Diarrhetic Shellfish Toxins in Tasmanian coastal waters: causative dinoflagellate organisms, dissolved toxins and shellfish depuration

by

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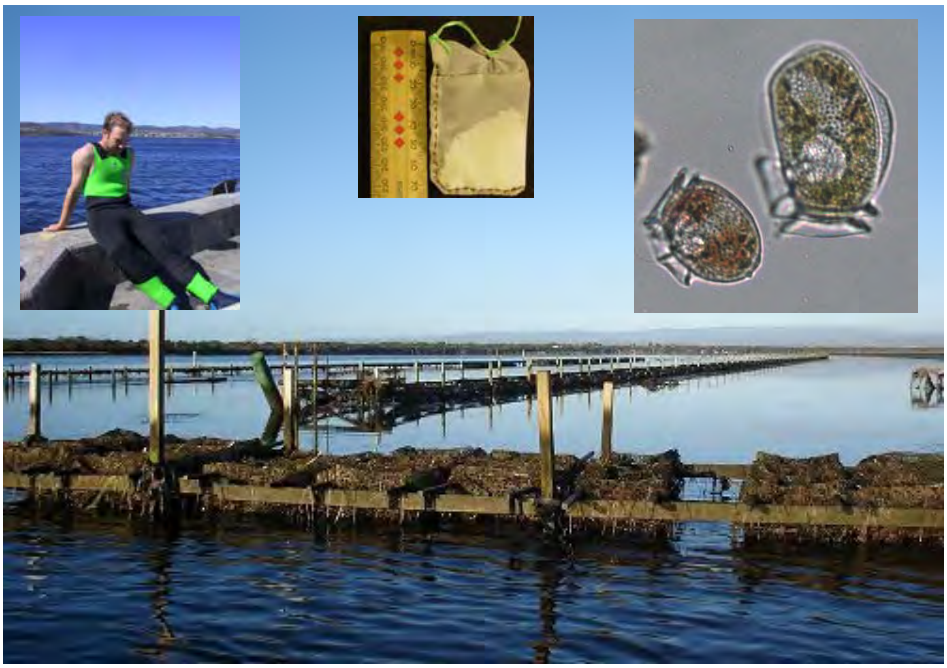

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Image of Freycinet Marine Farm taken from website www.wineglassbay.com. Insets from left to right: diving at Sullivans Cove; SPATT bags; and micrographs of *Dinophysis acuminata* (left) and *D. fortii* (right).

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Abstract

The Diarrhetic Shellfish Toxins (DST), okadaic acid (OA) + dinophysistoxin-1 (DTX-1), were detected above the regulatory limit of 0.20 µg/g of digestive gland (DG) in (non-commercial) blue mussels (*Mytilus edulis*) from Sullivans Cove, Tasmania. Pectenotoxin-2 (PTX-2), PTX-2 seco acids and *7-epi*-PTX-2 SA were also detected in mussels. This was associated with the occurrence of the toxic dinoflagellates, *Dinophysis acuminata* and *D. fortii,* which were seasonally prevalent at high cell densities (up to 7,380 cells/L for *D. acuminata*, 500 cells/L for *D. fortii*). A high density of *D. truncata* (1,850 cells/L) did not result in increased DST levels in *M. edulis* at Parsons Bay, Tasmania, suggesting that this may be a non- or weakly toxic dinoflagellate.

Subtle variations among *Dinophysis* morphotypes can pose problems for rapid and accurate identification. Tasmanian sequences of the D1-D3 region of the large subunit rDNA of *D. fortii* were indistinguishable from those of *D. fortii* from France and *D. acuta* from the North Atlantic, while Tasmanian *D. acuminata* was indistinguishable from European and New Zealand *D. acuminata*. Genetic sequencing of New Zealand *D. acuta* failed to discriminate between Tasmanian *D. fortii* and New Zealand *D. acuta* and neither did sequencing discriminate between European *D. fortii* and *D. acuta.*

A field depuration experiment was conducted in the Derwent River by placing *M. edulis* in 38 µm mesh size cages to screen out *Dinophysis* plankton cells. Mussels displayed biphasic depuration kinetics with a faster rate of PTX loss over the first 30 days followed by an increase of OA + DTX-1 depuration once there was no further change in PTX levels. The slow rate of depuration of OA + DTX-1 from day 15 to 30 followed by an increase in depuration may be attributed to mussels using lipid storage during a period of reduced food availability leading to a release of toxins in bound fractions. Solid Phase Adsorption Toxin Tracking (SPATT) detected dissolved DST in the Derwent River seawater medium at levels as high as 0.34 OA + DTX-1 µg/SPATT bag.

Cellular and exuded toxicity of *Prorocentrum lima* varied between two culture strains isolated from different locations in Tasmania, Australia. Cellular OA was greater in the Little Swanport (PLLSP) strain (36 pg/cell) compared to the Louisville Point (PLLV) strain (3.8 pg/cell), which was the only strain producing DTX-1. PTX-2 was produced by both strains at small concentrations up to 1.2 pg/cell. This is the first reported occurrence of PTX-2 produced by *P. lima*. The Louisville strain excreted higher concentrations of OA (reaching 18 μg/SPATT bag) in the first 20 days compared to the Little Swanport strain (11 μg OA/SPATT bag). For both strains this declined to 4 μg/SPATT bag on day 40. Both strains exuded higher dissolved toxin levels at low cell abundance of 1,200 cells/L (PLLV strain reaching 1.6 μg OA + DTX-1/SPATT bag) compared to at 2,400 cells/L (0.4 μg OA + DTX-1/SPATT bag). Tasmanian strains of *P. lima* were more toxic than other global strains and poses a potential DSP risk to Tasmanian shellfish farms.

In-vitro experiments with *Prorocentrum lima* suggest that dissolved toxins are exuded from DST producing dinoflagellates as well as from depurating mussels. Most of the DST was present dissolved in the seawater (94 %) when SPATT bags were exposed to *P. lima* cultures (6 % of DST in cells). Only a small amount of DST (1 %) was detected in the seawater medium when SPATT bags were exposed to contaminated mussels (99 % of DST in mussels). OA displayed an increase by more than 0.11 μg/g DG in mussels immersed in dissolved DST for 48 hrs indicating that mussels can accumulate DST in *in-vitro* conditions.

Dissolved DST can pose an additional threat to shellfish farms and can extend harvest closure periods after toxic dinoflagellate blooms. Toxicity differences among dinoflagellate species and strains can pose problems for shellfish monitoring programs and may require phytoplankton regulatory limits to be varied according to locality.

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- *P. lima* cultures (PLLSP and PLLV) were obtained from the aquatic botany culture facility at the University of Tasmania. *P. lima* cultures were established by Imogen Pearce in 2000. *Thalassiosira weissflogii* (CCMP1336 strain) was obtained from the CSIRO culture facility.
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Chapter 1:
General introduction

Definition of DST

Diarrhetic shellfish poisoning (DSP) is one of several classes of seafood poisoning caused by dinoflagellate biotoxins accumulated in commercial shellfish. Diarrhetic Shellfish Toxins (DST) are metabolites produced by dinoflagellates of the genera *Prorocentrum* Ehrenberg and *Dinophysis* Ehrenberg (Yasumoto *et al.* 1980, Murakami *et al.* 1982, James *et al.* 1999, Bravo *et al.* 2001, Pavela-Vrančič *et al.* 2001, 2002). The DSP syndrome is a human gastroenteritis caused by ingestion of shellfish contaminated with DST. This syndrome was first described in Japan by Yasumoto *et al.* (1978) as a seasonal shellfish poisoning that caused symptoms such as diarrhoea, abdominal pains, vomiting and nausea in humans that consumed contaminated shellfish. Since the first report, DSP has been demonstrated world-wide (Figure 1.1).

Figure 1.1. Known global distribution of Diarrhetic Shellfish Poisoning (DSP). Updated and modified from Hallegraeff (2010).

The seasonal occurrence of toxic shellfish and humans suffering DSP have been related to blooms of *Dinophysis* and *Prorocentrum*, which are the diet of filter feeding shellfish (Yasumoto *et al.* 1978, 1985, Kat 1983, Séchet *et al.*1990, Masselin *et al.* 1992, Belin 1993, Pitcher et al. 1993, Draisci *et al.* 1996, Uribe *et al.* 2001, Vale and Sampayo 2003). The toxins produced by these species are cyclic polyether compounds divided into two groups: the acidic group containing okadaic acid (OA), dinophysistoxins (DTX) and their derivatives; and the neutral polyether lactones

consisting of pectenotoxins (PTX) and their derivatives. OA and DTX exert their toxic effects by inhibiting serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (Carmody *et al.* 1996, Suoto *et al.* 2001, Traoré *et al.* 2001, Landsberg 2002). Inhibition of PP1 and PP2A increases protein phosphorylation which affects several cellular processes such as metabolism, cytoskeletal maintenance, gene transcription, cell division, membrane transport and secretion. Pectenotoxins have little or no oral toxicity (MacKenzie 2002, Miles *et al.* 2004) and therefore PTX levels are excluded from quarantine limits set by monitoring programs.

Global Distribution

Diarrhetic shellfish poisoning has a negative effect upon the shellfish industry in many regions of the world, necessitating monitoring programs in infected coastal regions. Outbreaks of DSP are financially costly to the shellfish industry of the contaminated region. The industry loses revenues through harvesting bans, embargo, recall and/or destruction of product, and legal fees. The estimated cost of DSP outbreaks in Canada has been estimated as $CAN93,000 annually (Todd 1995). In 1990 a case of DSP occurred in Chonos Archipelago and the adjacent fjords of Chile, resulting in an economical loss of $US100,000 impacting on three shellfish species (*Mytilus chilensis*, *Alacomya ater* and *Ameghinomya antiqua*) representing 87 percent of the national harvest (Lembeye *et al.* 1993).

Outbreaks of human shellfish poisoning can have serious impacts through loss of consumer confidence. This occurred in France, 1997, when sales of oysters and mussels were reduced by 70 % after a recall on a harvesting ban on shellfish growers in the Etang de Thau on the Mediterranean coast. The dilemma cost the state $US 350,000 in advertising to help recoup sales (Tournay 1999). In coastal waters of Sweden, Norway, Portugal and Spain surveillance systems have detected toxin levels over the regulatory limit leading to closures of shellfish farms. Shellfish farms have been closed for up to 6 months each year in Sweden and Norway since 1984; harvesting of French mussels was stopped for long periods due to high OA concentrations (Draisci *et al.* 1996) in 1989 and 1990; and throughout 1994 to 2001 OA exceeded the safety level of 0.2µg/g digestive gland (DG) or 20µg/100g of mussel meat, with toxin concentrations reaching 22-30µg (total DST)/100g in 1998 and 19µg

(OA+DTX-2)/g DG in 1995, in pipi (*Donax trunculus*) and mussel (*M. edulis*) in Portuguese farms, respectively (Vale and Sampayo 1999 and 2003).

Two known outbreaks of human DSP have occurred in Australia. In 1997, in Ballina, northern New South Wales, 200 people became ill after consuming local pipis (*Donax deltoides*). Fifty-six of these people required hospitalisation. Another incident occurred in March 2000 on North Stradbroke Island (Queensland) where an elderly woman became ill after consuming *Donax* pipis (Burgess and Shaw 2001). The Ballina incident resulted in prevention of pipi harvesting. Monitoring programs have led to prohibition of shellfish harvesting at various sites also in South Australia (Port Lincoln) and Victoria (Port Phillip Bay) in the year 2000 (Todd *et al.* 2001).

Analysis of DST

The presence of the DST producing algae in Tasmanian coastal waters poses a threat to sustainable shellfish industry. Biotoxin monitoring in Tasmanian shellfish farms was initiated in 1986 by the Tasmanian Shellfish Quality Assurance Program (TSQAP). The regulatory limit for DSP causative algae was established in Australia as *D. acuminata* (1,000 cells/L), *D. fortii* (500 cells/L) and *P. lima* (500 cells/L), matching practices in most countries where these toxic phytoplankton occur in commercial shellfish growing areas. Phytoplankton monitoring, regulatory levels and procedures for enumeration are consistent among countries, however the regulatory levels for shellfish toxicity and methods for detection can vary. Since the discovery of DSP most programs have set the quarantine level at 0.2µg/g of DG (or 20µg/100g of edible parts) or its mouse bioassay equivalence of 5 mouse units (MU)/100g of mussel meat (or per g of DG). The digestive gland of shellfish is a dark greenish-brown organ which is also known as hepatopancreas and it functions much like the liver and pancreas of higher animals. A mouse unit is the required toxin concentration to kill a female mouse (15 – 20 g) within 48 hours (Yasumoto *et al.* 1978). The mouse bioassay is no longer required by EU and is being phased out as this approach suffers from low sensitivity and interferences from fatty acids, where toxicity is due to total toxin composition but it does not detect which toxins are present. Therefore analytical tools such as chromatography coupled with mass spectroscopy have been developed and implemented to isolate, detect and identify DSP toxins. Most procedures for shellfish analysis only extract the toxins from the digestive gland (DG) since most

DST accumulates in this organ; the alternative is to extract the toxins from the edible parts. Toxins are extracted from plankton and shellfish with organic solvents that react differently to different chemical toxin structures. Some analyses may not detect all toxins present, and hence a universal solvent is required that extracts all known toxins with comparable efficiencies. In early studies the common solvent was acetone because it is easily evaporated, however acetone extracts can be very complex due to the presence of fats and other non-polar lipids that can enhance the potency of toxins in mouse bioassays (Quilliam 2003). Later studies have found methanol to be a better extraction solvent because it reduces the amount of lipids extracted.

Lee *et al.* (1987) were the first to implement the technique of high performance liquid chromatography (HPLC) with a fluorometric detector (FLD) using 9-anthryldiazomethane (ADAM) as a derivatising reagent that reacts with the free carboxyl group of OA/DTX toxins to introduce a fluorescence moiety that tags the molecule for detection. Modified procedures have been developed to overcome the problem associated with low purity of the ADAM reagent (resulting from poor stability), and the presence of other reactive co-extracts in shellfish tissue, which require a silica column cleanup following the derivatisation step. However, ADAM-HPLC is only selective for toxins with a free carboxyl group, whereas LC-MS can detect a wider range of toxins. Another form of HPLC is to introduce a UV-absorbing moiety into the molecule (HPLC-UV), though this method has limited application because only a few toxins have UV-absorbing chromophores such as the diene functions of some PTX.

OA and DTX-1 exert their toxic effects by inhibiting serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A) (Carmody *et al.* 1996, Suoto *et al.* 2001, Traoré *et al.* 2001, Landsberg 2002). This knowledge has led to the development of test kits such as a protein phosphatase (PP) inhibition assay and enzyme linked immunosorbent assays (ELISA) for quick detection of OA and/or DTX-1. These methods are based on the ability of PP2A to dephosphorylate a colourless substrate (*p*-nitrophenonylphosphate) to a yellow product (*p*-nitrolphenol) in alkaline medium. Toxin concentration is measured by the relative activity of PP2A in the presence of OA and DTX. The advantage of ELISA over HPLC is that it is faster to produce results but does not specify toxins present. This is a poor trade-off for determining

total toxin concentration when considering the risk of DSP to the public. Liquid chromatography seems to be the most valuable for research purposes because it has a superior range, can unambiguously identify new toxins and determine toxin structures and profiles. However the disadvantage of this method for monitoring purposes is that the procedure is time consuming and expensive compared to other techniques. Due to the complications associated with the various methods described, regulatory limits of DST vary from country to country (Table 1.1).

Monitoring DST in Australia

Monitoring of DST producers in Tasmanian and South Australian waters by State Shellfish Quality Assurance Programs, has detected the causative phytoplankton above the trigger level (>1,000 cells/L and >500 cells/L for *D. acuminata* and *D. fortii*, *D. acuta*, respectively) for toxin testing on 20 and 8 occasions in Tasmanian and South Australia, respectively. These results are summarised in Table 1.2 (courtesy of Alison Turnbull of TSQAP) and Table 1.3 (data courtesy of Clinton Wilkinson of SASQAP) along with results of DST testing. Toxicity testing may not have been conducted if the growing area was already closed for other reasons, or was delayed due to heavy weather conditions preventing sampling. DST was detected in Tasmanian mussels (23.8 and 17.9 µg/100g) on 2 occasions following counts of *D. fortii* exceeding the trigger level. Toxins (23.8 µg/100g) exceeded the TSQAP quarantine level on one occasion in Deep Bay. PTX-2 SA has been the only DST detected in Tasmanian oysters and scallops at low levels (0.5 and 1.9 – 3.8 µg/100g, respectively). DST was detected on 10 occasions in South Australian shellfish at very low levels (0.01 – 0.032 OA µg/100g). Oyster toxicity was at low levels (0.02 OA µg/100g) in the presence of DTX producers, and similar to levels detected in mussels, pipis and cockles (0.01 – 0.025 and 0.01 – 0.02 OA µg/100g, respectively) in the absence of DST producers. DST has been detected in Western Australian mussels at levels of 1.4, 2.8, 4 and 98 µg/100g for OA, DTX-3, PTX-2 and PTX-2 SA, respectively (Queensland Health Scientific Services pers. comm.). Toxin accumulation in shellfish species can vary as indicated in studies by Lindegarth *et al.* (2009), Vale (2004), Suzuki and Mitsuya (2001), Svensson *et al.* (2000). Literature data (Table 1.2, 1.3) suggest that mussels are more sensitive in accumulating toxins compared to scallops and oysters when exposed to similar conditions.

Table 1.1. International regulation levels of diarrhetic shellfish toxins in bivalve mollusc species and action levels of causative species monitored in countries that have established monitoring programs.

Country	Toxin level to initiate closure of shellfish harvesting.	DSP causative species monitored.[1]	Shellfish species analysed for toxin levels.
Australia[2]	20µg/100g (now expressed as 0.2 mg/kg)	*D. acuminata*, *D. acuta,* *D. fortii,* *P. lima*	shellfish
Canada Bauder *et al.* (2001)	0.2µg/g (equivalent to 20µg/100g whole tissue)	*D. acuminata*, *D. norvegica*, *P. lima*	mussels and scallops
France Wright (1995)	0.2MU[3]/g DG	*D. acuminata*, *D. acuta*, *D. norvegica*, *D. sacculus*	
Ireland Carmody *et al.* (1996)	0.8µg/g (OA + DTX2) HP	*D. acuminata*, *D. acuta,* *D. fortii*, *P. lima*,	mussels

Table 1.1. Continued

Country	Toxin level to initiate closure of shellfish harvesting.	DSP causative species monitored (action level).	Shellfish species analysed for toxin levels.
Japan Lee *et al.* (1987); Yasumoto and Murata (1990)	0.2 µg/g (5 MU/100g)	*D. acuminata*, *D. acuta*, *D. fortii*, *D. mitra*, *D. norvegica,* *P. lima*	mussels and scallops
New Zealand[2]	20µg/100g (now expressed as 0.2 mg/kg) (= 5 MU/100g EP)	*D. acuta* (500 cells/L), *D. acuminata* (500 cells/L), *P. lima* (500 cells/L)	mussels and scallops
Portugal Vale and Sampayo (2002a, 2003)	2µg/g DG (equivalent to 20µg/100g EP)	*D. acuminata*, *D. acuta*, *D. fortii*	mussels, oysters, clams and cockles
Spain Vale and Sampayo (2002, 2003)	2µg OA/g DG (equivalent to 20µg/100g EP)	*D. acuminata*, *D. acuta*, *D. norvegica*, *P. lima*	mussels
Sweden Svensson *et al.* (2000)	40 – 60 µg OA/g mussel meat	*D. acuminata*, *D. acuta*, *D. norvegica*	mussels and oysters

Table 1.1. Continued

Country	Toxin level to initiate closure of shellfish harvesting.	DSP causative species monitored (action level).	Shellfish analysed for toxin levels.
USA[4]	80µg toxin/100g	*D. acuminata*, *D. norvegica*, *P. minimum*	mussels and Sand Gapper
Europe (EC regulations) Lee *et al.* (1987)	5MU[5]/100g EP equivalent to 0.5MU/g HP) or 20 - 60µg/100g	Depends on the European country	Depends on the European country
Svensson *et al.* (2000)	4 – 5MU or 16 – 20µg OA /100g mussel meat		

DG = digestive gland equivalent to HP = hepatopancreas, EP = edible parts, MU = mouse units.

[1] = If a toxic phytoplankton exceeds a specific density (set by monitoring programs), known as the action levels, shellfish will be tested for toxicity.

[2] = Regulation limit set by Australia New Zealand Food Standards Code (FSANZ 2003).

[3] = Corresponds to mouse survival time of 5 hours.

[4] = Regulation set by the US Health Department of food and drug administration.

[5] = Corresponds to mouse survival time of 24 hours.

Mussels and cockles contained 0.025 and 0.019 µg OA/g, respectively during the absence of *Dinophysis*, while it required 1,400 – 1,500 cells/L of *D. acuminata* for oysters to reach 0.014 – 0.026 µg OA/g. During a bloom of *D. fortii* (2,100 cells/L) in Tasmania, scallops contained 0.038 µg/g of PTX, while no other DST was detected. Accumulation and depuration kinetics are important in establishing species specific harvest and closures in relation to regulatory levels of DST producing phytoplankton. Species specific harvest and closures for DST are currently not practiced, however some farms do practice this for Paralytic Shellfish Toxins (PST). This practice can be advantageous to shellfish farms if it allows harvesting of one species while closures are in place for another shellfish product.

Table 1.2. Potential DST producers in Tasmania found above trigger levels in Tasmania since Jan 2001 (courtesy of Alison Turnbull of TSQAP).

Algal Site	**Date**						**Shellfish toxins (mg/kg)**		
		Dinophysis acuminata	***Dinophysis acuta***	***Dinophysis fortii***	***D. tripos***	***Prorocentrum lima***	**DST total**	**PTX2 seco-acid**	**Shellfish**
Blackman Bay	28/2/03					1000			
Deep Bay	2/2/05	520		4000					
Deep Bay	3/5/05			700			0.238	0.14	mussels
Dunalley Bay	7/10/01			1000				0.057^{M}	mussels
Great Oyster Bay	24/10/01			3086					
Hastings Bay	18/1/05			4000					
Moulting Bay	30/3/03				1000				
Nubeena	6/5/03			1200			0.179	0.25	mussels
Nubeena	16/12/03			830					
Pipe Clay Lagoon	30/10/01			3333					
Pipe Clay Lagoon	6/12/01			1449					
Pipe Clay Lagoon	13/9/04	690					n.d	0.005	oysters
Port Arthur	13/11/01			2151					
Port Esperance	11/4/02			510					
Port Esperance	16/1/05			2100			n.d.	0.038	scallops
Recherche	5/11/01		3205						
Spring Bay	24/1/02			3704			n.d.		scallops
Stringers Cove	11/4/03			510					
Stringers Cove	16/1/05			1200			n.d	0.019	oysters
Triabunna	7/11/01			898					
Maximum		**690**	**3205**	**4000**	**1000**		**0.238**	**0.25**	**mussels**
Average ± std. dev.				**1917 ± 1293**			**0.21 ± 0.04**	**0.15 ± 0.1**	**mussels**
							n.d	**0.1 ± 0.1**	**oysters**
							n.d	**0.038**	**scallops**

n.d = not detected.

Table 1.3. Potential DST producers found above trigger levels in South Australia since March 2002 (data courtesy of SASQAP).

Algal site	Date	Cells/L		Toxins (µg/100g)				Shellfish
		D. acuminata	*D. acuta*	OA	DTX-1	PTX-2	PTX-2 SA	
Bickers Island	11/06/2002		31	0.016	0.016	0.01	0.043	oysters
Coorong	2/12/2002			0.019			0.019	pipis/ cockles
Coorong	5/01/2004			0.01		0.046	0.3	pipis/ cockles
Kellidie Bay	4/03/2004	1408		0.026		0.19	2	oysters
Kellidie Bay	15/03/2004	1505		0.014		0.068	0.42	oysters
Mount Dutton Bay*	8/03/2004	15276, 17073, 4096		0.032		0.18	1	oysters
Port Lincoln	14/01/2008					0.021	0.25	mussels
Smoky Bay	1/12/2003	1760				0.075	0.85	oysters
Smoky Bay*	9/12/2003	3168, 2380				0.090	2.7	oysters
Smoky Bay*	16/12/2003	18250, 10943, 10286, 20800		0.023		0.11	0.79	oysters
Smoky Bay	3/12/2007	2220				0.11	0.81	oysters
Streaky Bay- The Hummocks	21/11/2002	3225		0.02		0.62	0.77	oysters
Wallaroo	27/09/2004			0.01			0.045	mussels
Wallaroo	28/01/2005			0.025				mussels
Wallaroo	27/09/2005			0.014			0.037	mussels
Maximum		**18250**	**31**	**0.32**	**0.016**	**0.62**	**2.7**	
Average ± std. dev.		**8028 ± 7174**	**31**	**0.02 ± 0.01**		**0.021**	**0.11 ± 0.11**	**mussels**
				0.02 ± 0.01	**0.016**	**0.16 ± 0.16**	**1.1 ± 1.1**	**oysters**
				0.01 ± 0.006	**0.046**		**0.03**	**pipis/cockles**

* multiple counts at various sites within the bay.
Note: Enumeration samples not provided for the Coorong and Wallaroo sites for the above dates.

Extracellular DST

Natural dinoflagellate blooms have also been associated with dissolved DST in the seawater medium during high densities of *Dinophysis* species (MacKenzie *et al.* 1998, 2004). Toxic phytoplankton have been observed to release toxins into the seawater medium (Rausch De Traubenberg and Morlaix 1995) which may be exuded from cells in an allelopathic role to inhibit growth of co-occurring species (Windust *et al.* 1996,

1997, Snugg and VanDolah 1999). Dissolved toxins may pose an additional threat to shellfish farms if these toxins can be taken up and accumulated by shellfish. The concept of Solid Phase Adsorption Toxin Tracking (SPATT) was first developed by MacKenzie *et al.* (2004) to monitor dissolved DST in the field and to potentially be utilised as a tool to provide a more sensitive and cost effective means of monitoring biotoxins. SPATT bags are mesh bags that contain adsorbent resin beads (HP20) which have an affinity for binding DST and thus can be used to detect levels of dissolved toxins in the seawater medium. SPATT bags could provide early warnings of toxic plankton blooms and accumulation of toxins by shellfish.

