

Table 7.2: Summary of the bioconversion of PSP toxins observed in the tissues of the bivalves

Species	Tissue	Reaction	Final product
P. canaliculus (mussel)	Digestive gland	Epimerisation	Increase GTX1, GTX2, C2
	Other tissues	Epimerisation	Increase GTX1
P. donacina (clam)	Digestive gland	Epimerisation	Increase GTX1
		Decarbamoylation	Increase dcGTX2, dcSTX
	Other tissues	Decarbamoylation	Increase dcGTX2, dcGTX3, dcSTX
D. anus (clam)	Whole tissue	Epimerisation	GTX1
P. novaezelandiae (scallop)	Digestive gland	Epimerisation	Increase GTX1
	Adductor muscle	Reduction	Increase GTX3
	Other tissues	Reduction	Increase GTX3
		Epimerisation	Increase GTX1
O. chilensis (oyster)	Whole tissue	Epimerisation	GTX1

At the bottom of the sensitivity ranking to PSP toxins were the most resistant species; the mussel, *Perna canaliculus* and the clam, *Dosinia anus*. Both species fed actively on toxic dinoflagellates and accumulated toxins in the tissues. The intoxication and detoxification rate of mussels was faster than the other species of bivalves studied (*P. donacina* and *P. novaezelandiae*) which confirmed mussels as a good sentinel species for early warning of toxic algal blooms.

In the middle of this ranking was the clam, *Paphies donacina* which decreased its clearance rate when fed on *Alexandrium* species. Although the effect of the PSP toxin was not confirmed in this study, this species was sensitive to the presence of an *Alexandrium*

bloom. The ability of a species to retain high levels of PSP toxins for extensive periods is critical in public health management decisions. It may take only a short time to raise the toxicity above the critical level of 80 µg STX di-HCl equiv. 100 g^{-1}. Authorities need to be aware that while some bivalves (e.g. *Perna canaliculus*) are safe for human consumption others (e.g. *Paphies donacina*) could still remain toxic for long periods of time.

At the top of the PSP toxin sensitivity ranking was the scallop, *Pecten novaezelandiae* where the clearance rate was significantly lower in the presence of the toxic dinoflagellate *A. tamarense*. Despite the low clearance rate scallops accumulated PSP toxins in the tissues. They detoxified slowly and this finding would affect the market for this bivalve in the presence of a toxic algal bloom because aquaculture areas would need to be closed for longer periods of time than for other species such as mussels.

It is unclear where the oyster, *Ostrea chilensis* stands in the sensitivity ranking. It accumulated more toxins than the sensitive species, but it had been exposed to two more days of feeding with *A. tamarense* than the other species. It may therefore have a similar sensitivity to PSP toxins as *P. novaezalandiae* and *P. donacina*. Further studies will be necessary to confirm the intoxication/detoxification patterns in *O. chilensis.*

7.3.2 Considerations in future research

First, it is evident that New Zealand bivalve species differ greatly in the kinetics (toxin uptake and elimination rate constants), anatomical distribution, and capacity for biotransformation of the PSP toxins. The differential sensitivity of the bivalve species to the PSP toxins, can be determined on the basis of physiological (feeding) and behavioural (e.g. burrowing) and neurological responses (Bricelj and Shumway, 1998).

Differences were found in the toxin composition and toxin body burden among shellfish species from the same area, even though they had been exposed to the same toxic bloom (Cembella et al., 1993). It is therefore, necessary to establish the suite of

physiological responses characteristic of each species, rather than relying on extrapolation of data from other species.

Previous research has shown that sensitive species can also become more resistant when they are exposed to more recurrent HABs blooms (Bricelj et al., 2005). This suggests that constant sampling over different PSP toxin events should be carried out in order to detect possible changes in the physiology of the shellfish and the rate of accumulation of PSP toxins in the tissues.

It is important to point out that there are intrinsic (e.g. physiological responses and biotransformation of toxins) and extrinsic factors (e.g. toxic cell density, duration of exposure, toxicity of the phytoplankton species and their relative abundance) involved in determining the rate of toxin uptake and detoxification of shellfish during a toxic algal bloom (Bricelj and Shumway, 1998). In the presence of a toxic algal bloom, the location of the bivalves could also influence the rate of accumulation of toxins in the tissues. Species living close to the ocean surface (e.g cultured mussels) would be more exposed to the toxic algae than species inhabiting sediment in deeper water (e.g. wild scallops) or those that live intertidally (e.g. clams). In the presence of a toxic algal bloom, data collected *in situ* and under controlled laboratory conditions will give more information about the ability of the bivalves to accumulate and eliminate PSP toxins.

Fortunately in New Zealand, PSP blooms occur irregularly and to date studies have concentrated on relatively few species of edible shellfish. In the presence of a bloom it would be useful to collect spatial and temporal samples of a wide range of invertebrates and algae. These are needed to understand the potential effects of PSP toxins in the marine environment.It would also be useful to compare the responses of the same species from different parts of New Zealand. Shellfish may have developed resistance to PSP toxins and could become a major problem due to the rapid rate of intoxication that has been reported in resistant species such as mussels.

Finally, the increasing of HABs has clearly affected the aquaculture industry worldwide. The best way to face this problem is to understand the different factors affecting the rate of accumulation of toxins in the tissues of bivalves. As shown in the present study, many factors affect the PSP intoxication and detoxification processes of

marine species of bivalves. This makes the management of these events complex . It is advisable, therefore, to complete a data record of marine bivalves in New Zealand and their behaviour when exposed to toxic algae. Future research in the field and under controlled laboratory conditions will contribute to this better understanding of the interactions between harmful algal blooms and marine shellfish. Generalities can not be made and information of specific species need to be gathered, especially in detoxification processes. A better understanding of detoxification processes (e.g. estimations of detoxification rates) would consequently improve the management of the effects of these toxic events on the aquaculture industry with positive effects on both a local and a country-wide context.

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