Aims of thesis

The aims of this thesis were to: 1. Assess the threat of seasonal toxic *Dinophysis* blooms as the cause of potential toxicity of blue mussels (*Mytilus edulis*) in south-east Tasmanian waters and to compare dinoflagellate cellular toxin content with overseas reports; 2. Determine LSU rDNA sequences of the two common dominant toxic *Dinophysis* species (*D. acuminata* and *D. fortii*) in Tasmanian waters and to assess their phylogenetic relationships with *Dinophysis* sequences from other global locations; 3. Characterise the depuration rate of mussels (*M. edulis*) when toxic *Dinophysis* are removed from their diet in natural field conditions, and; 4. Elucidate the origin of dissolved toxins in Tasmanian coastal waters and the potential of SPATT bags as a monitoring tool for DSP and using DST producer *P. lima* as a model to access the potential threat of shellfish ability to take up the dissolved toxins in the same manner as ingesting toxic algae.

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Chapter 2:

Diarrhetic Shellfish Toxins in Tasmanian shellfish and their causative dinoflagellate organisms

Abstract

Two Diarrhetic Shellfish Poisoning causative dinoflagellates, *Dinophysis acuminata* and *D. fortii,* were seasonally prevalent at two sites along the south-east coast of Tasmania, Australia. High cell densities were recorded (up to 7,380 cells/L for *D. acuminata*, up to 500 cells/L for *D. fortii*) in the Derwent River estuary, Tasmania, from October 1986 to March 1987, October 2003 to March 2004 and again from September 2005 to April 2006. These species embedded in natural seston were estimated to contain high cellular toxin (OA + DTX-1) levels (26 µg/L and 148 µg/L of seston containing *D. acuminata* and *D. fortii*, respectively). This resulted in the accumulation of diarrhetic shellfish toxins (DST) in (non-commercial) blue mussels (*Mytilus edulis*) with toxin levels of okadaic acid (OA) + dinophysistoxin-1 (DTX-1) twice the regulatory limit of 0.20 µg/g digestive gland. *Dinophysis* cells contained a higher proportion of PTX-2 to PTX-2SA and 7-epi-PTX-2SA, whereas the reverse situation was observed in blue mussels and indicate that shellfish convert PTX-2 to PTX-2SA and 7-epi-PTX-2SA. By comparison, *D. acuminata* was not detected at Parsons Bay, Tasmania, where *D. fortii* was only detected occasionally at low levels. A high density of *D. truncata* (up to 1,850 cells/L) did not result in increased DST levels in *M. edulis*, suggesting that this may be a non- or weakly toxic dinoflagellate. Detection of DST levels above the regulatory limit in local blue mussels demonstrates the importance of monitoring and management programs for Tasmanian shellfish industries to prevent DSP outbreaks.

2. 1. Introduction

Diarrhetic shellfish toxins (DST) are metabolites produced by selected dinoflagellates of the genera *Dinophysis* Ehrenberg and *Prorocentrum* Ehrenberg (Murakami *et al.* 1982, James *et al.* 1999, Bravo *et al.* 2001, Pavela-Vrančič *et al.* 2001, 2002). Inclusion of PTX as a human toxin is debatable due to lack of evidence of human oral potency, with administration of pectenotoxins in mice showing only mild to no diarrhetic effects (Burgess and Shaw 2001). Diarrhetic effects have only been proven for OA and dinophysistoxins (DTX) (Vieites *et al.* 1996, Berven *et al.* 2001, Ramstad *et al.* 2001a, b) by inhibiting serine/threonine protein phosphatases 1

(PP1) and 2A (PP2A) (Haystead *et al.* 1989, Traoré *et al.* 2001), and causing fluid accumulation in the small intestine and altering the tissue structure of the small intestine (Terao *et al.* 1993, Berven *et al.* 2001). In addition, okadaic acid and DTX may potentially be involved as tumor promoters as demonstrated on several human cancer cell lines (Sipia *et al.* 2000, Maynes *et al.* 2001, Traoré *et al.* 2001).

Diarrhetic shellfish poisoning can have a negative effect upon the shellfish industry necessitating monitoring programs of plankton and shellfish in infected coastal regions. For example, during an outbreak of DSP in Ballina (New South Wales, Australia) in 1997, over 100 people were poisoned following consumption of pipis (*Plebidonax deltoides* Lamarck). This episode resulted in a harvesting closure (Burgess and Shaw 2001) for two months. Monitoring programs have also led to prohibition of shellfish harvesting at various sites in Port Lincoln (South Australia; Madigan *et al.* 2006) and Port Phillip Bay (Victoria; Todd *et al.* 2001). No human DSP cases have been formally reported from Tasmania, but the seasonal presence of toxic species including *Dinophysis acuminata* Claparède et Lachmann, *D. acuta* Ehrenberg, *D. caudata* Saville-Kent, *D. fortii* Pavillard, *D. tripos* Gourret, and *Prorocentrum lima* (Ehrenberg) Dodge (Hallegraeff and Lucas 1988), could pose a threat to the sustainability of shellfish industries and the health of seafood consumers. The cellular toxin content produced by these dinoflagellates is species- specific and varies according to strain and physiological condition (Moroño *et al.* 2003). The present study aims to assess the threat of seasonal toxic *Dinophysis* blooms as the cause of potential toxicity of blue mussels (*Mytilus edulis*) in south-east Tasmania and to compare cellular toxin content with overseas reports.

2. 2. Material and Methods

2. 2. 1. Study Site

Phytoplankton and mussel sampling were conducted at two sites on the south-eastern Tasmanian coast. One site was the Princess Wharf at Sullivans Cove (42º 53´ S 147º 20´ E) in the Derwent River estuary. The other site was Parsons Bay (43º 6´ S 147º 43´ E) on the Tasman Peninsula (Figure 2.1). Sullivans Cove was selected because of the known seasonal occurrence of *Dinophysis* during spring and summer and the high

abundance of the blue mussel (*M. edulis*) growing on the rocks and wharf structures. The Derwent River estuary is affected by industrial and municipal wastewater discharges that have an impact on nutrient levels. Average water temperatures and salinity at the site are 13.5 ºC and 33 ppt, respectively during experimental period. Weekly samples were collected at this site from August 1, 2003 to March 31, 2004 and September 2, 2005 to April, 27, 2006. The Tasman Peninsula is an area of commercial importance containing shellfish and other aquaculture farms. Parsons Bay represents an area moderately exposed to oceanic conditions of the Southern Ocean and Tasman Sea. Salinities were influenced by discharge of freshwater from a nearby creek, varying from 25 to 34 PSU. Weekly samples were collected at this site from January 12, 2004 to April 5, 2004.

2. 2. 2. Sample collection

2. 2. 2. 1. Phytoplankton

Weekly phytoplankton samples were collected with a depth-integrated sampling tube (length 7 m; hose diameter 24 mm (Sullivans Cove) and 14 mm (Parsons Bay), respectively) and vertical plankton net hauls (mesh size 20 μm) to a depth of 8 m. One litre of the unconcentrated sea water was fixed with Lugol's iodine solution, settled for 12 to 24 hours, the bottom 50 mL was collected via pipette and sub-samples counted under a Zeiss Axiovert25 inverted microscope. A quantitative record of *Dinophysis* and other plankton taxa was made by multiplying cells counted by a concentration factor of 20. A 20 μm net tow was passed through the water column (8 m) to concentrate the phytoplankton for cell counts and toxin analyses.

2. 2. 2. 2. Mussels

Blue mussels (*M. edulis*) were sampled for DST at Sullivans Cove on November 12, 2003, January 13, 2004, fortnightly from February 18 to March 31, 2004 and again on April 22, 2004. Fifteen mussels (ranging from 36 to 98 mm in length) were collected from concrete pylons on each sampling date. Long-line mussels samples from the

salmon farm lease at Parsons Bay were taken weekly from January 5 to April 5, 2004. Mussels were stored at -20ºC until further processing.

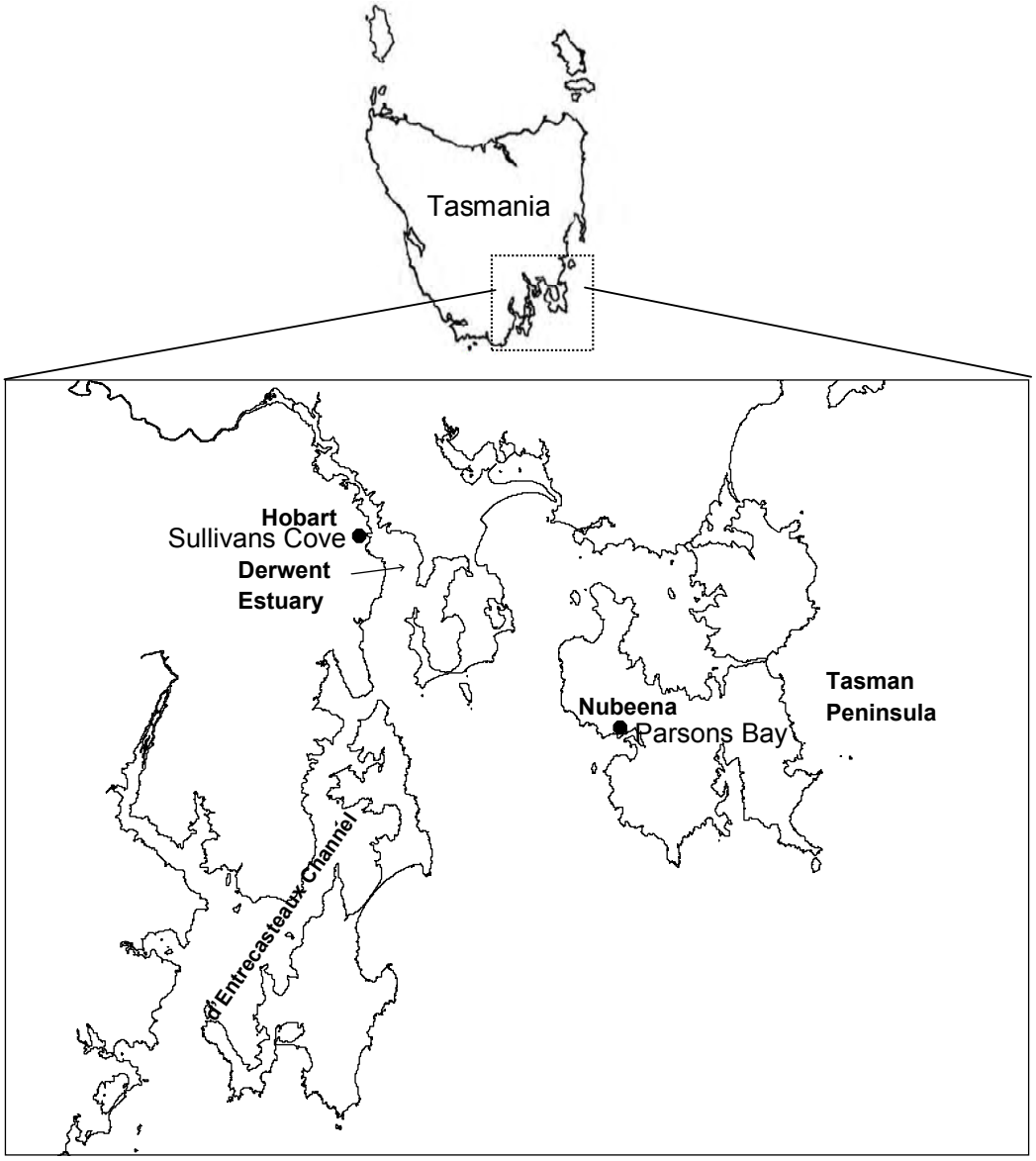

Figure 2.1. Southeast coast of Tasmania, Australia, showing location of sampling stations (●) in the Derwent Estuary and the Tasman Peninsula.

2. 2. 3. Toxin extraction from *Mytilus edulis* and phytoplankton

Mussels were thawed, the soft tissue removed from the shell and the digestive glands dissected. Digestive glands were weighed and placed into a 50 mL sample container and homogenised using a commercial hand-held blender (AFK stabmixer or Braun

multiquick). Approximately 4 g of homogenate was weighed into 50 mL plastic sample containers and homogenised with 16 mL of methanol. Samples were centrifuged for 10 min at 652 g. The supernatant was then transferred to a sample container. Phytoplankton 20 μm net tow samples were snap frozen until further analysis when they were thawed to disrupt cells. After centrifugation at 652 g for 10 min, the supernatant was decanted and the tubes containing pellets were immersed in boiling water for 5 min to inactivate enzymes of *Dinophysis* cells, preventing potential hydrolysis of conjugated forms of OA. Cellular toxins were extracted in 1 mL of 90 % methanol, evaporated to dryness under a nitrogen stream then resuspended in 1 mL of 90 % methanol. Final extracts of mussels and phytoplankton were filtered through an Acrodisc® syringe 0.2 μm Supor® membrane filter into vials and stored in a freezer until analysed by HPLC-MS/MS at Queensland Health Scientific Services.

2. 2. 4. HPLC-MS/MS analysis of toxins

Diarrhetic shellfish toxins were analysed using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord. On, Canada) equipped with a turbo-ionspray (high flow electrospray) interface coupled to a Shimadzu LC-10ADvp HPLC (Shimadzu Corp., Kyoto, Japan). Separation was achieved using a 150 x 4.6 mm Alltima C_{18} column (Alltech (Aust.), Baulkam Hills, NSW) run at 35 ^{o}C, and flow rate of 0.8 mL/min with a linear gradient starting at 5 % B for 5 min, ramped to 100 % B in 8 min, held for 5 min then to 5 % B in 1 min and equilibrated for 5 min (A = 10 % acetonitrile /HPLC grade water, B = 90 % acetonitrile/ HPLC grade water, both containing 0.1 % formic acid and 2 mM in ammonium formate.

The mass spectrometer was operated in both positive and negative ion modes. Analytes were confirmed (normally using the single ion monitoring mode, SIM) by characteristic ions and retention times (rt. in minutes) as described in Table 2.1.
Quantitation was achieved by comparing areas of peaks from samples to appropriate standards using the external standard method with a relative standard deviation (precision) of 3.3 – 12.7 % (Appendix Table A.1). Certified standards of pectenotoxin-2 and okadaic acid were obtained from Institute of Marine Biosciences, National Research Council, Canada. Pectenotoxin-2 seco acid concentrations were determined by comparison to the PTX-2 standard and assuming a similar response

factor. Dinophysistoxin-1 and DTX2 concentrations were determined by comparison to the OA standard and assuming a similar response factor.

Table 2.1. Mass spectrometer operating parameters.

Toxin	SIM	m/z	Retention time (min)
PTX-2	positive mode	876.6	14.62
PTX-2 SA	positive mode	894.6	13.73
7-epi PTX-2 SA	positive mode	899.6	14.15
OA	negative mode	803.5	11.90
DTX-1	negative mode	817.5	13.40
DTX-2	negative mode	803.5	12.28

2. 2. 5. Statistical Analyses

All statistical analyses were conducted using SAS Windows© 6.12 software. A linear regression was used to determine if there was a significant relationship between toxin concentration (okadaic acid + dinophysistoxin-1) of mussels and *Dinophysis* cells. A one-way model-1 Analysis of Variance (ANOVA) was used to determine if toxin concentration differed significantly over the time period of the study, where time was the fixed factor.

2. 3. Results

2. 3. 1. Sullivans Cove mussel toxin content

Figure 2.2 exemplifies HPLC analyses of PTX-2-SA, OA and DTX-1 from digestive glands of blue mussel (*M. edulis*) in positive and negative ionisation modes. OA and DTX-1 were detected in both positive and negative ionisation modes. PTX-2-SA was detected in positive mode of ionisation eluting before OA (8.79 min) at 8.61 min (Figure 2.2). Pectenotoxin-2 (PTX-2) derivatives (PTX-2 seco acids and *7- epi*-PTX-2-SA (0.61 and 0.43 µg/g digestive gland, respectively) and okadaic acid (OA) (0.43 µg/g DG) were the main toxins in *Mytilus edulis* from Sullivans Cove. PTX-2 derivatives displayed higher peaks compared to other DST in mussels (Figure 2.3A). Okadaic acid + dinophysistoxin-1 (DTX-1) were above quarantine levels of 0.2 µg/g

DG in *M. edulis* during November, January and mid-March (Figure 2.4A). Toxin levels then decreased to 0.06 µg/g DG from March 19 to April 22, 2004. During this same period *D. acuminata* and *D. fortii* were the most abundant *Dinophysis* species present (86 and 9 % of total *Dinophysis*, respectively) exceeding the Tasmanian Shellfish Quality Assurance Program (TSQAP) action level of 500 cells per litre during the period from October 23, 2003 to March 31, 2004. *D. acuminata* and D. *fortii* densities reached 7,380 and 500 cells/L, respectively, in the Derwent River during October 2003 to March 2004 (Figure 2.4A). No simple relationship was found between mussel toxin content and *Dinophysis* cell densities.

2. 3. 2. Parsons Bay mussel toxin content

Pectenotoxin-2 (PTX-2) derivatives (PTX-2 seco acids and *7- epi*-PTX-2-SA (0.01 to 0.50 µg/g DG and 0 to 0.07 µg/g DG, respectively) and okadaic acid (OA) (0.07 to 0.12 µg/g DG) were the main toxin compounds in *Mytilus edulis* from Parsons Bay. PTX-2 derivatives displayed higher peaks compared to other DST in mussels (Figure 2.3B). Toxin levels of 0.06 to 0.1 µg/g DG in *M. edulis* were consistently below the quarantine level and decreased with time (Figure 2.4B). *Dinophysis truncata* was the most abundant (83 %) *Dinophysis* species present with *D. tripos* co-dominant (16 %), while *D. acuminata* was not detected throughout the study period. *D. truncata* densities were above 500 cells from January 26 to March 1, 2004 but this did not appear to cause significant toxicity (OA + DTX-1) in *Mytilus edulis*.

2. 3. 3 Sullivans Cove phytoplankton toxin content

The Sullivans cove phytoplankton community was largely composed of diatoms (> 82 % of total cells) during October 2005 to April 2006. *Dinophysis* represented less than 13 % of total microalgae community during spring to early autumn, 2005 – 06 (Table 2.2). *D. acuminata* was the most abundant of the DST producers except during April, 2006 when *D. fortii* was the dominant *Dinophysis* species. *D. acuminata* and *D. fortii* densities ranged from 50 – 2,150 and 25 – 850 cells/L, respectively, during the spring to early autumn of 2005 – 06.

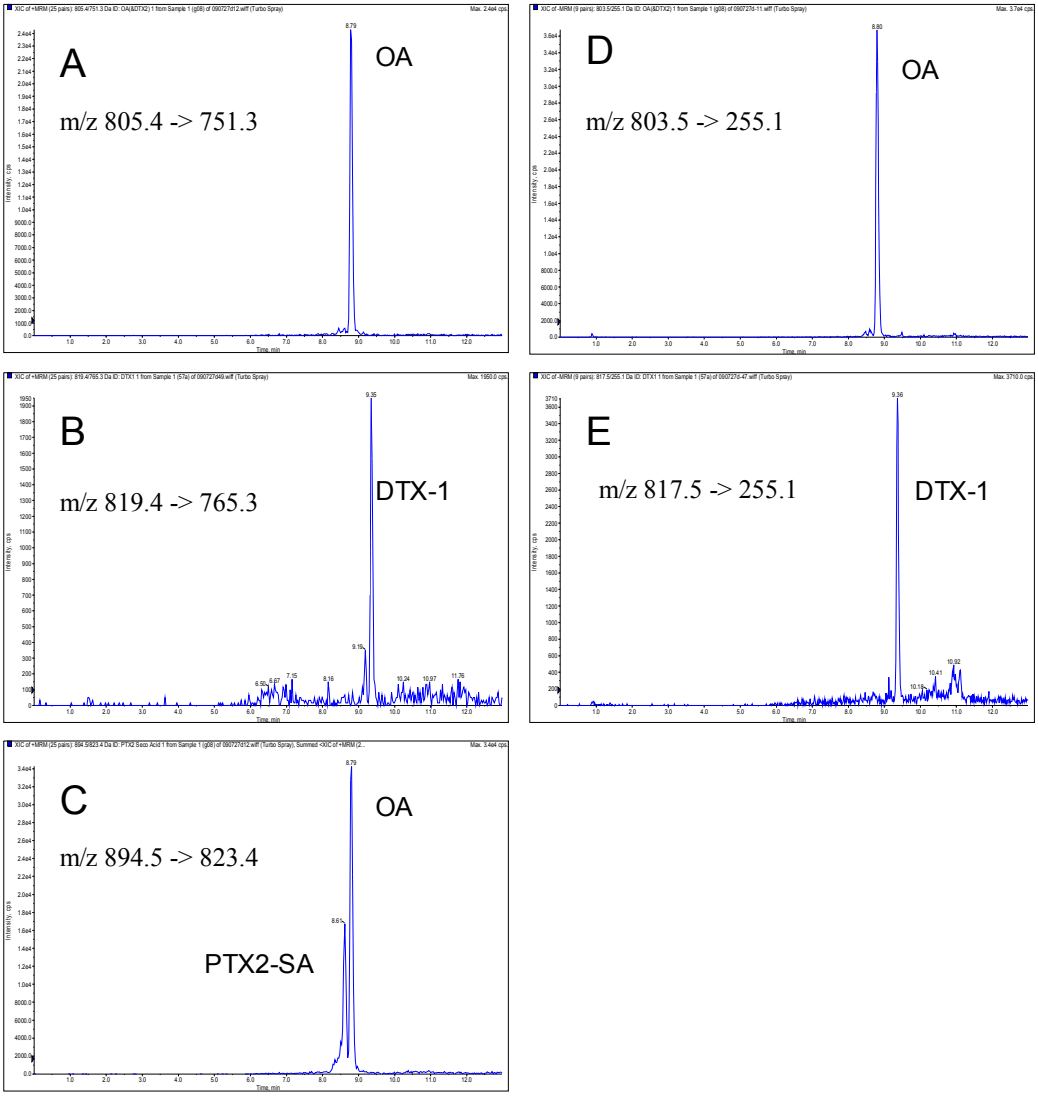

Figure 2.2. DST chromatograms of Tasmanian blue mussel (*M. edulis*) digestive glands. (A – C) Analyses performed in positive ionisation mode; (D – E) Analyses performed in negative ionisation mode. Conditions: flow rate of 0.8 mL/min, eluent A = 10 % acetonitrile /HPLC grade water, B = 90 % acetonitrile/ HPLC grade water, both containing 0.1 % formic acid and 2 mM in ammonium formate gradient elution starting at 5 % B for 5 min, ramped to 100 % B in 8 min, held for 5 min then to 5 % B in 1 min and equilibrated for 5 min. Scan range (m/z) for each analyte displayed in figures.

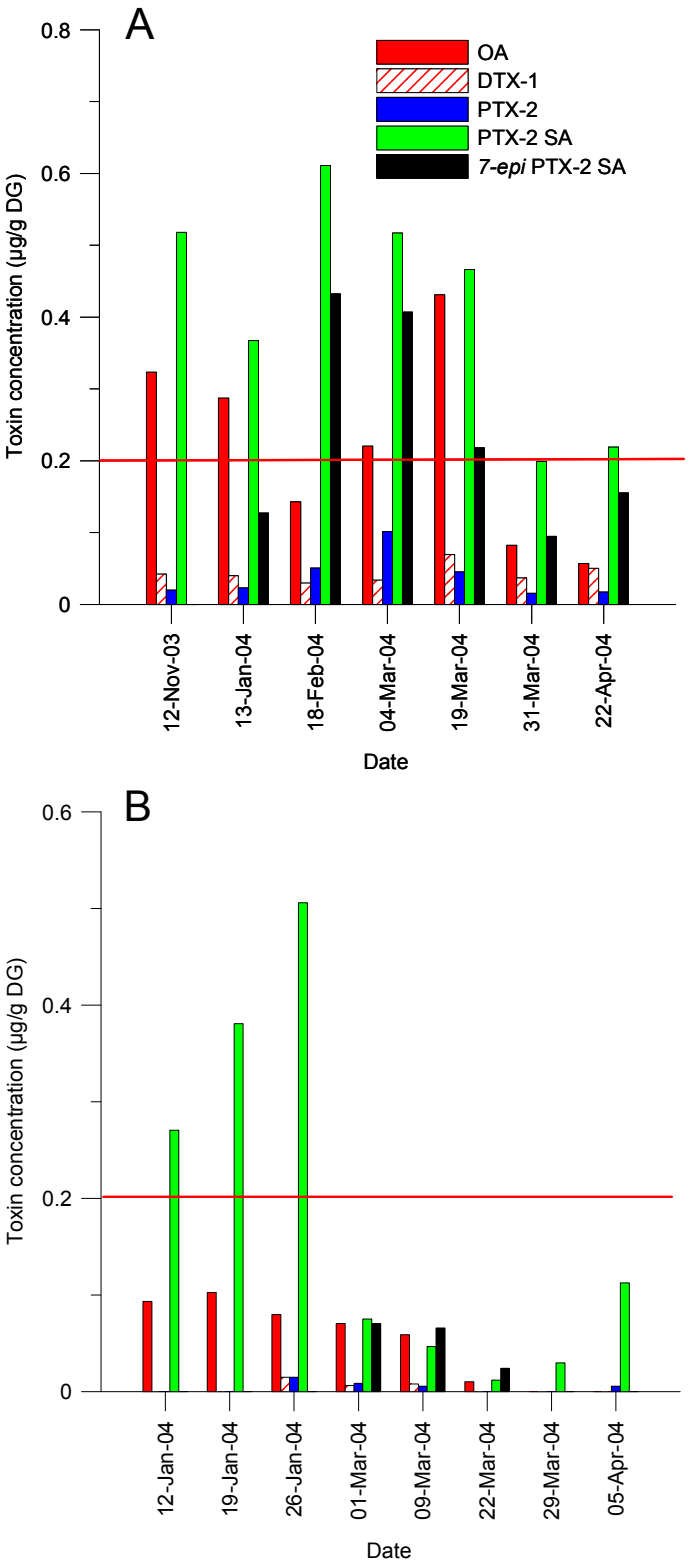

Figure 2.3. Toxin profiles of diarrhetic shellfish toxins in *Mytilus edulis* from (A) Sullivans Cove, and (B) Parsons Bay. DST quarantine level of 0.2 µg/g OA equivalents is indicated by the solid line.

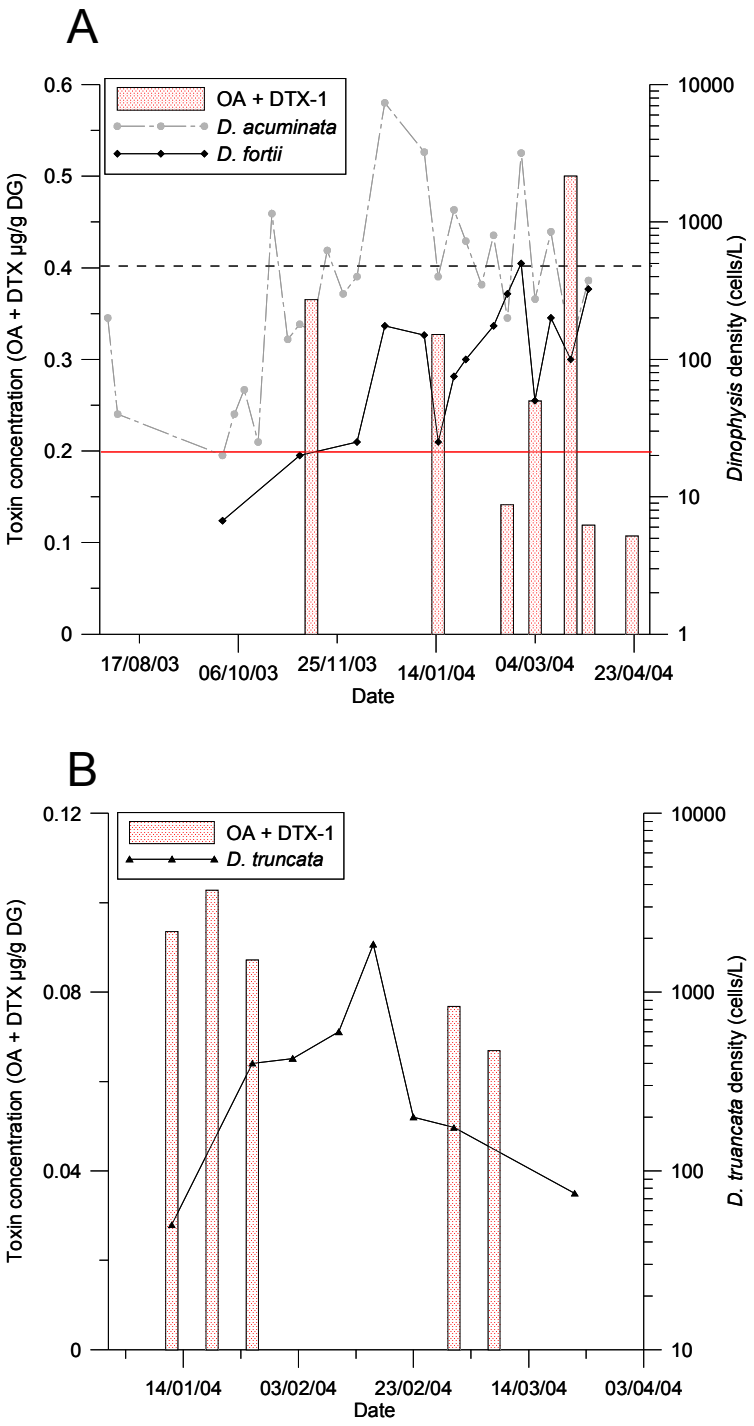

Figure 2.4. (A) Toxin levels of OA+DTX-1 in shellfish (µg/g DG) and cell densities of *D. acuminata* and *D. fortii*, at Sullivans Cove from August 1, 2003 to March 31, 2004. *Dinophysis* action level of 500 cells/L is indicated by the dashed line. DST quarantine level of 0.2 µg/DG is indicated by the solid line. (B) Levels of OA+DTX-1 and cell densities of *D. truncata* at Parsons Bay.

PTX, PTX derivatives, OA and DTX-1 were detected in phytoplankton net tow samples from Sullivans Cove throughout the spring to mid autumn period of 2005-06 when *D. acuminata* and *D. fortii* were the only known toxic *Dinophysis* species present in phytoplankton (Figure 2.5A). PTX and PTX derivatives were the predominant toxins up to 6 times greater than OA and DTX-1 (Figure 2.5, Table 2.3). In the first weeks of sampling *D. acuminata* was the only *Dinophysis* species detected until late September, 2005, when *D. fortii* began co-occurring with *D. acuminata* and DST levels displayed a small increase in the first week of co-occurrence. In mid March, 2006 to the end of April, 2006, *D. fortii* was the only *Dinophysis* detected and OA and DTX-1 concentrations were greater compared to periods where *D. acuminata* was the only *Dinophysis* species detected in. Table 2.3 highlights greater OA and DTX-1 levels in *D. fortii* compared to *D. acuminata*, although PTX-2 and their derivatives displayed similar levels for both species.

There did not appear to be any clear relationship between phytoplankton net tow DST levels and *Dinophysis* densities from depth-integrated samples.

2. 4. Discussion

2. 4. 1. Sullivans Cove mussel toxin content

Pectenotoxin-2 derivatives and okadaic acid were the main toxin compounds in *Mytilus edulis* during occurrences of *Dinophysis acuminata* (up to 7,380 cells/L) and *D. fortii* (up to 500 cells/L). Levels of OA and dinophysistoxin-1 in *M. edulis* were above quarantine level on November 12, 2003, January 13, March 4 and 19, 2004. *D. acuminata* and *D. fortii* have been widely confirmed to produce DST in Asian-Pacific and European waters (Yasumoto *et al.* 1980, Blanco *et al.* 1995, Draisci *et al.* 1996, Vale and Sampayo 2000, Uribe *et al.* 2001, Imai *et al.* 2003).

In the Derwent River these two species occurred at concentrations above the action level from October 23, 2003 to March 31, 2004. This compares to spring-summer *Dinophysis* blooms and diarrhetic shellfish toxicity in Japan (Yasumoto et al. 1978 and 1985), Spain and Portugal (Reguera *et al.* 1993, Martinez *et al.* 1993, Vale and Sampayo 2003), Italy (Draisci *et al.* 1996), France (Masselin *et al.* 1992, Belin

1993), Norway (Séchet *et al.* 1990), Chile (Lembeye *et al.* 1993, Uribe *et al.* 2001) and South Africa (Pitcher et al. 1993). A different seasonal toxicity occurred in Sweden, where DST levels were highest during the autumn and winter months, but this has been related to slow winter depuration of toxins (Svensson *et al.* 2000, Svensson 2003).

Toxin content of Tasmanian *M. edulis* was much lower than toxin levels of European *Mytilus* species that have measured as high as 6 to 50 times greater than the regulatory limit. However, different DST regulatory levels and analytical methods are practised among countries (Fernández *et al.* 2003), rendering precise comparisons difficult.

Dinophysis acuminata was the most abundant *Dinophysis* species for the duration of the study period and peaked in Austral summer. *Dinophysis fortii* blooms followed the *D. acuminata* peak on December 19, 2003 and both species were present until March 12, 2004. The toxin profile of *M. edulis* has been associated with *D. acuminata* blooms in Europe, Japan and New Zealand. *Dinophysis acuminata* is usually the abundant species in spring and summer in the Northern Adriatic Sea (Della Loggia *et al.* 1993) and Norway (Séchet *et al.* 1990, Maestrini 1998) followed by *D. fortii* in autumn (Della Loggia *et al.* 1993). However, the extent of the shift from *D. acuminata* to *D. fortii* in Tasmania was not as marked as that reported in the Northern Adriatic Sea, where monospecific *D. fortii* blooms tend to occur. When *D. fortii* was present in Tasmania, density was low and it always co-occurred with *D. acuminata*.

The decline in Tasmania of *D. acuminata* in mid-January to mid-February resulted in a reduction of *M. edulis* toxin content below quarantine level, when *D. acuminata* densities were still above 1,000 cells/L. The following rise in *D. acuminata* and *D. fortii* cells caused increased toxin levels in *M. edulis* which peaked in mid-March while both *Dinophysis* species were present.

Total toxin content in *M. edulis* may have been underestimated as esterified toxins were not quantified. The relationship between *Dinophysis* densities and mussel toxicity may become clearer with the inclusion of esterified toxins.

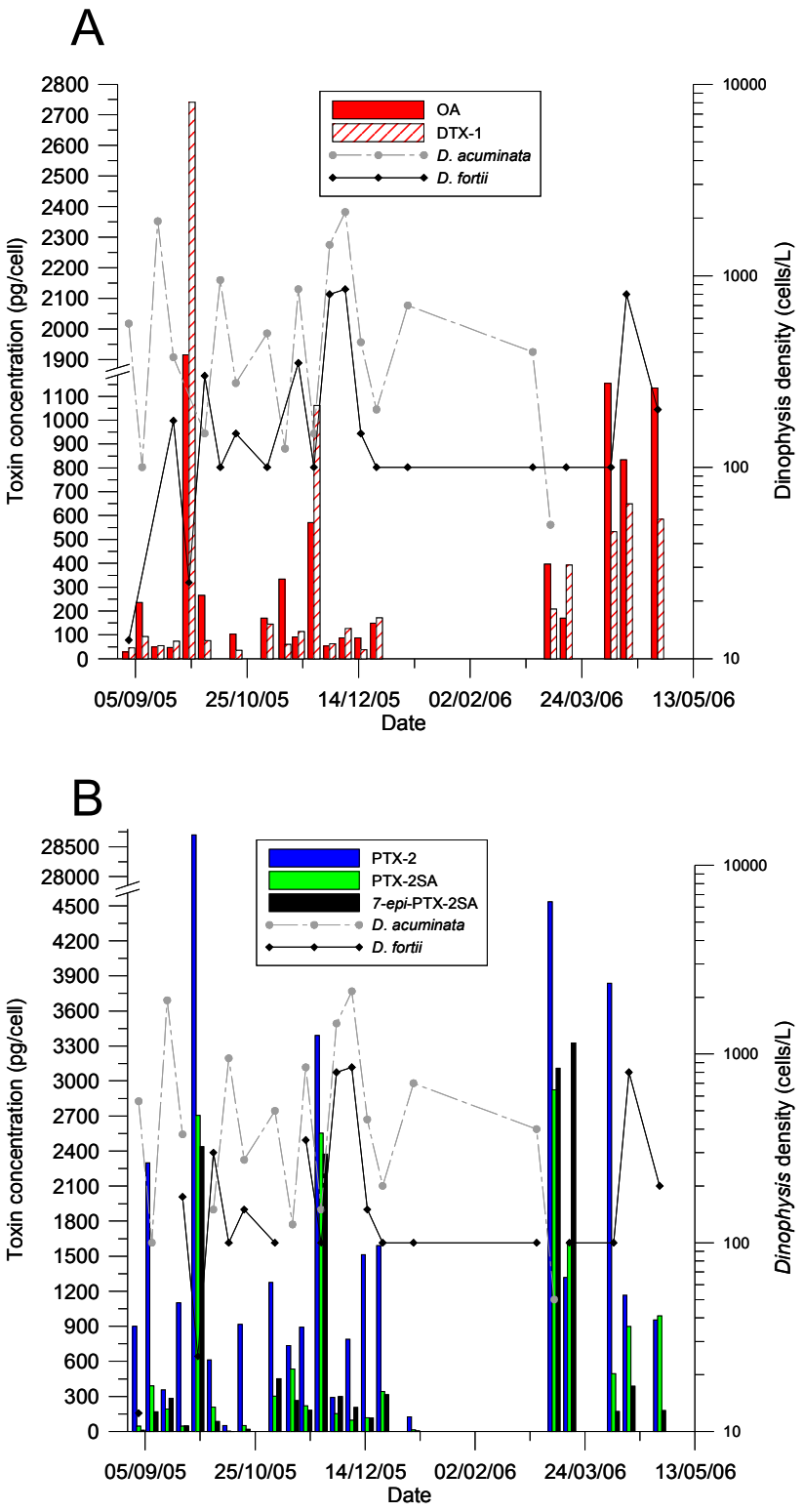

Figure 2.5. Toxin profiles of diarrhetic shellfish toxins (A) OA and DTX (B) and PTX's in phytoplankton samples (pg/cell), compared with cell densities of *Dinophysis acuminata* and *D. fortii* at Sullivans Cove.

Table 2.2. Monthly percentage of phytoplankton species from total cells in depth integrated samples during October 2005 to April, 2006 at Sullivans Cove.

	Oct	Nov	Dec	Jan	Feb*	March	April
Dinophysis sp.					No data		
D. acuminata	12.1	0.2	0.2	0.4		> 0.1	0
D. fortii	0.2	> 0.1	> 0.1	> 0.1		0	1.5
D. tripos	0	0	0	0		> 0.1	0
D. truncata	0	0	0	0		> 0.1	0
Total	**12.3**	**0.2**	**0.2**	**0.4**		**> 0.1**	**1.5**
***Protoperidinium* sp.**							
P. divergens	0	0	> 0.1	0		> 0.1	0
P. pellucidum	0	> 0.1	> 0.1	> 0.1		> 0.1	
Protoperidinium spp.	0.1	0	0	0		0	0.1
Total	**0.1**	**> 0.1**	**0.1**	**0.2**		**0.1**	**0.1**
Other dinoflagellates							
Alexandrium sp.	0	0	> 0.1	0.2		0	0
Ceratium sp.	2.4	0.5	0.5	0.4		0.1	13.4
Gymnodinium catenatum	> 0.1	> 0.1	> 0.1	0		> 0.1	0
Karenia umbella	0	0	0	0		> 0.1	0
Noctiluca scintillans	0	0	0	0		> 0.1	0
Prorocentrum gracile	0.2	> 0.1	> 0.1	> 0.1		> 0.1	1.4
Others	0.5	> 0.1	> 0.1			> 0.1	1.4
Total	**3.1**	**0.5**	**0.6**	**0.8**		**0.1**	**16.3**
Diatoms	**84.4**	**99.3**	**99.1**	**98.6**		**99.7**	**82.3**

* samples were not collected during February, 2006.

Table 2.3. Toxin levels of DST of Tasmanian *Dinophysis* species compared to other countries.

Species/ Location	Toxins pg/cell (ng/mL) ± standard error						Ratio	Reference (method)
	OA	**DTX-1**	**DTX-2**	**PTX-2**	**PTX-2SA**	**7-epi PTX-2SA**		
Australia (Tasmania)								
D. acuminata /D. fortii	75 ± 24 (32 ± 8)	88 ± 47 (37 ± 13)		558 ± 129 (254 ± 62)	172 ± 115 (58 ± 20)	171 ± 108 (74 ± 28)	OA:DTX1; 5:6 OA:PTX-2; 1:7	Current study (conc. plankton)
D. acuminata	127 ± 38 (15 ± 6)	52 ± 18 (11 ± 8)		991 ± 475 (104 ± 44)	505 ± 321 (53 ± 25)	478 ± 359 (64 ± 42)	OA:DTX1; 2:1 OA:PTX2; 1:7	Current study (conc. plankton)
D. fortii	411 ± 115 (87 ± 49)	270 ± 27 (62 ± 39)		909 ± 339 (140 ± 62)	501 ± 117 (96 ± 50)	508 ± 386 (59 ± 29)	OA:DTX1; 4:3 OA:PTX2; 2:5	Current study (conc. plankton)
D. acuminata								
Canada	25.5							Cembella 1989 (isolated cells)
Denmark	40							Andersen *et al.* 1996 (conc. plankton)
France	1.6							Lee *et al.* 1989 (isolated cells)
		158						Marcaillou *et al.* 2005
Japan		252						Suzuki *et al.* 1997 (conc. plankton)
		2.5 – 4.8		14.8				Kamiyama and Suzuki 2008 (culture)
New Zealand	1.2	2.4		25.8	1.7	27.5	OA:DTX1; 1:2 OA:PTX2; 1:21	MacKenzie *et al.* 2005 (conc. plankton)
Spain	37							Blanco *et al.* 1995 (conc. plankton)
Sweden	23.2	0.2						Johansson *et al.* 1996 (isolated cells)
USA	18 fg/cell	203 fg/cell						Hacket *et al.* 2009 (culture)

Table 2.3. cont.

Species/ Location	Toxins pg/cell (ng/mL) ± standard error						Ratio	Reference (method)
	OA	**DTX-1**	**DTX-2**	**PTX-2**	**PTX-2SA**	**7-epi PTX-2SA**		
D. cf. acuminata/D. sacculus								
France	0.6 – 29							Masselin *et al.* 1992 (isolated cells)
D. acuta								
New Zealand	2.7	0.1		107.5	5.8	113.3	OA:DTX1; 120:1 OA:PTX; 1:9	MacKenzie *et al.* 2005 (conc. plankton)
Ireland	58 – 85		78					Fernández-Puente *et al.* 2004
	9.5		6.6	31			OA:DTX2; 3:2 OA:PTX2; 1:3	Pizarro *et al.* 2009 (isolated cells)
	27		17	29			OA:DTX2; 5:3 OA:PTX2; 1:2	Pizarro *et al.* 2009 (plankton net)
	11 – 58		8 – 37	10 – 38			OA:DTX2; 3:2	Pizarro *et al.* 2008 (conc. plankton)
Portugal	15.6		10	48	0.5		OA:DTX2; 3:2 OA:PTX2; 1:3	Vale 2004 (conc. plankton)
Spain	0.6 – 94		0.5 – 169				OA:DTX2; 2:3	Blanco *et al.* 1995 (conc. plankton)
	9.4							Lee *et al.* 1989 (isolated cells)
Sweden	14	7						Johansson *et al.* 1996 (isolated cells)
D. fortii								
Japan		209						Suzuki and Mitsuya 2001 (conc. plankton)

Toxin levels of Tasmanian cells are averages calculated from several samples.

Mussel toxicity has been suggested to occur when *D. fortii* densities are as low as 200 cells/L (Yasumoto *et al.* 1985, Della Loggia *et al.* 1993), indicating this larger species (33 - 40 μm wide and 60 – 70 μm long) to be more toxic than *D. acuminata* (30 to 38 μm wide and 38 to 58 μm long). Toxin content of individual *Dinophysis* cells may be affected by environmental and physiological conditions such as light conditions, toxin storage area and availability of algae species which *Dinophysis* feed upon. Dynamics of accumulation and reduction in *M. edulis* toxicity is later discussed in Chapter 4.

2. 4. 2. Parsons Bay mussel toxin content

Toxin content was lower in *M. edulis* at Parsons Bay compared to mussels at Sullivans Cove, except for levels of PTX-2-SA which were similar at each site (0.50 and 0.61 μg/g DG, respectively). Toxin composition of *M. edulis* was also similar at each site, suggesting that toxins derived from the same biological source. Okadaic acid and PTX-2 derivatives (PTX-2-SA and *7-epi*-PTX-2-SA) were the main toxin compounds, 0.06 to 0.1 μg/g DG and 0.06 to 0.50 μg/g DG, respectively, compared to DTX-1 and PTX-2 (0.01 μg/g DG for each toxin). Total OA + DTX-1 concentrations were below quarantine levels during the study period. Toxin content was highest during mid-January 2004 when *Dinophysis* species were not detected, suggesting that *M. edulis* toxin content may have been the result of a bloom of toxic species immediately prior to the sampling period. Similarly, DST in mussels from Sweden (Haamer *et al.* 1990), Norway (Dahl and Ynderstad 1985, Underdal *et al.* 1985, Séchet *et al.* 2001), France (Belin 1993), Spain (Moroño *et al.* 2003) and from the Mediterranean coast of Italy (Boni *et al.* 1993, Della Loggia *et al.* 1993) have been shown to persist for several weeks or even months after the collapse of toxic *Dinophysis* blooms.

Only one *Dinophysis* species of proven toxicity, *Dinophysis fortii*, was detected in Parsons Bay at a density of 250 cells/L, and only in one sample (February 16, 2004). *D. truncata* was the dominant *Dinophysis* species in this location, and was present at densities ranging from 50 to 1,850 cells/L. This species has not been previously shown to produce DST. The presence of the Subantarctic species *D. truncata* (Burns and Mitchell 1982) matches the presence/absence of *D. truncata* in previous surveys conducted in 1992/3. Despite the absence of toxic *Dinophysis*

species, toxins were present in *Mytilus edulis* at low levels and continued to decrease with time, indicating *M. edulis* were naturally depurating at a rate of approximately 1 % per day over a 70-day period. *D. truncata* is unlikely to be the cause of low toxin levels in *M. edulis* as DST concentrations were decreasing over time, despite periods of increasingly high *D. truncata* densities. The similar levels of PTX-2-SA in Parsons Bay and Sullivans Cove *M. edulis* suggest that mussels were more efficient at retaining PTX-2-SA in the digestive gland while detoxifying or efficient at metabolising small amounts of PTX-2 to PTX-2-SA.

2. 4. 3. Sullivan Cove phytoplankton toxin content

This is the first known study in Australia to provide a comparative analysis of toxin profiles of the two most common and abundant toxic *Dinophysis* species, *D. fortii* and *D. acuminata*. Comprehensive DST profiles of individual *Dinophysis* species are increasingly possible now through the advancement of sensitive LC-MS technology and success in culturing (Park *et al.* 2006, Hacket *et al.* 2009). The estimated toxin levels per cell for *D. acuminata* and *D. fortii* in Tasmania, Australia, appear to suggest that the species are greater than 10-fold more toxic than the same species in other countries (Table 2.3). Only the mixed bloom of Tasmanian *D. acuminata*/*D. fortii* displayed *7-epi*-PTX-2-SA levels similar to levels of New Zealand *D. acuta* (Table 2.3). There appears to be a lack of quantitative pectenotoxin levels in the literature. PTX appears to be non-toxic to mildly toxic to humans (Hamano et al. 1985, Terao *et al.* 1993, Burgess and Shaw 2001); therefore the majority of DSP studies are only concerned with OA and DTX.

The high toxin content of *D. acuminata* and *D. fortii* in Tasmania compared to other locations (Table 2.3) may be due to differences in strains and physiological conditions. This may also explain the lack of relationship between phytoplankton DST levels and *Dinophysis* densities in Tasmania. Estimated toxin levels per cell for *D. acuminata* and *D. fortii* in Tasmania, Australia, appear 10-fold higher than for the same species in other countries (Table 2.3). Mixed blooms of Tasmanian *D. acuminata*/*D. fortii* displayed lower toxin levels compared to mono-specific *Dinophysis* blooms, except for DTX-1 which was greater in the mixed bloom compared to the *D. acuminata* bloom. Natural Tasmanian blooms dominated by *D.*

acuminata were estimated to contain 127 ± 38 pg OA/cell. This compares with culture estimates of 18 fg/cell (Hacket *et al.* 2009) ranging up to 40 pg/cell in Danish plankton concentrates (Andersen *et al.* 1996). Tasmanian blooms dominated by D. *fortii* were estimated to contain 411 ± 115 pg OA/cell and 270 ± 27 pg DTX-1/cell. This compares with plankton concentrate estimates of 209 pg DTX-1/cell in Japanese *D. fortii*, however, OA was not detected in their plankton sample (Suzuki and Mitsuya 2001). DST toxin profiles of Tasmanian were more consistent with literature values. Tasmanian OA:DTX-1 ratios of 2:1 were the inverse of the ratio of 2:1 in New Zealand (Mackenzie *et al.* 2005). OA:PTX-2 ratios of 2:5 in Tasmanian *D. fortii* and 1:9 in New Zealand *D. acuta* (but no DTX-2 present, Mackenzie *et al.* 2005) contain larger quantities of PTX-2 to OA. OA:PTX ratios of 1:3 in *D. acuta* from Ireland and Portugal (Pizarro *et al.* 2009, Vale 2004) are lower compared to New Zealand *D. acuta*.

Varying results may be due to differences in method of collection which may have an effect on toxin content in the cell. Collection methods of individual *Dinophysis* cells usually involve a concentration step as *Dinophysis* normally appear at low densities. Such concentration procedures may agitate and rupture cells causing a loss of toxins and producing underestimates of cellular toxin concentration. On the other hand, higher *Dinophysis* cellular toxin concentrations in phytoplankton concentrates compared to picked cells are likely to be due to toxin adhering to particulate matter (such as excreted in mussel faeces (Blanco *et al.* 1999)) or DST contained in heterotrophic phytoplankton such as *Protoperidinium* (Miles *et al.* 2004a) which was abundant in the study area. Pizarro *et al.* (2009) reported higher DST content per *Dinophysis* cell in plankton concentrates compared to picked cells, likely caused by extracellular toxins in the seston. Extra toxins associated with seston would require monitoring programs to measure seston toxin levels rather than *Dinophysis* abundance.

D. fortii OA and DTX-1 levels were up to 5 times greater than *D. acuminata* levels. Differences in cell volume between the two *Dinophysis* species undoubtedly contributed to the observed five-fold differences in toxin levels: the average cell volume of Tasmanian *D. fortii* was 50 μm^3 (range: 48 – 62 μm^3) and for *D. acuminata* this was 28 μm^3 (range: 8 – 32 μm^3). However, considering that the *D. acuminata*

average cell volume was only 44 % smaller than that of *D. fortii*, this could only account for a small proportion of the difference in toxins. As such it appears that some other factors such as differences in chloroplast density and/or prey feeding activity could have contributed to toxin levels. Cell volumes were estimated as described in Olenina *et al.* (2006). It has been suggested by Zhou and Fritz (1994) that toxins may be compartmentalised away from cytoplasmic phosphatases into regions not associated with protein phosphatases, such as chloroplasts. Larger cells have a greater area to store and produce more chloroplasts, hence have the ability to store more toxins. There were only a few occasions where only one of the target *Dinophysis* species was present at the sampling site and therefore more data is required to improve the accuracy of estimating average toxin levels of individual *Dinophysis* species.

Dinophysis cells contained a higher proportion of PTX-2 to PTX-2SA and 7-epi-PTX-2SA (Figure 2.5), whereas the reverse situation was observed in blue mussels (Figure 2.2). Similar toxin proportions in various *Dinophysis* species and shellfish have been observed by several authors (Suzuki *et al.* 2001a, b, MacKenzie *et al.* 2002, Vale and Sampayo 2002, Miles *et al.* 2004b) and indicate that shellfish convert PTX-2 to PTX-2SA and 7-epi-PTX-2SA. Potency of the pectenotoxin seco acids is significantly lower than PTX-2 (Daiguji *et al.* 1998), therefore the conversion of PTX-2 to pectenotoxin seco acids may be a defensive mechanism by the shellfish to reduce toxicity and at the same time is beneficial for human seafood safety.

2. 5. Conclusion

The present study established that two known diarrhetic shellfish toxin producing dinoflagellates, *Dinophysis acuminata* and *D. fortii*, occurred at high densities (up to 7,380 cells/L and up to 500 cells/L, respectively) at Sullivans Cove during the spring to early autumn seasons. These two species were the cause of diarrhetic shellfish toxin (DST) levels above the quarantine level of 0.20 µg OA + DTX-1/g DG in local (non commercial) blue mussels (*Mytilus edulis*) rendering shellfish unsafe for human consumption. Although there exist no commercial shellfish farms in the upper Derwent River, future surveys of commercial farms in the neighbouring

d'Entrecasteaux Channel are warranted. Toxin levels were considerably lower than toxic levels of European shellfish, however differences in regulation limits and analytical methods makes it difficult to compare toxicity on a global basis, thus an international standard is warranted to improve the trade regulations as they relate to biotoxins in exported shellfish.

By comparison, *D. truncata* was present at high densities (up to 1,850 cells/L) at Parsons Bay, and was considered to be non- or weakly toxic. No toxic blue mussels were present in from January 12 to April 5, 2004, and therefore these commercial mussels were safe for human consumption. A low level of DST (below 0.12 μg/g DG) was present in Parsons Bay *M. edulis* and was likely due to a previous bloom of a known toxic *Dinophysis* species. Toxin levels of Tasmanian *D. acuminata* and *D. fortii*, embedded in natural seston, appeared to be 10-fold greater than *Dinophysis* toxin levels in other regions.

The results indicate that DST in south-east Tasmania could pose a threat to public health and further understanding of factors that affect *Dinophysis* toxicity and refinement of reliable extraction and analytical DST methods are warranted to improve shellfish management programs.

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Chapter 3:

Morphotaxonomy and Genetic affinities of Tasmanian *D. fortii* and *D. acuminata*

Abstract

Diarrhetic Shellfish Poisoning (DSP) is a recognised problem world-wide that is associated with the occurrence of *Dinophysis* species. The cellular toxin content produced by *Dinophysis* is species – specific and varies according to strain and physiological condition. Accurate identification and knowledge of toxin profiles of *Dinophysis* species is important in establishing regulatory limits for each species and to identify levels of threat to which shellfish farms are exposed. Subtle variations among *Dinophysis* morphotypes pose problems for rapid and accurate identification, which is vital to DSP monitoring programs. The aim of this study was to determine ribosomal DNA sequences of the most common occurring toxic *Dinophysis* species in Tasmanian waters to define their phylogenetic relationships with other global populations. Tasmanian sequences of the D1-D3 region of the large subunit rDNA of *D. fortii* were indistinguishable from those of *D. fortii* from France and from *D. acuta* from the North Atlantic. Tasmanian *D. acuminata* was indistinguishable from European and New Zealand *D. acuminata*. Genetic sequencing of New Zealand *D. acuta* failed to discriminate between Tasmanian *D. fortii* and New Zealand *D. acuta* and neither did sequences discriminate between European *D. fortii* and *D. acuta.* Furthermore, European *D. acuta* is the only known *Dinophysis* to produce DTX-2. This toxin has never been detected in Australian and New Zealand waters in association with *D. acuta* or *D. fortii*.

3. 1. Introduction

Dinophysis species identification is primarily based on cell form and size, sulcal list form and length, shape and position of spines, presence or absence of chloroplasts, plate pattern and thecal ornamentation (Edvardsen et al. 2003, Hart et al. 2007). These characters can vary substantially during cell division and between populations and individuals. Classical morphology based taxonomy within the *Dinophysis* genus reveals highly variable morphotypes within species (Hart *et al.* 2007) which can produce problems in phytoplankton monitoring programs. For example, the morphologies of *D. acuminata*, *D. baltica*, *D. borealis*, *D. lachmanni*, *D. granii*, *D. skagi* and *D. subcircularis* were found to be overlapping and these taxa are now all

classified as *D. acuminata* (Rehnstam-Holm *et al.* 2002). Morphologies of *D. acuminata* and *D. sacculus* are also similar and may pose a problem for species identification (Edvardsen *et al.* 2003).

Molecular approaches have been developed to improve identification methods for toxic algae such as *Dinophysis* which can benefit monitoring programs. The nuclear rDNA contains regions with substantial differences in evolution rate which have been used to analyse higher level taxonomic relationships in algae. The large subunit (LSU) and small subunit (SSU) rDNA are highly conserved and also variable within the same molecule (Edvardsen *et al.* 2003). Conversely, highly divergent areas of the molecule are suitable for phylogenetic studies of closely related species. The conserved regions provide targets for universal sequencing primers for phylogeny at higher taxonomic levels. The internal transcribed spacer (ITS) 1 and ITS2 in contrast are useful at close phylogenetic (i.e. species and population) levels (Edvardsen *et al.* 2003).

The aim of this study was to determine rDNA sequences of common dominant toxic *Dinophysis* species (*D. acuminata, D. acuta* and *D. fortii*) in Tasmanian and New Zealand waters and to compare and determine their morphotaxonomic and phylogenetic relationship within the genus from known *Dinophysis* morphologies and sequences from other global locations.

3. 2. Material and Methods

3. 2. 1. Study Site and phytoplankton sample collection

Vertical plankton net hauls (mesh size 20 μm) to a depth of 8 m were used to collect and concentrate phytoplankton from the seawater medium. *D. acuminata* and *D. fortii* cells were collected from the south-eastern Tasmanian coast at Sullivans Cove (42º 53′ S 147º 20′ E) in the Derwent River estuary (see Figure 2.1) during September 2005 and at Marlborough Sound on the South Island of New Zealand during August 2004. *D. acuta* cells were collected at Menzies Bay, New Zealand on November 11, 2010. Sullivans Cove was selected because of the known seasonal occurrence of *Dinophysis* during spring and summer.

3. 2. 2. PCR amplification and cycle sequencing

Three individual *D. fortii* and three *D. acuminata* cells were isolated from Derwent River, Tasmania, Australia and three *D. acuminata* and three *D. acuta* cells isolated from Marlborough Sound and Menzies Bay, NZ, net tows into each of two 0.2 mL PCR tubes. These cells were used directly as a template to amplify approximately 900 bases of the D1-D3 regions of the large subunit ribosomal gene (LSU rDNA), using primers D1R and D3Ca (Scholin *et al*. 1994). Reaction conditions and cycle sequencing parameters are described in detail in de Salas *et al*. (2003).

3. 2. 3. Sequence alignment and phylogenetic analysis

Corrected sequences were aligned to other *Dinophysis* sequences (Table 3.1) using ClustalX (Thompson *et al.* 1997), and the resulting automatic alignment was refined manually. Phylogenetic analyses using minimum evolution (ME) and maximum parsimony (MP) search criteria were carried out using PAUP* 4.0b10 (Swofford 1999), and Bayesian analysis (likelihood) was carried out using MrBayes (Huelsenbeck & Ronquist 2001), both as described in de Salas *et al*. (2004).

3. 3. Results

3. 3. 1. Morphology of *Dinophysis* species

Dinophysis cells are frequently flattened laterally with a small epitheca and large hypotheca. The distinguishable features of *Dinophysis* are thecal projections attached to sails, which border the top of the cell, and the lists of variable length along the hypotheca (Balech 1976). Species diagnostics include size (Table 3.1) and shape of cells and sulcal lists. *D. fortii* is best distinguished by appearing to be broad with rounded antapices (Figures 3.1A – R). *D. acuta* is similar in size to *D. fortii* (Table 3.1) and is best identified by prominent concavities on both the dorsal and ventral posterior margins of the hypotheca (MacKenzie *et al*. 2005) and being widest below the middle (Figures 3.1S – T). *D. acuminata* is distinguished from *D. fortii*, *D.*

norvegica and *D. acuta* by its small size (Table 3.1) and usually regular oval cell form (Figure 3.2A – D) with some cells displaying minute bottom protrusions on the hypotheca (Figure 3.2A, C). *D. norvegica* overlaps in size with *D. acuminata* and *D. acuta*. *D. norvegica* and *D. acuminata* both have protrusions on the hypotheca, however *D. norvegica* has a more pointed hypocone which tapers to a triangular shape and a straight to convex ventral side on the posterior side. *D. norvegica* differs from *D. acuta* by widest near the middle (Edvardsen *et al*. 2003). *D. caudata*, *D. tripos* and *D. odiosa* are large species possessing antapical spines. *D. caudata* is irregularly subovate with long ventral hypotheca projections and is widest at the base of the sulcal list (Figure 3.2F). *D. tripos* is anterior-posteriorly elongated with distinctive long antapical projections and short dorsal projections (Figures 3.2G – H). *D. odiosa* is round with extended triangular projection or spine on the hypotheca.

Table 3.1. Size range of *Dinophysis* species.

***Dinophysis* species**	**Size range**		**References**
	Length (μm)	Width (μm)	
D. acuminata	38 – 58	30 – 38	Balech 1976
D. acuta	65 – 75	47 – 55	Balech 1976
D. caudata	70 – 110	37 – 50	Hallegraeff and Lucas 1988
D. fortii	60 – 70	35 – 40	Burns and Mitchell 1982; Hallegraeff and Lucas 1988
D. norvegica	57 – 71	39 – 53	Balech 1976
D. odiosa	63 – 65	50 – 53	Balech 1976
D. tripos	90 – 120	60	Burns and Mitchell 1982
D. truncata	61 – 70	37 – 49	Burns and Mitchell 1982

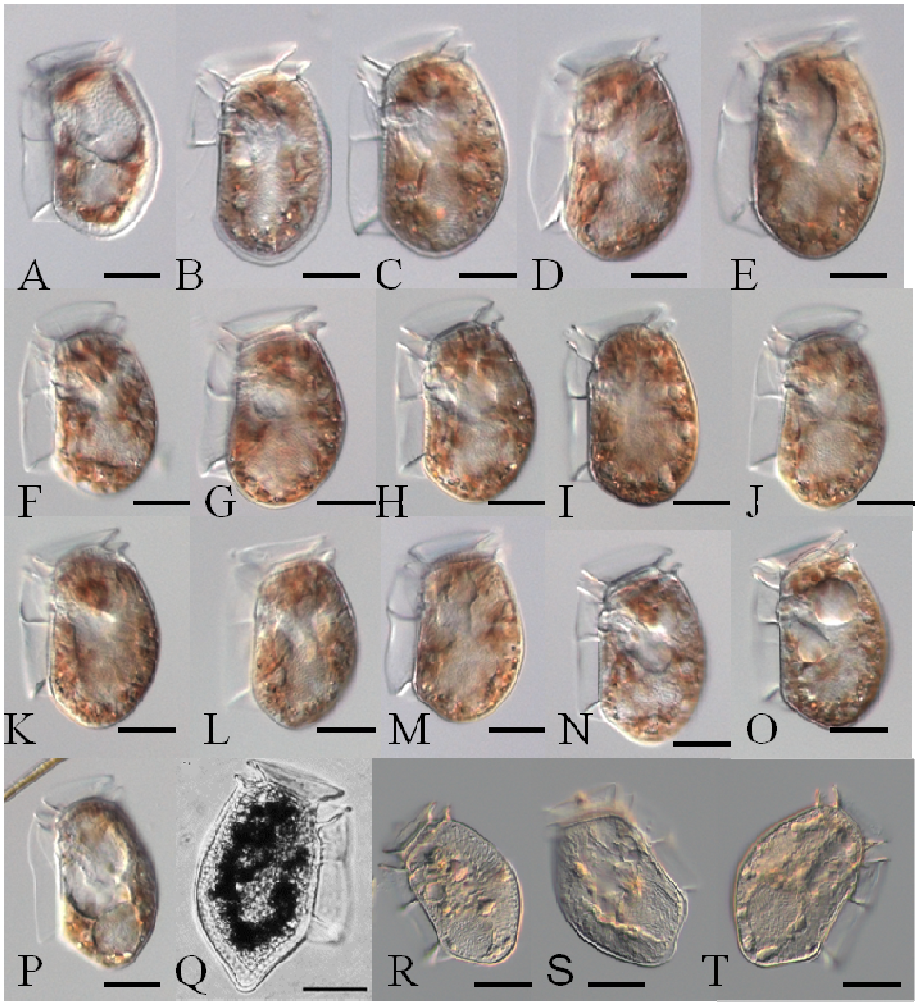

Figure 3.1. (A – R) Variations in *D. fortii* morphotypes from Tasmania, Australia. Images (A – P) taken by Miguel de Salas. Figure M - P display a slight concavity on the ventral posterior margin. Figure A - N represents the more typical subovoid shaped *D. fortii*; (Q) Typical *D. acuta* from Baltimore, Ireland (image from MacKenzie *et al.* 2005); (R – T) *D. acuta* from Menzies Bay, New Zealand. Scale bar = 25 μm.

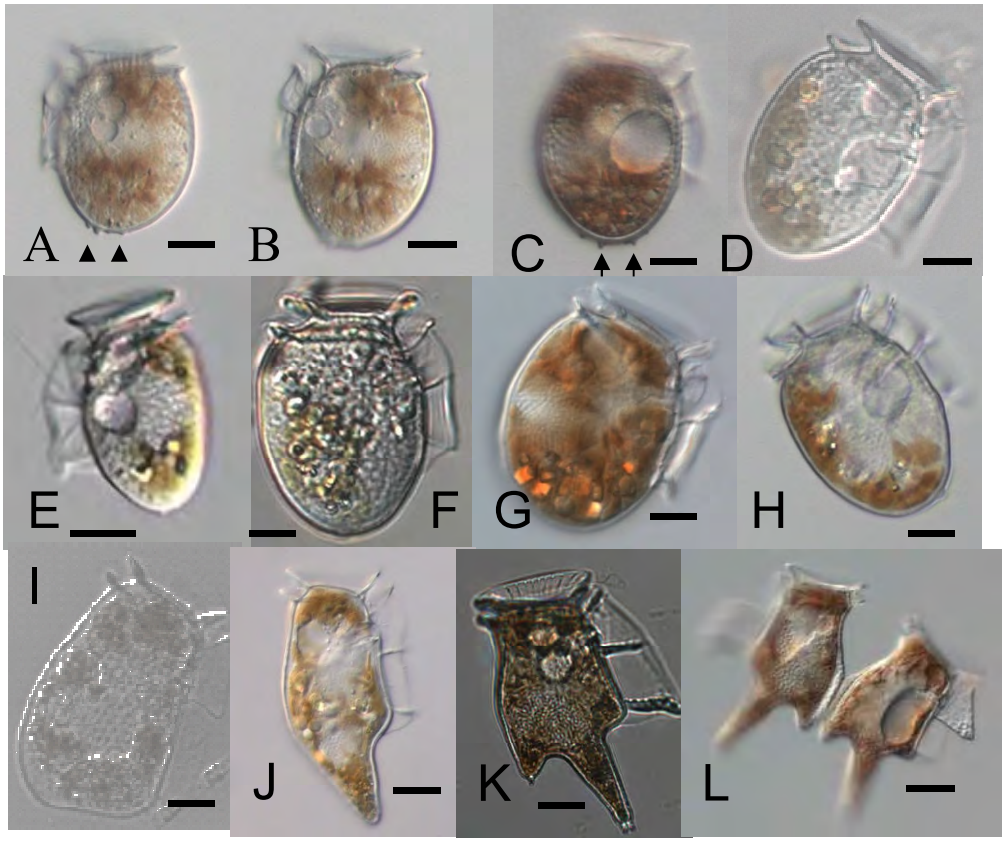

Figure 3.2. *Dinophysis* species from Tasmanian, Australia; (A – H) *D. acuminata*. Figure A and C display morphotype with minute bottom protrusions (arrows); (I) *D. truncata*; (J) *D. caudata*; (K – L) *D. tripos*. Scale bars: A – H = 12 µm; I = 15 µm, J – L = 25 µm. Images A – C taken by Miguel de Salas.

3. 3. 2. Sequencing of the LSU gene

Direct PCR amplification of the D1-D3 regions of the 28S ribosomal DNA gene of *Dinophysis* was successful using the primers D1R and D3C (Scholin *et al*. 1994). Phylogenetic analyses using maximum parsimony (MP), minimum evolution (ME) and maximum likelihood all agreed in placing Tasmanian *D. acuminata* with other isolates of this species from diverse geographic locations (Figure 3.3), with high support values. The clade containing *D. acuminata* consists of the smaller *Dinophysis* species *D. dens*, *D. norvegica* and *D. sacculus*. Tasmanian *D. fortii*, New Zealand *D. acuta*, Mediterranean *D. fortii* and northern European *D. acuta* all clustered into a single clade, and could not be differentiated into species based on their partial LSU sequences. The final clade consisted of larger *Dinophysis* species *D. caudata*, *D. tripos* and *D. odiosa* which all possess characteristic antapical spines.

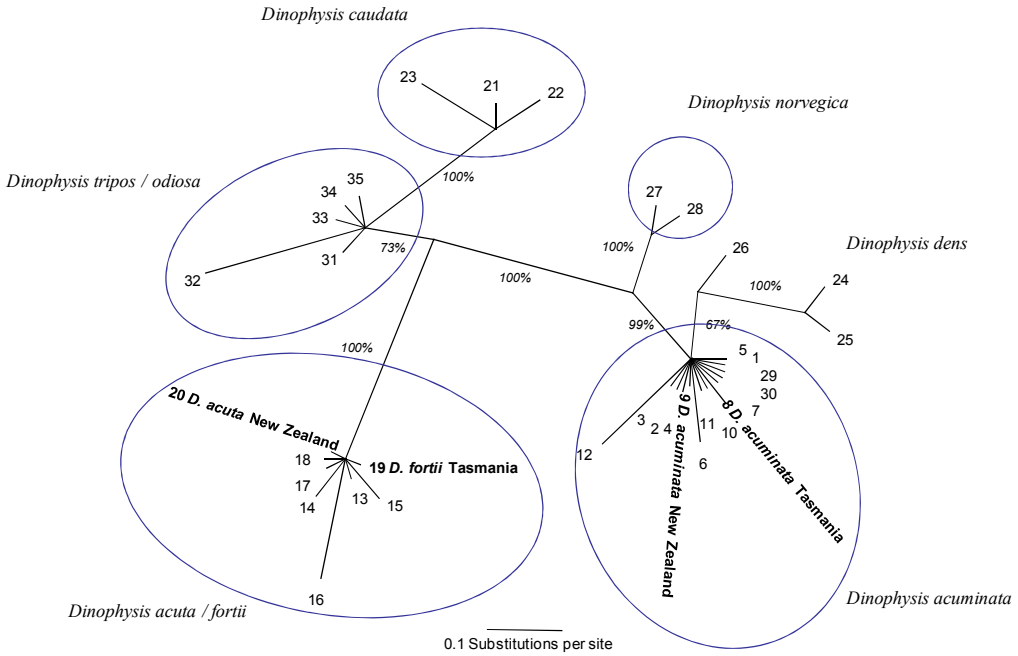

Figure 3.3. Bayesian unrooted phylogeny of *Dinophysis* species. The analysis consisted of 500,000 generations in two simultaneous runs of four chains each. The tree was sampled every 100 generations to produce 5,000 trees. The first 4,000 trees of each run were discarded as a burning, and a 50% majority rule consensus tree drawn with the remaining 2,000 trees (100 from each run). Compare with Table 3.2 for geographic origin of GenBank sequences used.

3. 4. Discussion

Partial 28S rDNA gene sequences of Tasmanian *D. fortii* were indistinguishable from those of *D. fortii* from France and from *D. acuta* from the North Atlantic. Genetic sequences of New Zealand *D. acuta* were also indistinguishable from Tasmanian *D. fortii* and from European *D. fortii* and *D. acuta*. However, *D. fortii* from Tasmania and Japan appear to be morphologically distinct from what is referred to as *D. acuta* in New Zealand (Figure 3.1T). The Tasmanian and Japanese *D. fortii* are similar in size to the New Zealand *D. acuta* but *D. fortii* tends to be broader with more rounded antapices. A full scanning microscopic characterisation of Tasmanian and New Zealand *Dinophysis* has been previously provided by Hallegraeff and Lucas (1988) and Burns and Mitchell (1982), respectively. The New Zealand *D. acuta* resembles Tasmanian and Japanese *D. fortii* though to a greater or lesser extent they display the *D. acuta* characteristics of prominent concavities on the dorsal and ventral posterior margins of the hypotheca (MacKenzie *et al*. 2005). It seems likely that these local

species designations have been confounded and in fact refer to the same taxon *D. fortii*.

Table 3.2. Sequenced LSU rDNA of *Dinophysis* species from world-wide locations.

N°	Species	Isolate	Origin	GenBank accesion Number
1	*D. acuminata*	DacmF5-17	Norway	AJ506976
2	*D. acuminata*	-	France	AY040576
3	*D. acuminata*	-	Scotland	AY040574
4	*D. acuminata*	36/3	France	AF318244
5	*D. acuminata*	MH1	Scotland	AY259228
6	*D. acuminata*	SC12	Scotland	AY259229
7	*D. acuminata*	SC9	Scotland	AY259231
8	***D. acuminata***	**DADE01**	**Tasmania**	**to be submitted**
9	***D. acuminata***		**Nelson, N.Z.**	**to be submitted**
10	*D. sacculus*	-	Corsica	AF318242
11	*D. sacculus*	-	France	AY040582
12	* *D. acuta*	-	Portugal	AY040569
13	*D. acuta*	DactF8-12	Norway	AJ506977
14	*D. acuta*	IC3T	Ireland	AY277641
15	*D. acuta*	LC114	Scotland	AY259233
16	*D. acuta*	SC179	Scotland	AY277644
17	*D. fortii*	12_4/4	South Africa	AF318237
18	*D. fortii*	23/4	France	AF318236
19	***D. fortii***	**DFDE01**	**Tasmania**	**to be submitted**
20	***D. acuta***	**-**	**New Zealand**	**to be submitted**
21	*D. caudata*	-	France	AY040584
22	*D. caudata*	24/1	France	AF318240
23	*D. caudata*	8/1	Corsica	AF318241
24	*D. cf. dens*	-	France	AF318239
25	*D. dens*	-	France	AY040572
26	*D. dens*	-	Portugal	AY040571
27	*D. norvegica*	-	Denmark?	AY571375
28	*D. norvegica*	DnorF8-4	Norway	AJ506985
29	*D. norvegica*	LC18	Scotland	AY259238
30	*D. norvegica*	LC24	Scotland	AY259239
31	*D. odiosa*	SC427	Scotland	AY259241
32	*D. odiosa*	SC430	Scotland	AY277651
33	*D. tripos*	-	France	AY040585
34	*D. tripos*	-	France	AF318238
35	*D. tripos*	SC359	Scotland	AY259242

* Putative misidentification of *D. acuminata*.

Further support for this is provided by similarity of toxin profiles of New Zealand *D. acuta* with Tasmanian *D. fortii* as distinct from European *D. acuta*. Lassus *et al.* (1998) noted similar differences between European and Chilean *D. acuta*

morphologies but concluded that the difference was insufficient to designate them as separate species.

The cellular toxin content produced by *Dinophysis* is species – specific and varies according to strain and physiological condition (Moroño *et al.* 2003) and therefore accurate identification and knowledge of toxin profiles of *Dinophysis* species is important in establishing regulatory limits for each species and to identify the level of threat to which shellfish farms are exposed to toxic *Dinophysis* species. The New Zealand *D. acuta* is not known to produce DTX-2, a toxin which thus far has only been characterised from *D. acuta* in Europe (Blanco *et al.* 1995, Carmody *et al.* 1996, Vale and Sampayo 2000), suggesting that the New Zealand species may not be *D. acuta* or that the toxins and ratio of toxins produced may vary according to strains and physiological conditions (Moroño *et al.* 2003).

Previous problems with morphologies among several *Dinophysis* have resulted in the species being grouped. For example, morphological variations of *D. acuminata*, *D. baltica*, *D. borealis*, *D. lachmanni*, *D. granii*, *D. skagi* and *D. subcircularis* ultimately led to them being grouped into the one species, *D. acuminata*. Morphologies of *D. acuminata* and *D. sacculus* are also very similar and may pose a problem for species identification. LSU rDNA sequences of *D. acuminata* isolated from Norway, France and South Africa are identical to *D. sacculus* from the Mediterranean (Edvardsen *et al.* 2003). This suggests that these two species may be synonymous. Tasmanian *D. acuminata* is genetically and morphologically indistinguishable from European and New Zealand *D. acuminata*. Tasmanian *D. truncata* also appears morphologically similar to *D. fortii* and New Zealand *D. acuta*. It is of similar size, broad like *D. fortii* but is morphologically distinct by having a flattened antapex (Figure 3.2I). Unfortunately molecular sequences for *D. truncata* are not yet available. The indistinguishable rDNA and slight differences in morphologies of *Dinophysis* species may be due to a recent divergence, low rate of evolution within the group/clade, highly conserved rDNA regions and/or a low level hybridisation through sexual coupling between species which generates intermediate/variable morphologies.

3. 5. Conclusion

Tasmanian *D. fortii* and New Zealand *D. acuta* sequences of the 28S rDNA gene were indistinguishable and also were identical to those of *D. fortii* from France and *D. acuta* from the North Atlantic. New Zealand *D. acuta* strongly resembles Tasmanian and Japanese *D. fortii* morphologically, suggesting that local species designations have been confounded and it seems likely that these two dinoflagellates belong to the same taxon. The ratio and content of toxins produced by *D. acuta* in New Zealand appear to be similar to Tasmanian *D. fortii*, lending further support that these species designations have been confounded. Tasmanian *D. acuminata* is genetically and morphologically indistinguishable from European and New Zealand *D. acuminata*. Due to subtle variations among *Dinophysis* morphotypes accurate identification and knowledge of toxin profiles of *Dinophysis* species is important in establishing regulatory limits for each species and to identify levels of threat to which shellfish farms are exposed to toxic *Dinophysis* species.

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Chapter 4:

Diarrhetic Shellfish Toxin accumulation and depuration in Tasmanian mussels (*Mytilus edulis*)

Abstract

Knowledge of depuration rates of shellfish contaminated with Diarrhetic Shellfish Toxins (DST) is important for estimating safe harvest periods and managing the impact on the shellfish industry. During the occurrence of *Dinophysis acuminata* and *D. fortii* from February 18 to April 22, 2004, in the Derwent River, Tasmania, a field depuration experiment was conducted by placing contaminated blue mussels (*Mytilus edulis*) in 38 μm mesh cages to screen out *Dinophysis* plankton cells. DST quantities from mussels were analysed by high performance liquid chromatography – mass spectrometry (HPLC-MS/MS). Mussels displayed biphasic depuration kinetics with a faster rate of PTX loss over the first 30 days followed by an increase of OA + DTX-1 depuration once there was no further change in PTX levels. The slow rate of depuration of OA + DTX-1 from day 15 to 30 followed by and increase in depuration may be attributed to mussels using lipid storage during a period of reduced food availability leading to a release of toxins in bound fractions.

4. 1. Introduction

Toxin accumulation and depuration may vary among shellfish species (Lindegarth et al. (2009), Vale (2004), Suzuki and Mitsuya (2001), Svensson et al. (2000)). Shellfish quality assurance monitoring programs in Australia indicate that toxin accumulation is greater in mussels compared to oysters and scallops when exposed to similar or lower densities of phytoplankton DST producers (Table 1.2 and 1.3 in Chapter 1). Such differences may be due to differing shellfish habitat preferences and filter feeding behaviour. Mussels tend to attach to substrates throughout the water column whereas scallops,and cockles inhabit the seabed. These habitat differences also suggest that different shellfish may be exposed to different phytoplankton communities. For example shellfish species such as scallops which bury themselves in the sand may be less likely to be exposed to planktonic dinoflagellates such as *Dinophysis* but can be exposed to benthic toxic algae such as *P. lima*. Toxin accumulation appears to be greater in mussels than oysters, even when grown under comparable intertidal (racks) or pelagic (long-line) habitats. There is no known procedure to stimulate depuration of toxic mussels. Most commonly this is left to occur naturally but the time required

may vary depending on the temperature-dependant metabolism of shellfish and the availability of non-toxic phytoplankton food (Haamer *et al.* 1990, Marcaillou-Le Baut *et al.* 1993, Haamer 1995, Blanco *et al.* 1999, Svensson 2003).

Understanding bivalve toxin accumulation and depuration of DST can be of great advantage to shellfish farms in establishing species specific harvest and closure in relation to regulatory levels of DST producing phytoplankton. Interspecific differences in levels of Paralytic Shellfish Toxins in shellfish are well known and some marine farms such as in Maine, practice species-specific harvest and closures of shellfish fisheries (Svensson *et al.* 2000). This study aims to assess the depuration rate of mussels (*M. edulis*) when toxic *Dinophysis* were removed from their diet in natural field conditions. Depuration rates are important in predicting the length of closure periods in shellfish farms.

4. 2. Methods

4. 2. 1. Field Depuration experiment

Availability of *Dinophysis* as a food source for *Mytilus edulis* growing underneath Princess Wharf in Sullivans Cove, Hobart, Tasmania, was reduced by removing mussels from the pylons and placing them inside five 400 x 400 x 300 mm PVC cages (90 mussels per cage) covered with a 38 μm nylon mesh (Figure 4.1). This mesh size excludes all *Dinophysis fortii* (56 – 83 μm long, 43 – 58 μm wide) cells and the majority of *Dinophysis acuminata* (35 – 58 μm long, 30 – 40 μm wide) cells. PVC fittings were glued together and 10 mm holes were drilled in the corners to allow the PVC pipes to fill with water and sink below the water surface. Inside the cage were two ropes surrounded by a 10 x 10 mm garden mesh in which the *M. edulis* were placed. Cages were attached to a 3 m rope (14 mm diameter) tied to 'D-shackles' on a chain suspended between two pylons underneath the edge of Princess Wharf. Cages were suspended in the upper 3 m depending on tides, approximately 1 m apart. Initial samples from the pylons were collected on February 18, 2004 (day 0) when *M. edulis* were placed in the cages. *Mytilus edulis* depurated in the cages for 15, 30, 43 and 60 days from February 18 to April 22, 2004. The water temperature during this period

ranged from 16 – 18 ºC. Fifteen *M. edulis* were collected per treatment at each time and placed in labelled plastic zip-lock bags and stored frozen. Mussels were sampled from the pylons on the same day when cages were sampled (control). A tear along the seam of the nylon mesh was observed on cage four when cages were sampled on day 30; therefore samples of cage four collected on day 30, 43 and 60 were omitted from the analysis.

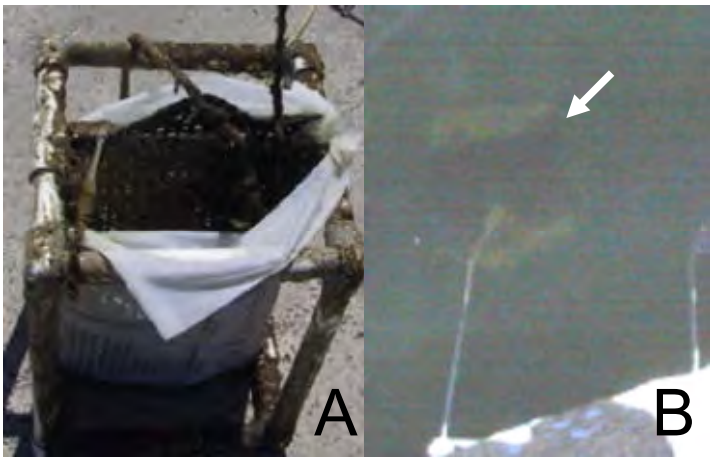

Figure 4.1. Depuration experiment. (A). Cage frame made of PVC (400 x 300 x 300 mm), the core is enclosed in a 10 x 10 mm garden mesh to hold the blue mussels (*Mytilus edulis*). The garden mesh was again enclosed with 38 µm nylon mesh. (B). Cages in the water suspended underneath the edge of Princess Wharf (arrows).

4. 2. 2. Toxin extraction from *Mytilus edulis*

Mussels were thawed, the soft tissue removed from the shell and the digestive glands dissected. Digestive glands were weighed and placed into a 50 mL sample container. Digestive glands were homogenised using a commercial hand-held blender at setting 1 (AFK stabmixer or Braun multiquick). Approximately 4 g of homogenate was weighed into 50 mL plastic sample containers and homogenised with 16 mL of 90 % methanol. Samples were centrifuged for 10 min at 652 g. The final extract was filtered through an Acrodisc® syringe 0.2 µm Supor® membrane filter into vials and stored in a freezer until analysed by HPLC-MS/MS at Queensland Health Scientific Services.

4. 2. 3. HPLC-MS/MS analysis of toxins

Diarrhetic shellfish toxins in mussels were analysed using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord. On, Canada) equipped with a turbo-ionspray (high flow electrospray) interface coupled to a Shimadzu LC-10ADvp HPLC (Shimadzu Corp., Kyoto, Japan). Separation was achieved using a 150 x 4.6 mm Alltima C_{18} column (Alltech (Aust.), Baulkam Hills, NSW) run at 35 ^{o}C, and using a flow rate of 0.8 mL/min with a linear gradient starting at 5 % B for 5 min, ramped up to 100 % B in 8 min, held for 5 min then to 5 % B in 1 min and equilibrated for 5 min (A = 10 % acetonitrile /HPLC grade water, B = 90 % acetonitrile/ HPLC grade water, both containing 0.1 % formic acid and 2 mM in ammonium formate).

The mass spectrometer was operated in both positive and negative ion modes. Analytes were confirmed (normally using the single ion monitoring mode, SIM) by characteristic ions and retention times (min) as described in Table 4.1.

Table 4.1. Mass spectrometer operating parameters.

Toxin	SIM	m/z	Retention time (min)
PTX-2	positive mode	876.6	14.62
PTX-2 SA	positive mode	894.6	13.73
7-epi PTX-2 SA	positive mode	899.6	14.15
OA	negative mode	803.5	11.90
DTX-1	negative mode	817.5	13.40
DTX-2	negative mode	803.5	12.28

Quantitation was achieved by comparing areas of peaks from samples to appropriate standards using the external standard method with a relative standard deviation (precision) of 3.3 – 12.7 % (Appendix Table A.1). Certified standards of pectenotoxin-2 and okadaic acid were obtained from Institute of Marine Biosciences, National Research Council, Canada. Pectenotoxin-2 seco acid concentrations were determined by comparison to the PTX-2 standard and assuming a similar response factor. Dinophysistoxin-1 and DTX-2 concentrations were determined by comparison to the OA standard and assuming a similar response factor.

4. 2. 4. Phytoplankton collection and enumeration

Samples for *Dinophysis* species identification and enumeration were collected with a depth-integrated sampling tube (hose diameter 24 mm) and vertical plankton net hauls (mouth diameter 215 mm, length 1000 mm, cod end diameter 50 mm, mesh size 20 μm), respectively. The depth-integrated sample tube (7 m) was passed into the water column (0 to 6 m) holding onto one end. It was then retrieved by a string attached to the other end of the tube so that both ends were positioned above the surface and the water trapped in the hose. Water was collected into a 1 L polyethylene bottle and fixed with Lugol's solution.

In the laboratory one litre of the depth-integrated water sample was transferred to a measuring cylinder and fixed with Lugol's iodine solution, and covered with aluminium foil. The preserved sample was left to stand for 12 to 24 hrs. After this time a subsample of 100 mL from the bottom of the cylinder was taken via a 2 x 50 mL pipette and placed into two 50 mL sample containers. Two mL from each sample jar was taken via pipette into a 50 mm petri dish. The species of *Dinophysis* cells in the petri dish were then counted under a Zeiss Axiovert25 inverted microscope.

4. 2. 5. Statistical Analyses

All statistical analyses were conducted using the SAS Windows© 6.12 software package. The field depuration experiment was analysed by a 1-way model-1 Analysis of Co-variance (ANCOVA), where cage was the covariant and time was a fixed factor. The 1-way ANCOVA saturated model was examined first to test for homogeneity of slope. The interaction between cage*days was insignificant, therefore the unsaturated model was used. Residuals were examined using normal probability plots to validate assumptions of ANCOVA and ANOVA models. If ANOVA and ANCOVA analyses were significant, a Ryan-Einot-Gabriel-Welsch (REGWQ) was conducted to test which groups were significantly different from other groups. REGWQ group results are shown in Apendix Table A.2.

4. 3. Results

4. 3. 1. OA + DTX-1

The saturated 1-way model I ANCOVA showed that there was no significant cage*days interaction effect on *Mytilus edulis* toxicity ($F_{(3,27)}$ = 2.11; P = 0.1223) from the raw data (Table 4.2), therefore the unsaturated model was conducted with the cage*days interaction omitted from the model. The results indicated an insignificant effect of cage and days on *Mytilus edulis* toxicity. Thus, toxicity of caged *M. edulis* were pooled and tested by a 1-way ANOVA against non-caged *M. edulis* (control).

Toxin content of caged *M. edulis* was significantly different compared to non-caged *M. edulis* (ANOVA Ryan-Einot-Gabriel-Welsch (REGWQ) groupings; $F_{(19,16)}$ = 258; P > F = <0.0001) which were exposed to *Dinophysis* and which showed a toxicity (OA + DTX-1) increase during the first 30 days (Figure 4.2A). REGWQ groupings (Appendix Table A.2) showed that OA + DTX-1 toxicity of caged *M. edulis* were similar on days 15 and 30 (0.12 µg/g DG) and at days 43 and 65 (0.04 µg/g DG) of containment and significantly different from non-caged (control) *M. edulis* which significantly increased with time. Non-caged mussels increased by 50 % (0.17 to 0.25 µg/g DG) over the first 15 days followed by a 97 % increase from day 15 to day 30 (0.25 to 0.5 µg/g DG). Day 30 to 43 displayed a 76 % decrease in toxicity (0.5 to 0.12 µg/g DG) after which no further change was observed (Figure 4.2A). Caged *M. edulis* depurated at a rate of 2 % per day over the first 15 days (0.17 to 0.12 µg/g DG), no further detoxification occurred from day 15 to 30, and this was followed by a further detoxification rate of 5 % per day from day 30 to 43 (0.12 to 0.04 µg/g DG).

4. 3. 2. PTX-2 and PTX derivatives

The saturated 1-way model I ANCOVA showed that there was no significant cage*days interaction effect on *Mytilus edulis* toxicity (PTX:2; $F_{(3,35)}$ = 1.25; P = 0.3054 and PTX-2 seco acids: $F_{(3,38)}$ = 1.63; P = 0.1994) from the raw data (Table

4.3), therefore the unsaturated model was conducted with the cage*days interaction omitted from the model. Thus, toxicity of caged *M. edulis* were pooled and tested by a 1-way ANOVA against non-caged *M. edulis* (control).

Toxin content of caged *M. edulis* was significantly different compared to non-caged *M. edulis* (ANOVA REGWQ groupings; PTX-2: $F_{(21,17)} = 3.18$; $P > F = 0.0093$ and PTX-2 seco acids: $F_{(21,19)} = 5.99$; $P > F = 0.0001$) which were exposed to *Dinophysis*. REGWQ groupings (Appendix Table A.2) showed that PTX-2 and seco acids of caged *M. edulis* were similar on days 30 to 60 and significantly different from non-caged. PTX-2 in non-caged mussels increased by 100 % (0.05 to 0.1 µg/g DG) over the first 15 days followed by a decrease of 55 % (0.1 to 0.05 µg/g DG) and 66 % (0.05 to 0.02 µg/g DG) from day 15 to 30, and 30 to 43, respectively. No further change occurred from day 43 to 60. Caged mussels decreased at 40 % (0.05 to 0.03 µg/g DG) during the first 15 days followed by 66 % (0.03 to 0.01 µg/g DG) from day 15 to 30 after which no further change occurred (Figure 4.2B). Caged mussels depurated at a rate of 3 % per day over the first 15 days followed by 4 % from day 15 to 30. This rate was faster than non-caged mussels which increased by 7 % per day over the first 15 days followed by 4 % from day 15 to 30, and 30 to 43.

PTX derivatives (PTX-2SA and *7-epi*-PTX-2SA) in non-caged mussels decreased at a slower rate to caged mussels during the depuration period. PTX derivates in non-caged mussels decreased by 11 % (1.04 to 0.92 µg/g DG) compared to 77 % (1.04 to 0.23 µg/g DG) in caged mussels from day 0 to 15, followed by a decrease of 25 % (0.92 to 0.67 µg/g DG) and 53 % (0.23 to 0.11 µg/g DG) in non-caged and caged mussels, respectively, from day 15 to 30. Non-caged mussels displayed a decrease of 57 % (0.67 to 0.29 µg/g DG) followed by a 27 % (0.29 – 0.37 µg/g DG) increase from day 30 to 43, and 43 to 60, respectively. No significant changes occurred in caged mussels after 30 days (Figure 4.2C). Caged mussels depurated PTX derivatives at a rate of 5 and 4 % per day from day 0 to 15, and 15 to 30, respectively, compared to non-caged mussels which depurated at a rate of 1, 2 and 4 % per day from day 0 to 15, 15 to 30, and 30 to 43, respectively.

Standard error bars are not shown for February 18, 2004 (day 0) due to damage of the duplicate toxin sample during transport).

Dinophysis was present at Sullivans Cove, Hobart, Tasmania, throughout the depuration experiment from February 18 to April 22, 2004. Densities reached and exceeded the regulatory limit (1,000 cells/L for *D. acuminata* and 500 cells/L for *D. fortii*) on February 26 (Table 4.4).

Table 4.2. Raw data of mussel OA + DTX-1 levels in depuration experiment at Sullivans Cove, Hobart, Tasmania.

	Toxin Concentration (OA + DTX-1 µg/g DG)				
Day	0	15	30	43	60
Control	0.17	0.25	0.50	0.12	0.11
		0.26	0.51	0.11	0.11
			0.50		
Average	**0.017**	**0.25 (n = 2)**	**0.50 (n = 3)**	**0.12 (n = 2)**	**0.11 (n = 2)**
Cages		0.11	0.07	0.04	0.04
		0.13	0.07	0.05	0.04
		0.12	0.15	0.04	0.02
		0.11	0.12	0.02	0.03
		0.13	0.12		0.02
		0.15	0.14		0.05
		0.13	0.14		
		0.09	0.13		
		0.10			
Average		**0.12 (n = 9)**	**0.12 (n = 8)**	**0.04 (n = 4)**	**0.03 (n = 6)**

Table 4.3. Raw data of mussel PTX-2 and PTX-2SA + 7-epi-PTX-2SA levels in depuration experiment at Sullivans Cove, Hobart, Tasmania.

	Toxin Concentration (µg/g DG)									
	PTX-2					PTX-2SA + 7-epi-PTX-2SA				
Day	0	15	30	43	60	0	15	30	43	60
Control	0.05	0.15	0.04	0.01	0.02	1.04	1.37	0.60	0.30	0.36
		0.05	0.05	0.02	0.01		0.48	0.70	0.29	0.39
			0.05					0.75		
Average	**0.05**	**0.10 (n = 2)**	**0.05 (n = 3)**	**0.02 (n = 2)**	**0.02 (n = 2)**	**1.04**	**0.92 (n = 2)**	**0.67 (n = 3)**	**0.29 (n = 2)**	**0.37 (n = 2)**
Cages		0.02	0.00	0.01	0.00		1.8	0.10	0.04	0.09
		0.02	0.00	0.00	0.00		0.20	0.11	0.05	0.09
		0.02	0.02	0.00	0.01		0.25	0.15	0.04	0.07
		0.02	0.01	0.00	0.00		0.24	0.12	0.04	0.06
		0.03	0.00	0.01	0.00		0.25	0.10	0.11	0.05
		0.03	0.01	0.01	0.02		0.29	0.11	0.11	0.21
		0.02	0.01	0.00			0.23	0.10	0.12	
		0.02	0.01	0.01			0.15	0.09	0.09	
		0.04					0.32			
Average		**0.03 (n = 9)**	**0.01 (n = 8)**	**0.01 (n = 8)**	**0.01 (n = 6)**		**0.23 (n = 9)**	**0.11 (n = 8)**	**0.08 (n = 8)**	**0.10 (n = 6)**

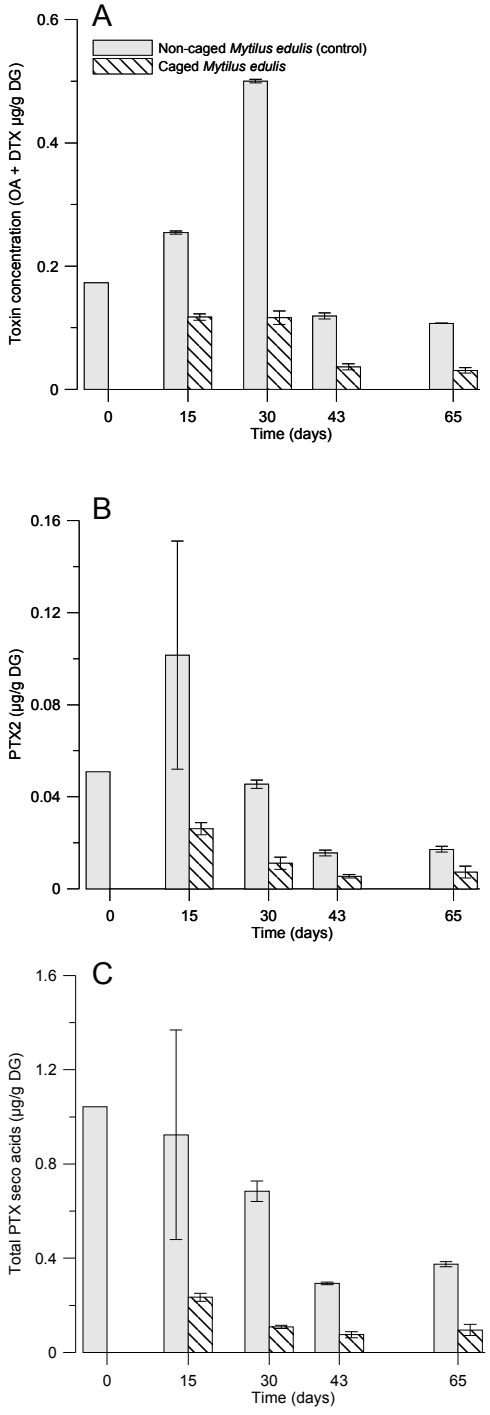

Figure 4.2. Diarrhetic shellfish toxin levels from the depuration experiment. Mean and standard error of toxicity of caged and non-caged (control) *Mytilus edulis* from Sullivans Cove during periods of high *Dinophysis* densities (up to 3,680 cells/L). (A) okadaic acid (OA) + dinophysistoxin-1 (DTX-1); (B) PTX-2 and (C) Total of PTX-2 derivatives (PTX-2SA and *7-epi*-PTX-2SA).

Table 4.4. *Dinophysis* density during the depuration period from February 18 to April 22, 2004.

	Dinophysis density (cells/L)	
Date	*D. acuminata*	*D. fortii*
Feb 19, 2004	200	300
Feb 26, 2004	3,180	500
March 4, 2004	280	50
March 12, 2004	850	200
March 22, 2004	130	100
March 31, 2004	380	330

4. 4. Discussion

Toxicity (OA + DTX-1) of caged *Mytilus edulis* (0.12 μg/g DG at 15 and 30 days and 0.04 μg/g DG at 43 and 65 days of containment) was significantly lower than the initial toxicity (0.17 μg/g DG) at the time mussels were placed in the cages. Toxicity of non-caged *M. edulis* significantly increased by 50 % after 15 days and by 97 % from 15 to 30 days due to the high density of *Dinophysis* (3,180 cells/L of *D. acuminata* and 500 cells/L of *D. fortii*). Toxicity significantly decreased by 76 % from day 30 to 43 and no further increase or decrease was observed from day 43 to 60. Caged *M. edulis* decreased by 35 % and did not depurate any further from day 15 to 30, a further decrease of 69 % occurred from day 30 to 43 after which no further change in toxicity was observed from day 43 to 60. The 38 μm mesh therefore successfully prevented DST from accumulating in *M. edulis*. The mesh size was large enough to exclude most *Dinophysis fortii* cells (56 – 83 μm long, 43 – 58 μm wide) with the exception of the smaller *D. acuminata* cells (35 to 58 μm long, 30 to 38 μm wide) while still allowing *M. edulis* to feed on smaller phytoplankton.

Caged mussels depurated PTX's at a faster rate than OA + DTX-1 over the first 30 days after which depuration of OA + DTX-1 increased when no further change was observed for PTX's. During day 0 to 15, PTX-2 and PTX derivatives decreased by 49 % (3 % per day) and 77 % (5 % per day), respectively, compared to OA + DTX-1 which decreased at 29 % (2 % per day).

PTX-2 and PTX derivatives decreased by 57 % (4 % per day) and 53 % (4 % per day), respectively, compared to no change in OA + DTX-1 levels from day 15 to 30.

After day 30 no further significant change occurred in PTX's, however, the depuration of OA + DTX-1 increased to 67 % (5 % per day) from day 30 to 43. These results indicate biphasic depuration in mussels.

Caged *M. edulis* detoxified OA + DTX-1 at a rate of 2 % per day in the first 15 days, while on day 30 *M. edulis* showed no further detoxification until day 43 when a further detoxification rate of 5 % per day (day 30 to 43) was observed. Similar depuration rates have been found in French mussels from the Atlantic (10 % per day) and Mediterranean coast (6.6 % per day) (Marcaillou-Le Baut *et al.* 1993), in *M. edulis* from Sweden (3.2 % per day) (Svensson 2003) and bay scallops (*Argopecten irradians*) from Canada (8.4 % per day) (Bauder *et al.* 2001). Depuration was greatest in the first 15 days, following which no further detoxification was observed, indicating biphasic depuration kinetics. This finding was consistent with previous studies (Bauder *et al.* 1996, Svensson 2003) that show detoxification is faster in the first 1 to 2 weeks and then slows down to almost 0 % in the next 1 to 2 weeks. It was suggested by these authors that toxins occur in two compartments with different depuration kinetics. Similarly, Masselin *et al.* (1992) and Moroño *et al.* (2003) have suggested that bi-phasic detoxification comprised of rapid loss of labile toxins followed by slow release of the bound fraction. This was observed in this study by the rapid loss of PTX's during the first 30 days followed by an increase in the depuration rate of OA + DTX-1. The increase in the higher depuration rate of OA + DTX-1 from day 30 to 43 following a slow depuration rate in this study may be attributed to the reduced food availability over an extended period of time leading to release of bound toxin fractions in storage.

There are two possible mechanisms that may explain depuration kinetics in shellfish. The first is starvation; if shellfish are not feeding they cannot accumulate toxins. The digestive gland is a major site for lipid storage in mussels and therefore the majority of the lipophilic toxins are accumulated in this organ. Usage of stored lipid increases during the later stages of starvation may enhance the rate of depuration. The second is the availability of nontoxic compared to toxic food. Several authors have proposed that depuration of DST in mussels is affected by the availability of nontoxic food (Haamer *et al.* 1990, Marcaillou-Le Baut *et al.* 1993, Haamer 1995, Blanco *et al.* 1999, Svensson 2003). Fecal deposition has been suggested by Blanco *et al.* (1993) and Bauder *et al.* (1996) to be the main route for

shellfish to eliminate DST; the toxins are eliminated at a higher rate during high feeding activity. It is likely that the depuration of caged *M. edulis* observed here was due to the availability of nontoxic food such as the dinoflagellates *Gymnodinium* sp., *Gonyaulax*, and the diatoms *Chaetoceros*, *Skeletonema* and *Pseudo-nitzschia* species. Although the mesh reduced the availability of larger nontoxic dinoflagellates such *Ceratium* species, importantly it increased the ratio of nontoxic to toxic food.

Under natural field situations, shellfish detoxification is likely to occur when the ingestion rate of toxic algae is reduced (Svensson 2003). This can be achieved by a decrease or disappearance of toxic algae in the water column while filtration is maintained, which is what this study was able to simulate. It can also be achieved by a high relative abundance of nontoxic phytoplankton accompanying toxic species. This would reduce filtration activities of shellfish in order to regulate their physiological needs, thus reducing ingestion rate of *Dinophysis* (Svensson 2003).

Temperature is also expected to effect filtration rate and hence metabolism in *Mytilus edulis* in laboratory conditions leading to changes in depuration rates. Jørgensen *et al.* (1990) and Riisgård (1991) observed an increase in filtration rate of *M. edulis* during rapid increases in temperature. However an increase in algal concentrations at the same temperatures caused a reduction in filtration rate over time. This would indicate that filtration rate is more dependant on food sources rather than temperature. Field studies of *M. galloprovincialis* by Blanco et al. (1999) found the opposite situation occurred in the later stages of depuration with lower temperatures related to increase in depuration. This was likely caused by the release of toxins from storage during the second phase of depuration. The temperature range in this present study varied by 2 ºC indicating that temperature would have had little effect on filtration. Since caged and non-caged mussels were subjected to the same environmental conditions the toxin differences observed are likely caused by the reduction of toxic phytoplankton.

Comparisons between depuration studies should be made with caution due to variety of toxin analysis techniques and methods used. Svensson's (2003) study was laboratory based in which algal species (*Isochrysis galbana* and *Thalassiosira pseudonana*) and rations fed to mussels were controlled. Marcaillou-Le Baut *et al.* (1993) study was a comparison between mussel depuration in laboratory conditions and an aquaculture pond, in which food species and rations were not controlled.

4. 5. Conclusion

Access of toxic *Dinophysis* species to the blue mussel diet at Sullivans Cove was reduced by placing mussels in cages with a mesh size of 38 μm to screen *Dinophysis* plankton cells. The reduction of toxic dinoflagellates from the mussel diet increased the ratio of nontoxic to toxic phytoplankton which prevented toxin accumulation and produced a depuration rate of 2 % per day of OA + DTX-1 and 3 and 5 % per day of PTX-2 and PTX derivatives over 15 days. No further depuration of OA + DTX-1 occurred from day 15 to 30, which was followed by a further detoxification rate of 5 % per day from day 30 to 43. PTX-2 and derivatives decreased at 4 % per day from day 15 to 30 after which no further change occurred. Mussels displayed biphasic depuration with a faster rate of PTX loss over the first 30 days followed by an increase of OA + DTX-1 depuration once there was no further change in PTX levels. Knowledge of depuration rates of contaminated shellfish are important to for managing the impact of DST for the shellfish industry and estimating safe harvest periods.

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Chapter 5:

Can mussels (*Mytilus edulis*) take up dissolved DST? *Dinophysis* field and *Prorocentrum lima* laboratory experiments using Solid Phase Adsorption Toxin Tracking (SPATT)

Abstract

Solid phase adsorption toxin tracking (SPATT) is a method involving porous synthetic resin filled sachets (SPATT bags) for binding biotoxins from the seawater medium. This study was the first in Australia to detect DST dissolved in natural seawater at levels as high as 0.27 μg/SPATT bag during the presence of *Dinophysis acuminata* and *D. fortii*. SPATT bags were also exposed to Tasmanian *Prorocentrum lima* cultures and contaminated blue mussels (*Mytilus edulis*) to determine the origin of dissolved toxins. Most of the Diarrhetic Shellfish Toxins were present in the dissolved seawater (94 %) when SPATT bags were exposed to *P. lima* cultures (6 % of DST in cells). Only a small amount of total DST (1 %) was present in the seawater medium when exposed to contaminated mussels (99 % of total DST). These results indicate that dissolved DST can be exuded from both toxic phytoplankton and depurating shellfish. It appears that mussels are able to take up DST with OA increasing by more than 0.11 μg/g DG in mussels immersed in dissolved DST for 48 hrs. This poses an additional threat to shellfish farms and can extend harvest closure periods after toxic phytoplankton blooms.

Cellular and exuded toxicity of *Prorocentrum lima* varied between culture strains isolated from different locations in Tasmania, Australia. Cellular OA was greater in the Little Swanport (PLLSP) strain (36 pg/cell) compared to the Louisville Point (PLLV) strain (3.8 pg/cell), which was the only strain producing DTX-1. The Tasmanian strains are newly associated here with PTX-2 production (0.4 – 1.2 pg/cell). The Louisville strain excreted higher concentrations of OA (reaching 18 μg/SPATT bag) in the first 20 days than the Little Swanport strain (11 μg OA/SPATT bag) and for both strains this declined to 4 μg/SPATT bag on day 40. Both strains exuded higher dissolved toxin levels at 1,200 cells/L (PLLV strain reaching 1.6 μg OA + DTX-1/SPATT bag) compared to 2,400 cells/L (0.4 μg OA + DTX-1/SPATT bag). Tasmanian strains produce a higher amount of toxin per cell compared to other global strains and is a potential DSP risk for Tasmanian shellfish farms. Dissolved DST caused a reduction in cells densities of the diatom *Thalassiosira weissflogii* suggesting that the dissolved DST may play an allelopathic role.

Toxicity differences among dinoflagellate species and strains can pose problems for shellfish monitoring programs and may require phytoplankton species regulatory limits to be adjusted regionally.

5. 1. Introduction

In Tasmanian waters, the seasonal presence of toxic species including *D. acuminata*, *D. acuta*, *D. caudata*, *D. fortii*, *D. tripos*, and *P. lima* (Hallegraeff and Lucas 1988, Pearce *et al.* 2001, Hallegraeff 2002), poses a threat to the reputation of shellfish industries and the health of seafood consumers. OA producing *P. lima* has previously been identified in Tasmanian waters by DNA sequences (Pearce and Hallegreaff 2004). Monitoring programs involve regular testing of shellfish meat for toxins and inspection of water samples to identify the presence and density of the toxic algal species. Depending on the monitoring programs implemented, shellfish testing for toxins may only be conducted if the problematic species exceed the regulatory limit. The advantage of phytoplankton monitoring is that it can avoid the cost of toxin analysis, although the disadvantage is that samples only provide a snap-shot in time and place of the algal composition which may not be a true representative of the area and may not necessarily forecast an algal bloom.

Natural dinoflagellate blooms have also been associated with dissolved DST in the seawater medium associated with high densities of *Dinophysis* species (MacKenzie *et al.* 1998, 2004). The concept of solid phase adsorption toxin tracking (SPATT) was developed by MacKenzie *et al.* (2004) to provide a more sensitive and cost effective integrated means of monitoring biotoxins. SPATT bags are mesh bags that contain adsorbent resin beads (HP20) which have an affinity for binding DST and thus can be used to detect levels of dissolved DSP toxins in the seawater medium.

In the present study SPATT bags were used in field and laboratory conditions to: 1. Determine the origin of dissolved toxins in Tasmanian coastal waters; 2. Explore its potential as a monitoring tool. The dissolved toxins in the seawater medium may also potentially pose an additional threat if shellfish are able to take up toxins in the same manner as ingesting toxic algae. Laboratory experiments were conducted to: 3. Assess the potential of this latter threat; 4. Determine the relationship between *P. lima* density and exudate toxicity; and 5. Observe the effect of dissolved

toxin on the diatom *Thalassiosira weissflogii* to assess possible allelopathic advantages of dinoflagellates exuding toxins into seawater.

5. 2. Methods and Materials

5. 2. 1. Study site

A SPATT bag field experiment and phytoplankton and mussel sampling were conducted on the south-eastern Tasmanian coast at Sullivans Cove (SC) (42º 53´ S 147º 20´ E) in the estuarine waters of the Derwent River. Sullivans Cove was selected because of the known seasonal occurrence of *Dinophysis* during spring and summer (as shown in Chapter 1) and the high abundance of the blue mussel (*Mytilus edulis*) growing on the rocks and wharf structures. The Derwent River is an estuary that is affected by industrial and municipal wastewater discharges that have an impact on nutrient levels. Average water temperatures and salinity at the site are 13.5 ºC and 33 ppt, respectively during experimental period. The field study was conducted from February 19 to April 22, 2004.

5. 2. 2. Phytoplankton collection and enumeration

Samples for *Dinophysis* species identification and enumeration were collected with a depth-integrated sampling tube (hose diameter 24 mm) and vertical plankton net hauls (mouth diameter 215 mm, length 1000 mm, cod end diameter 50 mm, mesh size 20 μm), respectively. The depth-integrated sample tube (7 m) was passed into the water column (0 to 6 m) holding onto one end. It was then retrieved by a string attached to the other end of the tube so that both ends were positioned above the surface and the water trapped in the hose. Water was collected into a 1 L polyethylene bottle and fixed with Lugol's solution.

In the laboratory one litre of the depth-integrated water sample was transferred to a measuring cylinder and fixed with Lugol's iodine solution, and covered with aluminium foil. The preserved sample was left to stand for 12 to 24 hrs. After this time a subsample of 100 mL from the bottom of the cylinder was taken via a 2 x 50 mL pipette and placed into two 50 mL sample containers. Two mL from each sample

jar was taken via pipette into a 50 mm petri dish. The species of *Dinophysis* cells in the petri dish were then counted under a Zeiss Axiovert25 inverted microscope.

5. 2. 3. Mussel collection

Blue mussels (*Mytilus edulis*) at Sullivans Cove were sampled for diarrhetic shellfish toxin measurements fortnightly from February 18 to March 19, 2004. Fifteen *M. edulis* (ranging from 36 to 98 mm in length) were removed from pylons underneath the Princess Wharf (Figure 3) on each sampling date. Mussels were placed in labelled zip-lock bags and stored frozen until further processing.

5. 2. 4. Solid Phase Adsorption Toxin Tracking (SPATT)

Adsorbent sachets (50 x 60 mm) were made from 38 μm nylon mesh, sewn with nylon thread as described by MacKenzie *et al.* (2004). Adsorbent resin beads (Diaion® HP20) were dispensed into the sachets (approximately 3 g dry weight). Into the flap a polyester string was sewn to fix the sachets to the frame of the submerged cages used in the depuration experiment. The resin beads adsorb dissolved DST from the seawater medium and toxins extracted from the beads with methanol (detailed in 5. 2. 6) to determine concentrations from HPLC-MS/MS (detailed in 5. 2. 7). Figure 5.1 displays a flow diagram demonstrating the use of SPATT bags for extraction and determining DST concentrations from the seawater medium.

Six sachets were deployed on February 18, 2004 and collected the following week, at which time six more sachets were deployed. This procedure continued until March 19, 2004. After retrieval the sachets were stored frozen prior to extraction and analysis.

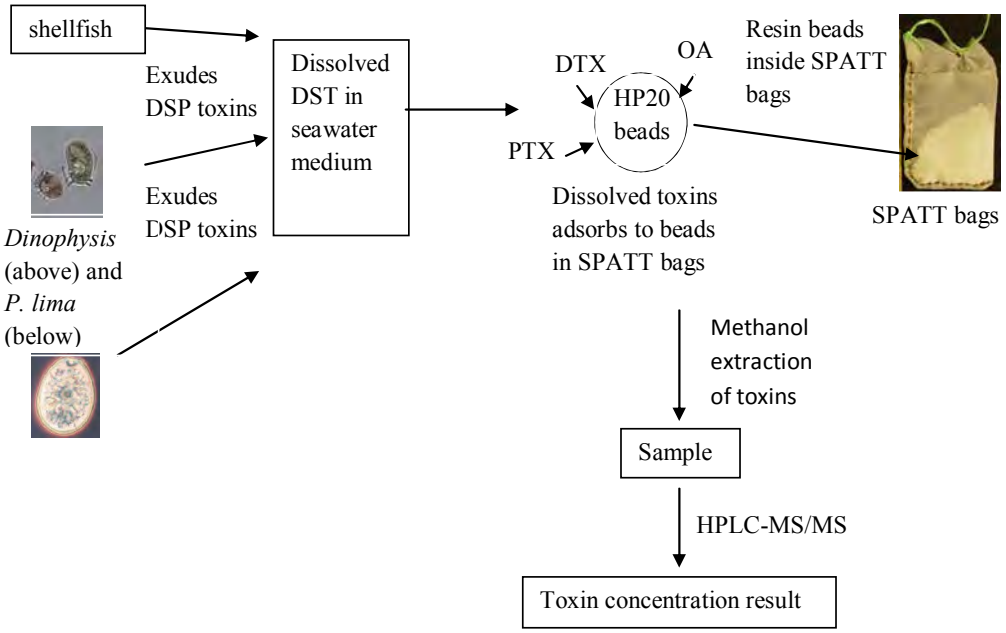

Figure 5.1. Flow diagram demonstrating the use of SPATT bags adsorbing dissolved DST from the seawater medium and extraction to determine toxin concentration. HP20 beads have an affinity for binding DST. Dissolved toxins in the seawater medium are adsorbed onto the Diaion® HP20 resin beads. Resin beads (HP20) are enveloped in 38 μm nylon mesh (SPATT bags).

5. 2. 5. Laboratory experiments

5. 2. 5. 1. Dissolved DST from *Prorocentrum* culture

Two laboratory experiments were conducted with SPATT bags exposed to *P. lima*. The first was to determine if the Tasmanian strain *P. lima* produced dissolved toxins; the second was to determine if a maximum adsorption capacity existed for SPATT bags over time. Two strains of *P. lima* were used for the experiments to determine if toxicity varied between strains and with density. Two strains isolated from the east coast of Tasmania (Little Swanport (PLLSP) and Louisville Point (PLLV)) by Pearce *et al.* (2001) were used for the experiments to compare toxicity among *P. lima* strains from different locations isolated in June 2000.

A total of 18 SPATT bags were placed in 3 x 8 L tanks (six bags per tank) of filtered seawater containing a cultured Tasmanian strain *P. lima* at densities of 600, 1200 and 2400 cells/L. Two bags from each tank were obtained at 7, 20 and 40 days.

Water samples were collected for toxin extraction from *P. lima* cells at the time SPATT bags were retrieved.

To determine if there was a maximum adsorption capacity of SPATT bags, the bags were exposed to a 20 L culture of *P. lima*. A total of six SPATT bags were added to 3 x 500 mL cultures of *P. lima* with two bags retrieved at 7, 20 and 40 days. Cultured *P. lima* cells were collected onto a 0.2 μm filter membrane. The filters were then pulped in 90% methanol and centrifugated at 652 g for 10 min, the supernatant was decanted and the tubes containing pellets were immersed in boiling water for 5 min to inactivate enzymes of *P. lima* cells, preventing potential hydrolysis of conjugated forms of OA. Cellular toxins were extracted in 1 mL of 90% methanol, evaporated to dryness under a nitrogen stream then resuspended in 1 mL of 90% methanol. Final extracts were filtered through a Acrodisc® syringe 0.2 μm Supor® membrane filter into vials and stored in a freezer (-20 ºC) until analysed by HPLC-MS/MS.

5. 2. 5. 2. Mussels and *T. weissflogii* exposed to dissolved DSP toxins

Filtered seawater containing dissolved toxins but without *P. lima* cells was created by passing 20 L of a *P. lima* (PLLSP strain) culture through a 0.2 μm filter membrane under vacuum to eliminate cells. The filtered water was collected into Schott bottles. Control groups consisted of placing SPATT bags into two beakers containing 400 mL of filtered seawater medium containing no known dissolved DST (Treatment 1) and two beakers containing dissolved DST (two SPATT bags per beaker) harvested from the *P. lima* culture (Treatment 2). The control groups ensured that dissolved toxins were appropriately harvested and allowed for initial dissolved concentration levels to be determined. Treatment 3 was a control that consisted of two beakers without mussels but containing dissolved toxins and *T. weissflogii* (2,500 cells/mL). A water sample from the beakers was collected for enumeration of *T. weissflogii* at 2, 13 and 18 hrs.

Mussels were exposed to the following treatments over a period of 48 hrs: 4. No dissolved toxins SPATT bags; 5. Beakers containing dissolved toxin and SPATT bags; 6. beakers containing dissolved toxin without SPATT bags; 7. Dissolved toxins with the diatom *Thalassiosira weissflogii* (CCMP1336 strain) (2,500 cells/mL) and

SPATT bags; 8. Dissolved toxins with *T. weissflogii* (2,500 cells/mL) and no SPATT bags. Treatment groups are summarised in Table 5.1.

Table 5.1. Summary of dissolved DST experimental treatments.

Treatment	
1	Control: Filtered seawater (FSW) + SPATT
2	Control: Dissolved DST from *P. lima* culture + SPATT
3	Control: *T. weissflogii* + dissolved DST
4	Mussels in FSW + SPATT
5	Mussels + dissolved DST + SPATT
6	Mussels + dissolved DST – SPATT*
7	Mussels + dissolved DST + *T. weissflogii* + SPATT
8	Mussels + dissolved DST + *T. weissflogii* – SPATT*

* Spatt bags were added immediately after the experimental period (48 hrs) and retrieved after 7 days to ensure that treatments contained dissolved DST in the medium.

Each treatment contained 3 beakers of 400 mL of medium with 5 mussels per beaker. An initial density of 2,500 cells/L of *T. weissflogii* was added to filtered seawater (control) and the following treatments containing dissolved toxins: with (Treatment 3) and without SPATT bags; and mussels immersed in dissolved DST with and without SPATT bags (Treatment 7 and 8). Treatments without mussels consisted of 2 beakers with 400 mL of medium. *T. weissflogii* density counts were conducted at 2, 13 and 18 hrs. The treatments without SPATT bags during the experimental 48 hr period had bags added immediately afterwards and were retrieved 7 days later. All treatment beakers contained air stones to ensure constant aeration throughout the experimental period.

5. 2. 6. Toxin extraction from *Mytilus edulis*, phytoplankton and SPATT bags

Mussels were thawed, the soft tissue removed from the shell and the digestive glands dissected. Digestive glands were weighed and placed into a 50 mL sample container. Digestive glands were homogenised using a commercial (AFK stabmixer or Braun multiquick) hand-held blender. Approximately 4 g of homogenate was weighed into 50 mL plastic sample containers and homogenised with 16 mL of methanol. Samples were centrifuged for 2 x 5 min at 652 g. The final extract was filtered through a Acrodisc® syringe 0.2 μm Supor® membrane filter into vials and stored in a freezer

until analysed by HPLC-MS/MS methods at the Queensland Health Scientific Services.

Phytoplankton 20 μm net tow samples were snap frozen until further analysis when they were thawed to disrupt cells. After centrifugation at 652 g for 10 min, the supernatant was decanted and the tubes containing pellets were immersed in boiling water for 5 min to inactivate enzymes of *Dinophysis* cells, responsible for potential hydrolysis of conjugated forms of OA. Cellular toxins were extracted in 1 mL of 90% methanol, evaporated to dryness under a nitrogen stream then resuspended in 1 mL of 90% methanol. Final extracts were filtered through a Acrodisc® syringe 0.2 μm Supor® membrane filter into vials and stored in a freezer until analysed by HPLC-MS/MS.

P. lima cells collected from the tanks were concentrated onto a 0.2 μm glass fibre filter (GFF) via gravity sedimentation. The filter was pulped in 1mL of 90% methanol, sonicated for 2 min then centrifuged at 652 g for 10 min. The supernatant was dried under a N_2 stream and re-suspended in 2 mL of 90% methanol. The sample was then filtered through an Acrodisc® syringe 0.2 μm Supor® membrane filter into vials and stored in a freezer until analysed by HPLC-MS/MS.

Adsorbent sachets were prepared for toxin analysis as described by MacKenzie *et al.* (2004). Extraction of the adsorbent sachets involved soaking them twice for 5 min each in 500 mL of MilliQ water to remove salts, and vigorously shaking them free of most water before removing the resin contents and soaking these for 2 hrs in 80 mL 100% methanol. The extract was filtered and washed through glass wool and an anhydrous $NaSO_4$ column (2 mL), to remove residual water, with an additional 80 mL of methanol. The sample was evaporated to dryness, re-suspended in 15 mL methanol transferred to a glass vial and again reduced to dryness on a heating block at 40 ºC under an N_2 stream. The sample was re-suspended in 80% methanol, filtered (0.2 μm) and dispensed into vials for analysis by HPLC-MS.

5. 2. 7. HPLC-MS/MS analysis of toxins

Diarrhetic shellfish toxins were determined at the Queensland Health Scientific Services by HPLC-MS using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord. On, Canada) equipped with a turbo-ionspray (high flow electrospray) interface coupled to a Shimadzu LC-10ADvp HPLC system (Shimadzu Corp., Kyoto, Japan). Separation was achieved using a 150 X 4.6 mm Alltima C_{18} column (Alltech (Aust.), Baulkam Hills, NSW) run at 35 ^{o}C, and flow rate of 0.8 mL/min with a linear gradient starting at 5% B for 5 minutes, ramped to 100% B in 8 minutes, held for 5 minutes then to 5% B in 1 minute and equilibrated for 5 minutes (A = 10% acetonitrile /HPLC grade water, B = 90% acetonitrile/ HPLC grade water, both containing 0.1% formic acid and 2 mM in ammonium formate. The mass spectrometer was operated in both positive and negative ion modes. Analytes were confirmed (normally using the single ion monitoring mode, SIM) by characteristic ions and retention times (rt., minutes) as described in Table 5.2.

Table 5.2. Mass spectrometer operating parameters.

Toxin	SIM	m/z	Retention time (min)
PTX-2	positive mode	876.6	14.62
PTX-2 SA	positive mode	894.6	13.73
7-epi PTX-2 SA	positive mode	899.6	14.15
OA	negative mode	803.5	11.90
DTX-1	negative mode	817.5	13.40
DTX-2	negative mode	803.5	12.28

Comparing areas of peaks from samples to appropriate standards using the external standard method achieved quantitation with a relative standard deviation (precision) of 3.3 – 12.7 % (Appendix Table A.1). Certified standards of pectenotoxin-2 and okadaic acid were obtained from, IMB, NRC, Canada. Pectenotoxin-2 seco acid concentrations were determined by comparison to the PTX-2 standard and assuming a similar response factor. Dinophysistoxin-1 and DTX2 concentrations were determined by comparison to the OA standard and assuming a similar response factor.

5. 3. Results

5. 3. 1. Sullivans Cove Solid Phase Adsorption Toxin Tracking (SPATT)

Pectenotoxin-2 (PTX-2) and OA at concentrations of 0.16 to 0.28 and 0.09 to 0.27 μg/SPATT bag, respectively, were the predominant DST compounds adsorbed by the resin beads from the water at Sullivans Cove. By contrast the dominant toxins PTX-2 derivatives and OA were found in local *M. edulis* at concentrations of 0.29 to 0.45 and 0.17 to 0.50 μg/g DG, respectively (Figures 5.1A, B). A negative relationship was observed between toxin uptake in SPATT bags and toxins in mussels from February 2 to March 19, 2004. While dissolved OA + DTX-1 in SPATT bags decreased, the toxins in mussels increased. No clear relationship was found when toxins in SPATT bags and mussels were compared to *Dinophysis acuminata* and *D. fortii* densities (Figures 5.1C, D).

5. 3. 2. *P. lima* cultures

OA concentration in the *P. lima* Little Swanport strain (36 pg/cell) was 10 times higher than the Louisville Point strain (3.8 pg/cell) which contained a higher concentration of DTX-1 (0.8 pg/cell) and PTX-2 (1.2 pg/cell) after 20 days (Figure 5.3A, B). The Little Swanport cells did not appear to contain any DTX-1 but in contrast the dissolved DST in the seawater medium showed DTX-1 at a concentration of 0.08 μg/SPATT bag. The Louisville Point strain contained a greater concentration of dissolved OA (18 μg/SPATT) and DTX-1 (3 μg/SPATT) after 20 days. OA concentrations in both strains were similar on day 40 (Figure 5.3C, D) while PTX-2 was not detected in any of the culture seawater media.

The dissolved OA + DTX-1 toxin from seawater medium of the Louisville Point strain was greater at each cell density over the 40 day period than for the Little Swanport strain (Figure 5.4A, B). The dissolved DST concentration from the Louisville Point strain increased over time and displayed a greater toxin concentration at the lower cell densities on day 7 and 20 (0.15 and 0.35 μg OA + DTX-1/SPATT bag at 600 cells/L, respectively, 0.05 and 0.2 μg OA + DTX-1/SPATT bag at 1,200 cells/L, respectively. The cell density of 1,200 cells/L produced a maximum of 1.6 μg

OA + DTX-1/SPATT bag on day 40 (Figure 5.4B). The dissolved DST from the Little Swanport strain displayed a small increase over time at 600 cell/L (0 to 0.05 µg OA + DTX-1/SPATT bag from day 7 to 40).

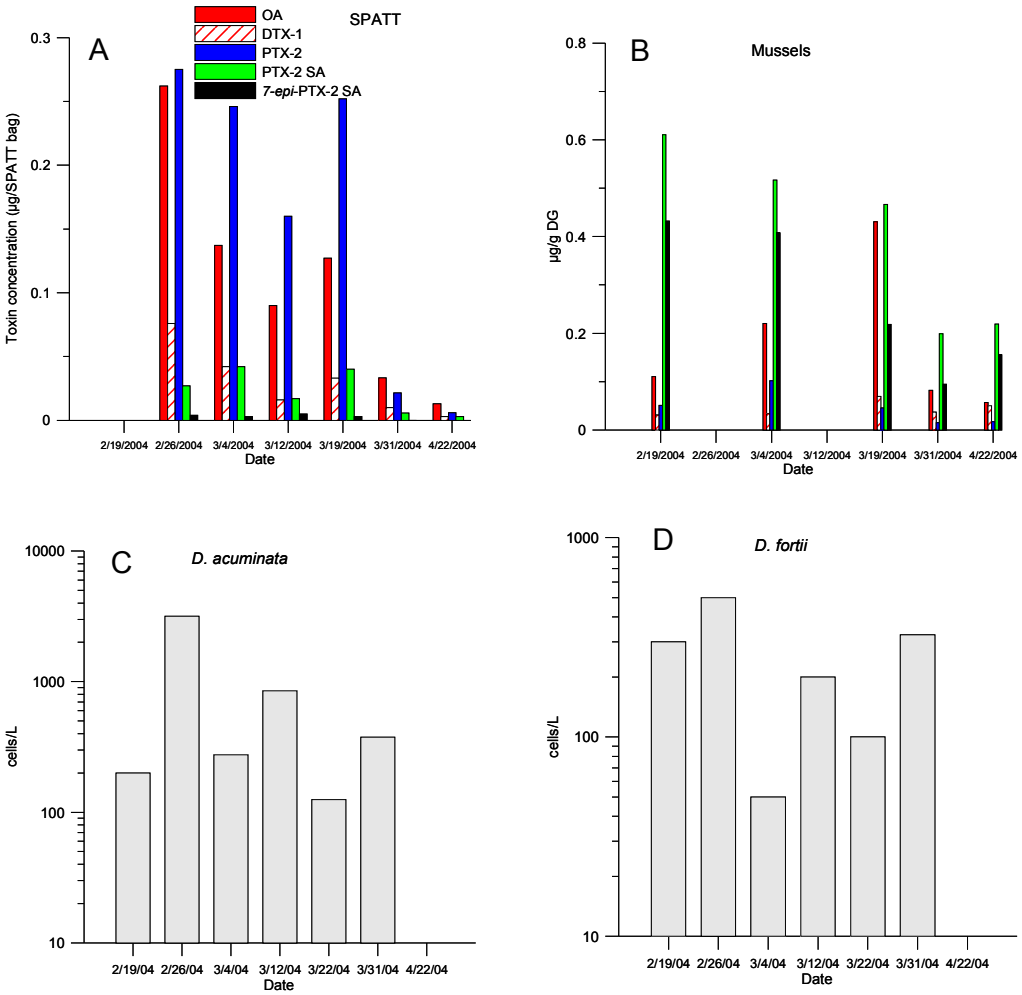

Figure 5.2. (A) Diarrhetic shellfish toxin profile in seawater solution (SPATT bags) compared to (B) toxin profile in *Mytilus edulis*, in relation to (C) *Dinophysis acuminata* and (D) *D. fortii* densities at Sullivans Cove from February to April 2004.

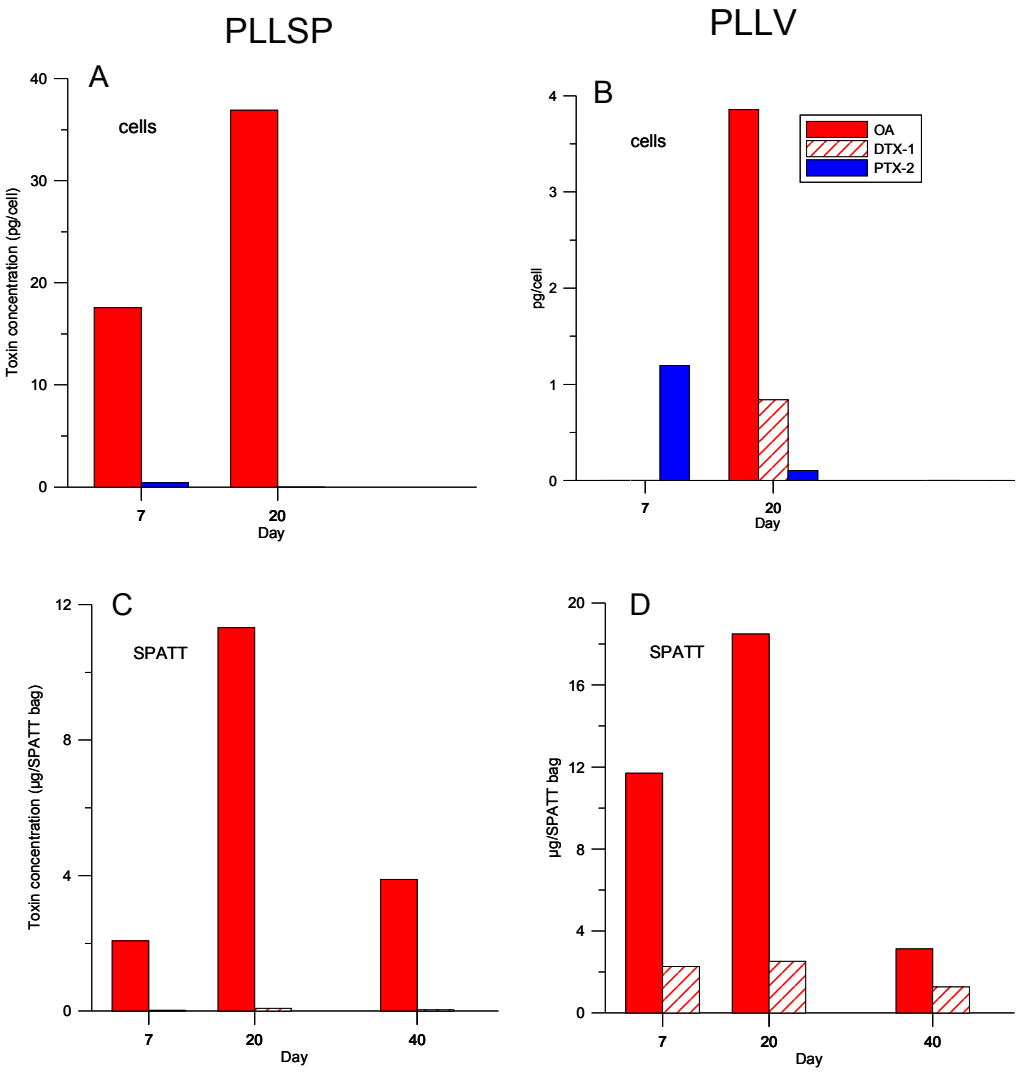

Figure 5.3. DST profile of *Prorocentrum lima* cells and dissolved toxin (SPATT bags) from the culture medium of two strains of *P. lima*; A, C; Little Swanport (PLLSP), Tasmania: B, D: Louisville Point (PLLV), Tasmania.

The higher cell densities (1,200 and 2,400 cells/L) displayed a small decrease in toxin concentration over time (0.05 to 0.03 OA + DTX-1 µg/SPATT bag at 1,200 cells/L and 0.03 to 0 OA + DTX-1 µg/SPATT bag at 2,400 cells/L from day 20 to 40. Dissolved DST were not detected on day 7 for each of the densities or on day 40 from the seawater medium containing 2400 cells/L of the Little Swanport strain (Figure 5.4A). PTX-2 and their derivatives were not detected in the seawater medium during the experiment.

5. 3. 3. Dissolved DST uptake by laboratory mussels

OA in all treatments was the predominant dissolved DST (Figure 5.5). PTX-2 derivatives (PTX-2 SA and *7-epi* PTX-2 SA) were present at concentrations up to 0.17 μg/SPATT bag in laboratory treatments with *M. edulis*. Dissolved OA and PTX-2 derivatives were detected at low concentrations (0.01 and 0.03 μg/SPATT bag, respectively) from the media when mussels were immersed in filtered seawater containing no dissolved toxins (Treatment 4). DST content in SPATT bags immersed in dissolved toxins (Treatment 5) and with *T. weissflogii* (Treatment 7) were very similar containing 0.12 and 0.09 μg of OA/SPATT bag, respectively, and 0.02 and 0.01 μg of PTX-2 derivatives/SPATT bag, respectively. SPATT bags were added after the removal of mussels exposed to dissolved toxins with and without *T. weissflogii* in the absence of SPATT bags (Treatment 6 and 8 as shown in Table 5.1) contained OA at concentrations of 0.44 and 0.67 μg/SPATT bag, respectively and PTX-2 derivatives at concentrations of 0.17 and 0.11 μg/SPATT bag, respectively. DTX-1 was detected in Treatment 8 at a low concentration of 0.01 μg/SPATT bag. No PTX-2 was detected in the dissolved medium from any treatment.

OA concentration was similar in the presence and absence of mussels immersed in seawater medium containing dissolved DST (Treatments 2, 3 , 5 and 7). However treatments containing mussel (Treatment 4 - 8) displayed the presence of PTX-2 at low concentrations up to 0.02 μg/SPATT bag after 48 hr incubation in the dissolved toxin medium. Figure 5.5 summarises the toxin content of SPATT bags from the treatment groups shown in Table 5.1.

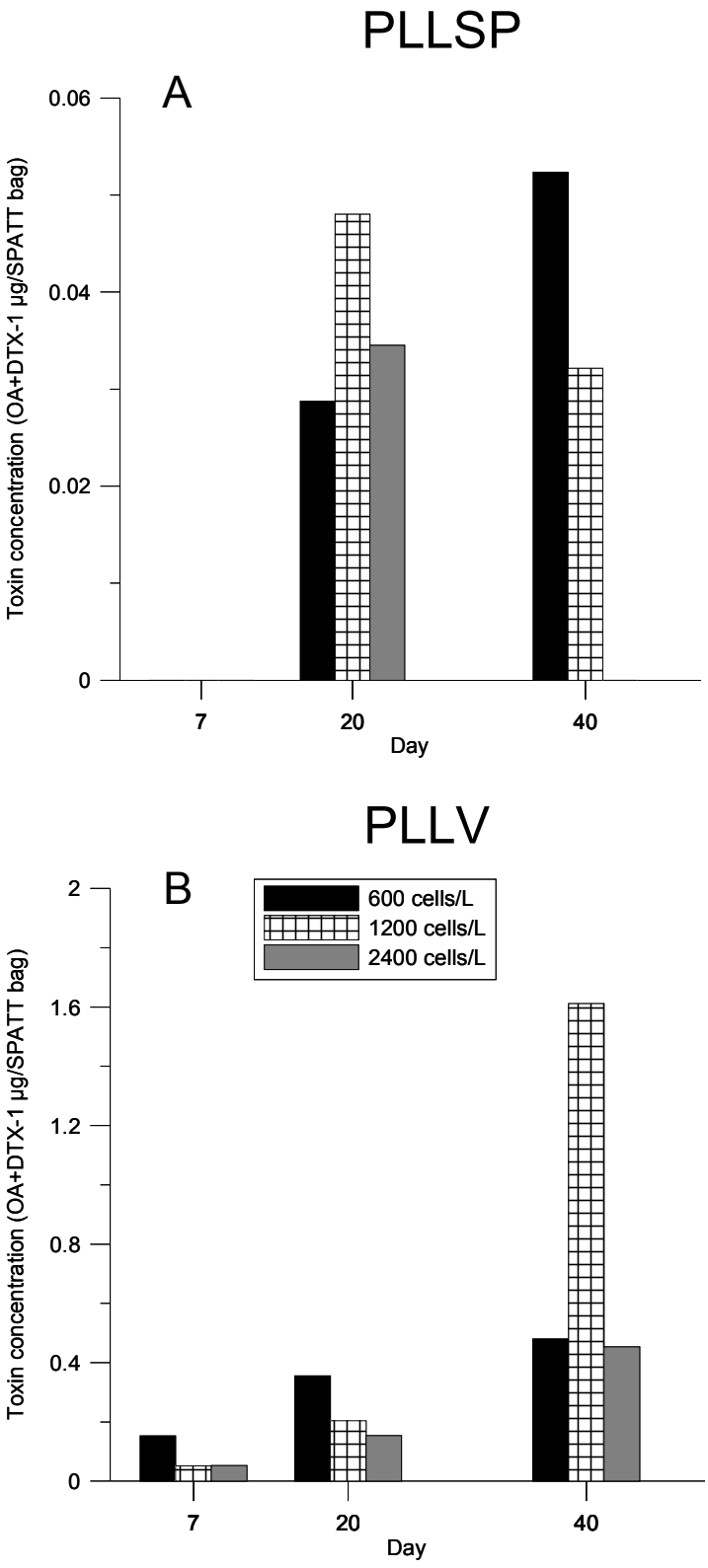

Figure 5.4. Dissolved DST (SPATT bags) in the seawater medium of two strains of *Prorocentrum lima*; (A) Little Swanport; (B) Louisville Point, at varying cell densities (600, 1200 and 2400 cells/L).

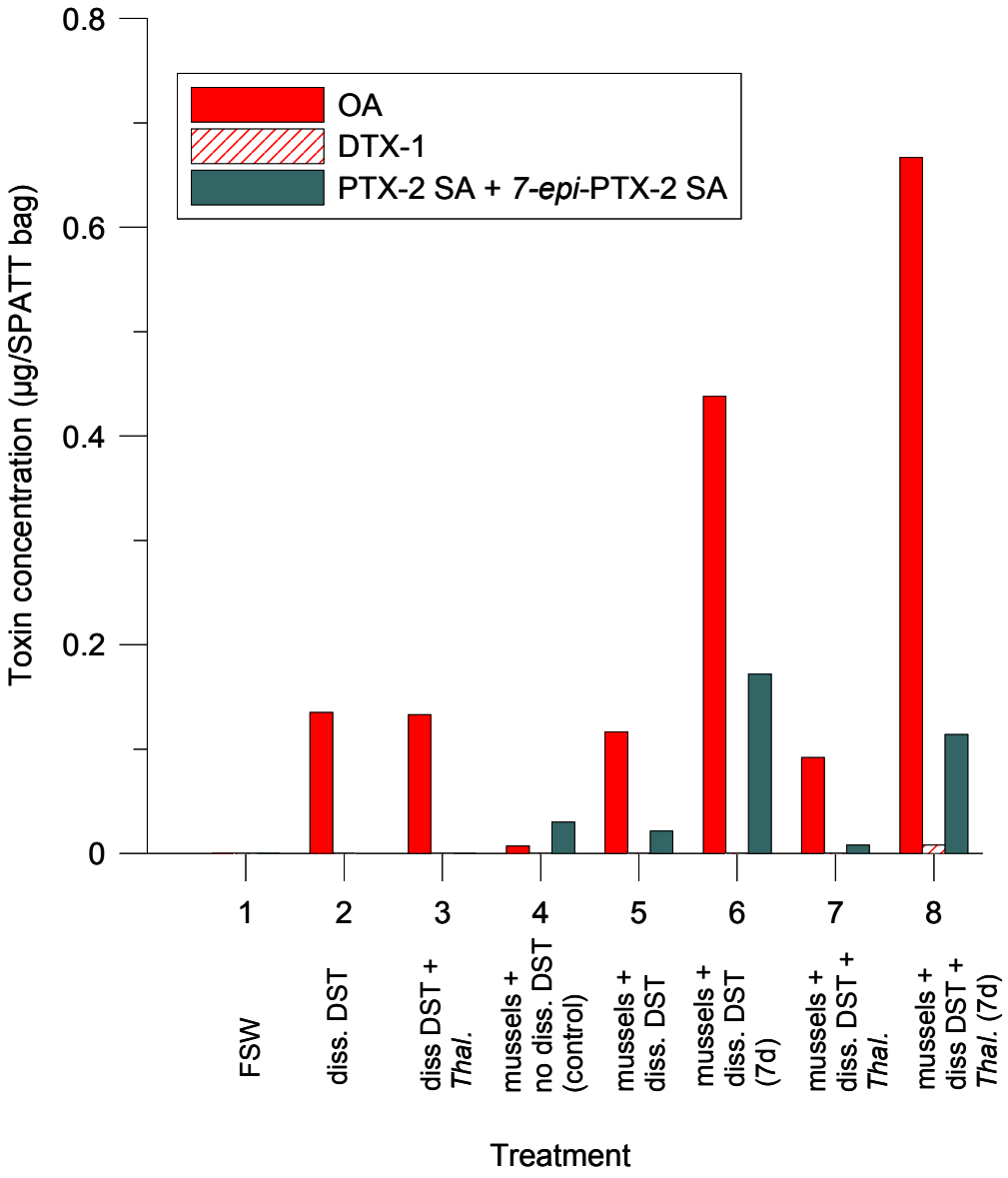

Figure 5.5. DST profile in *in-vitro* seawater solution (SPATT bags). Treatments refer to the conditions SPATT bags were exposed to in beakers containing 400 mL of filtered seawater. FSW = filtered seawater; diss. = dissolved; *Thal.* = *Thalassiosira weissflogii*.

PTX-2 derivatives (PTX-2 SA and *7-epi* PTX-2 SA) were the dominant DST in *M. edulis* at concentrations from 0.079 to 1.9 µg/g DG and 1.62 to 2.34 µg/g DG, respectively from the various treatments (Figure 5.6). OA, DTX-1 and DTX-2 were detected in all mussels at concentrations from 0.26 to 0.39 µg/g DG, 0.04 to 0.08 µg/g DG and 0.45 to 1.04 µg/g DG, respectively (Figure 5.6). OA increased by more than 0.11 µg/g DG in all mussels immersed in dissolved DST (Treatments 5 – 8) compared to mussels not subjected to dissolved DST (Treatment 4). The other toxins did not

vary greatly in mussels exposed to dissolved toxins compared to control mussels. The toxins *7-epi* PTX-2 SA and a possible DTX-2 compound were greater in mussels exposed to dissolved toxins (Treatment 5) and when containing *T. weissflogii* (Treatment 7), respectively. Treatments 5 and 7 contained both mussel and SPATT bags whereas Treatments 4 and 6 did not contain SPATT bags while the mussels were immersed in the dissolved DST seawater medium. Toxin content did not display a notable variation in mussels exposed to dissolved toxins with *T. weissflogii* in the presence (Treatment 7) and absence (Treatment 8) of SPATT bags. However the concentration of the DTX-2 like compound was double in the treatment with SPATT bags compared to the treatment which did not contain SPATT bags while the mussels were immersed in the dissolved DST seawater medium.

Thalassiosira weissflogii density remained constant over 18 hrs when not subjected to dissolved DST in the seawater medium. By contrast *T. weissflogii* density was reduced by half the initial concentration when subjected to dissolved DST in the seawater medium, particularly in the treatment that contained SPATT bags (Table 5.3). There was no difference in ingestion rate in the presence or absence of SPATT bags, therefore the results were pooled. *M. edulis* diatom cell ingestion rate declined from 1,064 cells/hr in the first 2 hrs to 128 cells/hr in 18 hrs.

5. 4. Discussion

5. 4. 1. Field Study

The results of the present study show that significant concentrations of DST (ranging from 0.11 to 0.34 μg OA+DTX-1/SPATT bag) can be found dissolved in Derwent River seawater in the presence of *D. acuminata* and *D. fortii*. These values are comparable to those found by Fux *et al.* (2009) and MacKenzie *et al.* (2004) (1.3 – 17 μg OA/SPATT bag and 0.01 – 0.06 μg OA/SPATT bag, respectively) on the west coast of Ireland and at Queen Charlotte Sound, New Zealand, respectively, in the presence of *D. acuta* and *D. acuminata*. DTX-2 was present in SPATT from Ireland (0.09 – 3.4 μg/SPATT bag) (Fux *et al.* 2009).

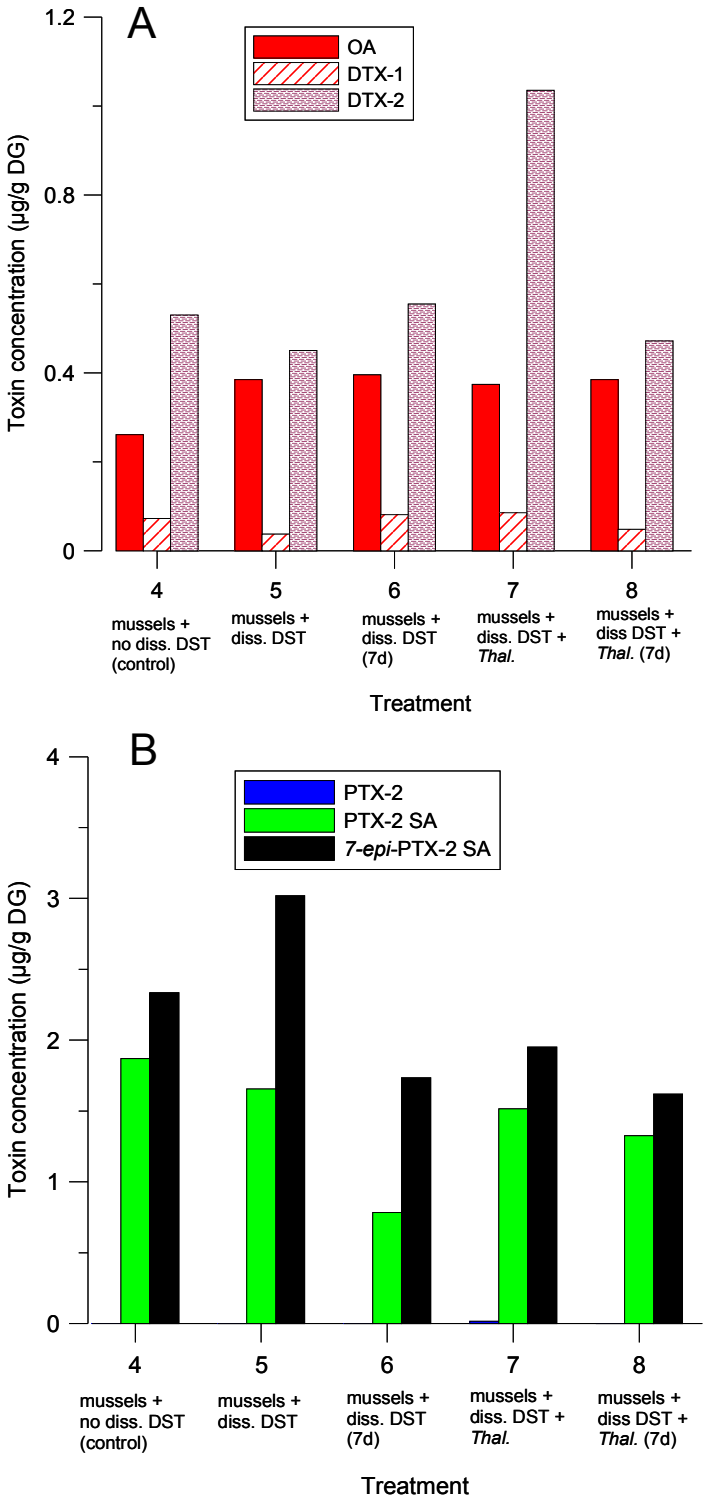

Figure 5.6. DST profile of A) OA, DTX-1and a possible DTX-2 compound; B) PTX-2 and PTX-2 derivatives; of *M. edulis* subjected to various treatments. Treatments refer to the condition mussels were exposed to in beakers containing 400 mL of filtered seawater medium. FSW = filtered seawater; diss. = dissolved; *Thal.* = *Thalassiosira weissflogii*; d = days.

Table 5.3. *Thalassiosira weissflogii* diatom cell density and mussel ingestion rate of *T. weissflogii* ± standard error when exposed to dissolved toxins.

Time (hrs)	0	2		13		18	
Treatment	Initial density (cells/mL)	Density (cells/mL)	Mussel ingestion rate (cells/hr)	Density (cells/mL)	Mussel ingestion rate (cells/hr)	Density (cells/mL)	Mussel ingestion rate (cells/hr)
Filtered seawater	2500			3070		2460	
Dissolved toxins without SPATT bags	2500			2180		1940	
Dissolved toxins with SPATT bags	2500	2910		1140		1180	
Mussels in dissolved toxins	2500		1064 ± 84*		185 ± 8*		128 ± 2*

* Ingestion rate results from mussels in dissolved toxins with and without SPATT bags were pooled as there was no significant difference between the two treatments: n = 3.

A negative relation between uptake of OA+DTX-1 in SPATT bags and those found in *Mytilus edulis* was observed. Compared to *M. edulis* DST levels at Sullivans Cove, the toxin composition in the seawater medium included a higher PTX-2 to PTX-2-SA ratio (9:1) compared to that found in mussels (1:13). A high ratio of PTX-2 to PTX-2-SA in the seawater medium compares well to toxin composition of *D. acuta* (10:1 and 12:1) and *D. acuminata* (34:1) cells in New Zealand (MacKenzie *et al.* 2005, Suzuki *et al.* 2001a,b). The lower ratio of PTX-2 to PTX-2-SA in *M. edulis* can be explained by the ability of shellfish to metabolise PTX-2 to PTX-2-SA and the capacity of the digestive gland to retain PTX-2-SA (Suzuki *et al.* 2001a,b).

These results indicate that *M. edulis* are able to take up the DST in dissolved form. Although there was no clear correlation between *Dinophysis* density and dissolved DST in the seawater medium, the likely origin of the dissolved toxin was either from the *Dinophysis* cells or depurating mussels.

5. 4. 2. *P. lima* laboratory experiments

P. lima cultures are known to produce OA, DTX-1, DTX-2 (rarely and in trace amounts) and DTX-4 (Hu *et al.* 1993, 1995; Bravo *et al.* 2001) and have been observed to release toxins into the seawater medium (Rausch De Traubenberg and Morlaix 1995). The DTX-2 compound is unique to European strains of *D. acuta* (Vale and Sampayo 2000) and *P. lima* (Hu *et al.* 1993). This study established that Tasmanian *P. lima* (strains PLLSP and PLLV) contain OA, DTX-1 and PTX-2. This is the first reported occurrence of PTX-2 associated with *P. lima*. Tasmanian strains produce a higher amount of toxin per cell compared to other strains examined (Table 5.4). The toxin concentration of OA in the *P. lima* Little Swanport strain was 10 times greater than that in the Louisville Point strain, which was the only culture containing DTX-1. The two strains exuded similar quantities of OA into the seawater medium ranging from 3 – 17 μg/SPATT bag after 40 days. The Louisville strain excreted higher concentrations of OA in the first 20 days than the Little Swanport strain and for both strains this declined to 4 μg/SPATT bag on day 40. DTX-1 was detected in the seawater medium of both cultures. Even though the Little Swanport cells did not contain DTX-1 the SPATT bags accumulated DTX-1 at low concentrations of 0.5 μg/bag. The OA levels in cells of the *P. lima* Louisville Point strain may have been

lower due to either a slower cellular reproduction or due to cellular resources redistributed to produce greater concentrations of DTX-1.

Table 5.4. Toxin levels in *Prorocentrum lima* from various global locations.

Strain origin	OA (pg/cell)	DTX-1 (pg/cell)	PTX-2 (pg/cell)	Reference
France (Marseille)	1.9	0.8	N/A	Barbier *et al.* 1999
Japan	4	N/A	N/A	Murakami *et al.* 1982
Mexico	5.2	N/A	N/A	Heredia-Tapia *et al.* 2002
New Zealand	6.3	N/A	N/A	Rhodes et al. 2006
Spain (Galician Coast)	0.19 – 12.87	1.01 – 12.45	N/A	Bravo *et al.* 2001
Spain	5 – 26	N/A	N/A	Lee *et al.* 1989
Spain	14.3	2.7	N/A	Barbier *et al.* 1999
Australia (Heron Is.)	1.31 – 5.88	N/A	N/A	Morton and Tindall 1995
Australia (Little Swanport – Tas)	25 – 53	2 – 14	N/A	Pearce *et al.* 2001
Australia (Little Swanport – Tas)	**1.7 – 36**	**N/A**	**0.4**	**Current study**
Australia (Louisville Point – Tas)	**3.8**	**0.8**	**0.1 – 1.2**	**Current study**

DST concentrations of *P. lima* cells and dissolved toxins in the seawater medium of the two strains increased (OA) or were stable (DTX-1) from day 7 to day 20, then declined by day 40. PTX-2 was detected in the cells of the *P. lima* strains but was not present in the seawater medium and appeared to decline over 20 days. Increased toxin concentration over the first 20 days may be due to a high rate of cellular reproduction immediately after inoculation. The decline in toxin concentration at day 40 may be due to a slower reproduction as the cell density approached a plateau due to limiting nutrients.

The controlled *P. lima* cell density experiment (Figure 5.4) displayed a general increase of OA + DTX-1 concentration in the seawater medium over time at 600, 1200 and 2400 cells/L. However the Little Swanport *P. lima* strain at 1200 and 2400 cells/L displayed a decrease in OA + DTX-1 concentration from day 20 to day 40. Lower densities of *P. lima* appeared to produce greater dissolved DSP toxins in the

seawater medium except for on day 20 of the Little Swanport strain and day 40 of the Louisville Point strain which show that dissolved toxins in the seawater medium of 1200 cells/L tank was higher in OA + DTX-1 concentration than the seawater medium from the 600 cells/L tank.

5. 4. 3. Mussels exposed to dissolved DSP toxins

The results from the eight treatment regimes (Table 5.3) to determine if mussels are able to take up dissolved DST from the seawater and/or if mussels are able to excrete dissolved toxins into seawater indicate that mussels depurate DST into seawater and may be able to take up dissolved DST. SPATT bags detected very low concentrations of OA (0.01 µg/SPATT bag) and PTX-2 SA + *7-epi* PTX-2 SA (0.03 µg/SPATT bag) in beakers that contained natural mussels immersed in filtered seawater (Treatment 4) which did not contain dissolved toxins in the seawater. It is possible that the mussels may have contained low concentrations of *Dinophysis* species in the byssial threads. The presence of PTX-2 derivatives (PTX-2 SA and *7-epi* PTX-2 SA) in the seawater medium strongly suggests that the DST originated from the shellfish as these toxins appear to be metabolites produced by shellfish (Suzuki *et al.* 2001a, b) while they were not detected in DST producing phytoplankton. PTX-2 SA and *7-epi* PTX-2 SA were also not detected in the medium from *P. lima* cultures (Treatment 2). The presence of PTX-2 derivatives in the seawater at Sullivans Cove (Figure 5.2A) confirm that the PTX-2 SA and *7-epi* PTX-2 SA were exuded from mussels. Although the DTX-2 like compound was not detected in *P. lima* cells or SPATT it was detected in mussels from all treatments. Mussels either accumulated the DTX-2 like compound from *P. lima* prior to collection as the toxin was present in the control (Treatment 4) or more likely it was a metabolite produced from DST storage in mussels during a period of stress/lack of food. Due to the lack of DTX-2 standard, the compound was identified based on known literature MS/MS transitions and analysis of a Certified Reference Material containing DTX-1 as a comparison. Estimated concentrations are based on applying a relative response factor (relative to OA) determined by McNabb *et al.* (2005).

DST concentration of OA in mussel immersed in dissolved toxins in seawater medium (Treatment 5 – 8) increased by more than 0.11 µg/g DG for all treatments

compared to the control (Treatment 4). There was no significant increase in DTX-1, DTX-2 like compound, PTX-2 SA and *7-epi* PTX-2 SA in mussel immersed in dissolved DST in the medium (Treatment 5 – 8) compared to the control (Treatment 4). Dissolved OA in the seawater medium containing mussels after 48 hrs (Treatment 5 and 7) displayed a very low decrease of more than 0.02 µg/SPATT bag compared to the control containing dissolved DST in the medium in the absence of mussel and algae (Treatment 2). However PTX-2 derivatives (PTX-2 SA + *7-epi* PTX- 2 SA) were detected only in the seawater medium that contained mussels and therefore provides further support that PTX-2 is metabolised to PTX-2 SA and *7-epi* PTX-2 SA in mussels and exuded into the seawater from shellfish.

It could be possible that the small increase in OA in mussels immersed in dissolved toxins may have been due to uptake of the dissolved OA from the seawater. The possible explanation for no observed increase of DTX-1, PTX-2 SA and *7-epi* PTX-2 SA in mussels immersed in dissolved toxins could be due to the absence of DTX-1 and PTX-2 in the seawater medium. Hence the production of these compounds in mussels most likely were from stored DST of a previous exposure to toxic phytoplankton retained by the digestive gland. Shellfish in marine farms in Ireland did not display any increase in DST in the presence of dissolved toxins in the seawater medium (Fux *et al.* 2009). Field studies conducted at Sullivans Cove and by Fux *et al.* (2009), MacKenzie *et al.* (2004) have shown that large quantities of dissolved DST (Table 5.5) can be present in seawater.

Table 5.5. Dissolved DST detected by SPATT bags at various field locations.

Location	Toxins µg/SPATT					Reference
	OA	DTX-1	DTX-2	PTX-2	PTX-2 SA	
Tasmania, Australia	**0.26**	**0.08**		**0.27**	**0.04 µg**	**Present study**
Ireland	17		3.4 µg	3.8	0.15	Fux *et al.* (2009)*
New Zealand	0.06	0.03		0.07	0.04	MacKenzie *et al.* (2004)*

*Note: Values referenced in Fux *et al.* (2009) and MacKenzie *et al.* (2004) were given as ng/g and ng, respectively.

SPATT bags in Treatments 6 and 8 were added after the mussels were removed from the beakers for two purposes. The first was to compare DST in mussel immersed in dissolved toxins with SPATT bags (Treatments 5 and 7) and without SPATT bags (Treatments 6 and 8) to determine if SPATT bags competed with mussels for toxin uptake. The second purpose was to ensure that the seawater medium that the mussels were exposed to contained dissolved DST. The higher toxin concentration in SPATT bags from Treatments 6 and 8 was likely to have resulted from the longer immersion time (7 days) in the medium compared to 48 hrs (Treatments 5 and 7). The DST in mussels showed no significant decrease in OA and DTX concentration when treatments with SPATT bags were compared to treatments without bags during the 48 hr period. This suggests that although SPATT bags are a useful monitoring tool for DST, even in confined laboratory experiments it did not appear to aid in the reduction of DST in shellfish by binding the toxins to the beads and therefore reducing the amount of dissolved DST in the seawater medium which may be available to be taken up by the shellfish.

5. 4. 3. Effect of food availability on mussel toxins

Availability of phytoplankton has been suggested to increase shellfish metabolism and remove toxins from the shellfish (Haamer *et al.* 1990, Marcaillou-Le Baut *et al.* 1993, Haamer 1995, Blanco *et al.* 1999, Svensson 2003) which make it difficult to determine if shellfish can take up dissolved DST from the seawater medium in natural conditions.

In the present laboratory study mussels were immersed in seawater containing dissolved DST with no algae (Treatments 5 and 6) and containing *Thalassiosira weissflogii* (Treatments 7 and 8) to determine if mussels could take up dissolved toxins from the seawater in *in-vitro* conditions and if the availability of food particles affected toxicity in mussels immersed in seawater containing dissolved toxins. The slight increase of OA concentration in mussels immersed in dissolved toxins may have been due to uptake of the dissolved OA in the seawater.

The addition of *Thalassiosira weissflogii* to the beakers did increase DTX-2 like compound levels but did not affect other toxins. The increase suggests that feeding increased metabolism and therefore increased the production of the DTX-2 compound. It has been suggested that availability of food particles may increase

shellfish metabolism and increase the rate of depuration (Haamer *et al.* 1990, Marcaillou-Le Baut *et al.* 1993, Haamer 1995, Blanco *et al.* 1999, Svensson 2003). Although the DTX-2 like compound increase was the only variation of toxin concentration in mussels observed between the absence and presence of available food particles in this study the time period of 48 hrs and the cell densities may not have been sufficient for the shellfish to feed, metabolise and depurify. The mussel ingestion rates of *T. weissflogii* during the first 18 hrs when mussels were immersed in the medium containing dissolved toxins and *T. weissflogii* declined over time (Table 5.3). This decline can be attributed to reduction in diatom cell densities which were not replenished during the 48 hrs. The density of *T. weissflogii* subjected to dissolved DST in the seawater medium treatment declined over 18 hrs compared to the treatment which were not subjected to dissolved DST and which remained at the initial concentration. This may indicate that the DST acted on *T. weissflogii* cells to reduce their numbers by inhibiting diatom growth and disrupting cell function causing cellular decay as indicated by the studies of Windust *et al.* (1996, 1997) and Snugg and VanDolah (1999). This reduction of diatom cell density does not appear to be due to lack of nutrients in the medium as densities did not decline in the control without dissolved toxins. The increase of 570 cells/mL during the first 2 hrs observed for *T. weissflogii* in filtered seawater may be attributed to cellular reproduction when the cells were transferred to the beakers.

5. 5. Conclusion

The present experiments using Solid Phase Adsorption Toxin Tracking have demonstrated that dissolved diarrhetic shellfish toxins in the seawater medium represent exudates from shellfish and toxic dinoflagellates *(Dinophysis acuminata*, *D. fortii* and *Prorocentrum lima*). The presence of PTX-2 derivatives (PTX-2 SA and *7-epi* PTX-2 SA) in the seawater medium would indicate that these toxins originate from shellfish. These products can only be produced as metabolites by shellfish as they have never detected in DST producing phytoplankton. The *in-vitro* experiments with *P. lima* exudates confirmed that dissolved DST are exudates from both shellfish and the toxic dinoflagellates and that shellfish were the only producers of dissolved PTX-2 derivatives (PTX-2 SA and *7-epi* PTX-2 SA) in the seawater medium.

OA and DTX-1 were the main toxins produced by *P. lima* cultures. *P. lima* also produced small concentrations of PTX-2. This is the first known study to associate *P. lima* with the production of PTX-2. Cellular and exuded toxicity of *P. lima* appear to vary significantly between strains isolated from different locations in Tasmania, Australia. Cellular toxins were greater in the Little Swanport (PLLSP) strain (36 pg/cell) compared to the Louisville Point (PLLV) strain (3.6 pg/cell), however exuded toxins were greater in the PLLV strain (18 µg OA/SPATT) compared to the PLLSP strain (11 µg OA/SPATT).

The DST appeared to have a detrimental effect on the diatom *T. weissflogii* causing a reduction in the density of the diatom thus confirming the suggestion by Windust *et al.* (1996, 1997) and Snugg and VanDolah (1999) that DST produced by dinoflagellates (*Dinophysis* species and *P. lima*) can act as allelochemical against other phytoplankton.

The *in-vitro* experiments with mussels immersed in dissolved DST produced from *P. lima* cultures led to an increase of OA by more than 0.11 µg/g DG in mussels and suggests that mussels can accumulate toxins dissolved in the seawater medium. A DTX-2 like compound was detected in mussels. The toxin was absent from *P. lima* and dissolved toxins in the seawater indicating that the compound was a metabolite produces by mussels. Such dissolved DST may pose a further risk to shellfish farms if mussels are able to take up the toxins in the dissolved form in the same manner as ingesting toxic algae although this has not yet been confirmed from field studies such as those conducted on the west coast of Ireland (Fux *et al.* 2009).

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Chapter 6:
Conclusions

DST in Tasmanian *Dinophysis* and mussels

The present study established that two known Diarrhetic Shellfish Toxin (DST) producing dinoflagellates, *Dinophysis acuminata* and *D. fortii*, occurred at high densities (up to 7,380 cells/L and 500 cells/L, respectively) at Sullivans Cove during the spring to early autumn seasons. These two species are the likely cause of DST levels reaching 0.5 µg/g DG of OA + DTX-1 (above the quarantine level of 0.20 µg/g DG) in local (non commercial) blue mussels (*Mytilus edulis*) rendering these shellfish unsafe for human consumption. PTX-2, PTX-2 SA and *7-epi*-PTX-2 SA were also detected in mussels, with the PTX-2 derivatives (PTX-2 SA and *7-epi*-PTX-2 SA) often producing higher peaks compared to OA and DTX-1. In the absence of evidence of human oral potency of PTX (Mackenzie 2002, Burgess 2003, Miles *et al.* 2004) this toxin is universally not regulated. The toxicity of mussels in this study was 2 and 20 times the levels previously reported by TSQAP (Table 1.2) and SASQAP (Table 1.3), respectively. These monitoring programs have shown that *Dinophysis* concentrations are regularly above the action level during spring – summer in both Tasmania and South Australia. Although there exist no commercial shellfish farms in the upper Derwent River, future surveys of the commercial farms in the neighbouring d'Entrecasteaux Channel are warranted. Toxin levels were considerably lower than levels in European shellfish (12.5 µg OA/g DG in mussels (Vale and Sampayo 2002); 6.3 µg OA + DTX-2/g DG in mussels (Carmody *et al.* 1996); 2.6 µg OA equiv./g in mussels (Svensson *et al.* 2000)), however differences in regulatory limits and continuing improving analytical methods make it difficult to compare toxicity on a global basis, thus a uniform international standard is warranted to improve trade regulations as they relate to biotoxins in exported shellfish.

By comparison, *D. truncata* was present at high densities (up to 1850 cells/L) at Parsons Bay, and was considered to be non- or weakly toxic. No toxic blue mussels were present there from January 12 to April 5, 2004, and therefore these commercial mussels were safe for human consumption. A low level of DST (below 0.12 µg/g DG) was present in Parsons Bay *M. edulis* and was likely due to a previous bloom of a known toxic *Dinophysis* species.

***Dinophysis* morphotaxonomy and phylogenetics**

Tasmanian *D. fortii* and New Zealand *D. acuta* sequences of the 28S rDNA gene were indistinguishable and also indistinguishable from those of *D. fortii* from France and *D. acuta* from the North Atlantic. New Zealand *D. acuta* strongly resembles Tasmanian and Japanese *D. fortii* morphologically, suggesting that local species designations have been confounded and it seems likely that these two dinoflagellates belong to the same taxon *D. fortii*. The OA:PTX ratio of 1:9 and the absence of DTX-2 (a toxin uniquely associated with European *D. acuta*) in New Zealand *D. acuta* compared to OA:PTX (2:5) in Tasmanian *D. fortii*, lends further support that these species designations have been confounded. OA:PTX ratios of 1:3 in European *D. acuta* are lower than New Zealand *D. acuta*. Tasmanian *D. acuminata* is genetically and morphologically indistinguishable from European and New Zealand *D. acuminata*. Due to subtle variations among *Dinophysis* morphotypes accurate identification and knowledge of toxin profiles of *Dinophysis* species is important in establishing regulatory limits for each species and to identify levels of threat to which shellfish farms are exposed.

Accumulation and depuration kinetics

Currently in Tasmanian shellfish farms toxin testing is only conducted with mussels which generally accumulate higher toxin levels. Monitoring of shellfish testing by TSQAP and SASQAP indicate that differences in species specific toxin accumulation exist and could have important implications for the harvest of shellfish. Monitoring data from TSQAP and SASQAP (Table 1.2, 1.3) suggest that mussel and cockles are more sensitive in accumulating DST compared to scallops and oysters. Mussels and cockles contained 0.025 and 0.019 µg OA/g, respectively during the absence of *Dinophysis*, while it required 1,400 – 1,500 cells/L of *D. acuminata* for oysters to reach 0.014 – 0.026 µg OA/g. During a bloom of *D. fortii* (2,100 cells/L) in Tasmania, scallops contained 0.038 µg/g of PTX, while no other DST was detected. Species specific differences raise the possibility that some shellfish species could be harvested during periods when other species more affected by DST algal producers are banned from harvest. However, further study of the mechanisms of species

specific toxin accumulation and depuration are required to determine any differences which could potentially be advantageous to the shellfish industry.

Access of toxic *Dinophysis* species to the blue mussel diet at Sullivans Cove was reduced by placing mussels in cages with a mesh size of 38 μm which screens out *Dinophysis* plankton cells. The reduction of toxic dinoflagellates from the mussel diet increased the ratio of nontoxic to toxic phytoplankton which prevented toxin accumulation and produced a depuration rate of 2 % per day of OA + DTX-1 over 15 days, no further depuration occurred from day 15 to 30, which was followed by a further detoxification of 5 % per day from day 30 to 43. PTX-2 and derivatives decreased at 4 % per day from day 15 to 30 after which no further change occurred. Mussels displayed biphasic depuration with a faster rate of PTX loss over the first 30 days followed by an increase of OA + DTX-1 depuration once there was no further change in PTX levels.

The depuration rate of 1 % per day in *M. edulis* at Parsons Bay in natural conditions was likely to be related to impoverished phytoplankton abundance available to *M. edulis*.

P. lima **cellular and dissolved DST**

The experiments using Solid Phase Adsorption Toxin Tracking demonstrated that dissolved diarrhetic shellfish toxins in the seawater medium are exudates from shellfish and toxic dinoflagellates *(Dinophysis acuminata*, *D. fortii* and *Prorocentrum lima*). The presence of PTX-2 derivatives (PTX-2 SA and *7-epi* PTX-2 SA) in the seawater medium would indicate that the toxins originated from the shellfish as these toxins appear to be metabolites exclusively produced by shellfish (Suzuki *et al.* 2001a, b) and do not appear to be detected in DST producing phytoplankton. *In-vitro* experiments confirmed that dissolved DST are exudates from both shellfish and the toxic dinoflagellates and that shellfish were the only producers of dissolved PTX-2 derivatives (PTX-2 SA and *7-epi* PTX-2 SA) in the seawater medium. The *in-vitro* experiments with mussels immersed in dissolved DST produced from *P. lima* cultures resulted in an increase of OA by more than 0.11 μg/g DG in mussel during 48 hrs and indicates that mussel may be able to accumulate toxins dissolved in the seawater medium. The dissolved DST may pose a further risk to shellfish farms if mussels are able to take up the toxins in the dissolved form in the same manner as ingesting toxic

algae although this has not been conclusively indicated from field studies (Fux *et al.* 2009). However, field studies of dissolved DST in the seawater medium and mussel toxicity are influenced by other factors such as currents, and the food availability of non-toxic algae which has been indicated to affect toxin accumulation and depuration rates in mussels (Haamer *et al.* 1990, Marcaillou-Le Baut *et al.* 1993, Haamer 1995, Blanco *et al.* 1999, Svensson 2003). The addition of the non-toxic diatom *T. weissflogii* to beakers containing mussel immersed in dissolved DST did not affect mussel toxicity during the experimental period. The DST appeared to have a detrimental effect on *T. weissflogii* causing a reduction in the density of the diatom supporting the suggestion by Windust *et al.* (1996 and 1997) Snugg and VanDolah (1999) that DST are produced by the dinoflagellates (*Dinophysis* species and *P. lima*) to act as allelopathic agents against other phytoplankton to reduce competition for available nutrients.

OA and DTX-1 were the main DST produced by *P. lima* cultures. Cellular and exuded toxicity of *P. lima* appear to vary between strains isolated from different locations in Tasmania, Australia. Cellular toxins were 10 times greater in the Little Swanport (PLLSP) strain compared to the Louisville Point (PLLV) strain, however exuded toxins were 7 – 10 µg/SPATT bag greater in the PLLV strain during the first 20 days. Tasmanian strains produce 2 – 12 times more OA per cell compared to other global strains examined (Table 5.4), suggesting a potential DSP risk for East Coast Tasmanian shellfish farms. Environmental factors such as nutrient and light conditions and the phytoplankton community of the region the cells originated may affect the cellular production of toxins and the rate at which the toxins are exuded from the cells.

A DTX-2 like compound was detected in *in-vitro* mussels. This toxin was absent from *P. lima* and dissolved toxins in the medium indicating that the DTX-2 like compound was a DST metabolite produced by mussels. This toxin needs further chemical characterisation to determine its biological origin.

In conclusion, these results indicate that DST in south-east Tasmania could pose a threat to public health and the economy of the shellfish industry but to a lesser extent than European DST contamination of shellfish industries. Thus increased monitoring for DST needs to continue and be expanded to all commercial Tasmanian shellfish growing leases. TSQAP is currently focused on PSP by *Gymnodinium*

catenatum but DSP by *Dinophysis* or benthic *P. lima* could pose new problems due to climate change induced shifts in harmful algal blooms. Water temperature, currents, rainfall and ocean acidification changes have already been shown to expand the range and change the dominance of some harmful algae in temperate regions (Hallegraeff 2010). Further understanding of factors that affect *Dinophysis* toxicity, dissolved DST toxicity in seawater solution and mechanisms for toxin accumulation and depuration kinetics in shellfish would be invaluable for future shellfish management programs.

Future Work

- DST accumulation in mussels during the presence of *D. truncata* at Parsons Bay warrants toxin and genetic characterisation of this little known dinoflagellate species to be conducted.
- DTX-2 is a toxin compound unique to *D. acuta*, some *P. lima* and shellfish in Europe. The detection of a DTX-2 like compound in *in-vitro* mussels suggests the toxin was a metabolite. Further work is required to characterise the compound and determine its origin and toxicity.
- *In-vitro* studies indicate that mussels can take up toxins dissolved in the seawater. Further trials are required in the field to determine the effect this hazard may have on shellfish farms.
- Significant toxicity differences among dinoflagellate species and strains indicated by the *P. lima* experiments in this study suggest that extensive cellular toxin studies are required for toxic algal strains and phytoplankton species regulatory limits to be potentially adjusted regionally.
- Toxins in the seston can cause significant overestimates of *Dinophysis* toxin content per cell and suggests that monitoring programs should measure seston toxin levels.
- Shellfish species specific toxin accumulation and depuration studies are required to aid shellfish farmers in establishing species specific regulation which can reduce the impact of DST on the shellfish industry.

These proposed studies would aid in minimising the risk of DST impacts on the shellfish industry and public health.

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Appendix

Table A.1. HPLC-MS/MS precision (% Relative Standard Deviation) of DSP toxins calculated from control standards.

	Control standards of toxins (ng/ml or ug/L)				
	OA	DTX-1	PTX-2	PTX-2 SA	*7-epi* PTX-2 SA
	1003.0	172.5	2.2	3.9	33.0
	1032.3	165.8	2.1	3.6	32.7
	946.6	125.7	1.9	3.4	31.4
	999.2	142.0	2.2	3.8	34.5
	1021.7	144.0	2.2	3.8	34.0
mean	1000.5	150.0	2.1	3.7	33.1
Standard Deviation	33.1	19.0	0.2	0.2	1.2
% Relative Standard Deviation	**3.3**	**12.7**	**7.4**	**4.8**	**3.7**

Table A.2. Ryan-Einot-Gabriel-Welsch (REGWQ) groupings of DST in caged and non-caged mussels.

Group		Mean (µg/g DG)			REGWQ groups		
		OA	PTX-2	Total PTX-2 SA	OA	PTX-2	Total PTX-2 SA
Day 0	Control	0.14	0.05	1.04	C	A B	A
Day 15	Control	0.22	0.10	0.92	B	A	A B
	Cage 1	0.11	0.02	0.19	D C E	B	C
	Cage 2	0.10	0.02	0.25	D C E	B	C
	Cage 3	0.12	0.03	0.27	D C E	A B	C
	Cage 4	0.10	0.02	0.19	D F E	B	C
	Cage5	0.08	0.04	0.32	D C E	A B	C
Day 30	Control	0.43	0.05	0.68	A	A B	A B C
	Cage 1	0.06	0.004	0.10	G F	B	C
	Cage 2	0.11	0.02	0.14	D C E	B	C
	Cage 3	0.12	0.01	0.10	D C E	B	C
	Cage 5	0.12	0.01	0.09	D C E	B	C
Day 43	Control	0.08	0.02	0.29	F E	B	C
	Cage 1	.	0.01	0.04	.	B	C
	Cage 2	.	0.004	0.04	.	B	C
	Cage 3	0.04	0.01	0.11	G H	B	C
	Cage 5	0.03	0.004	0.10	G H	B	C
Day 65	Control	0.06	0.02	0.37	G F	B	B C
	Cage 1	0.02	0.004	0.09	H	B	C
	Cage 2	0.01	0.01	0.07	H	B	C
	Cage 3	0.02	0.004	0.05	H	B	C
	Cage 5	0.02	0.02	0.21	H	B	C