

Risk Assessment of Marine Algal Toxins on Humans and Dugongs

A thesis submitted by

Eri Takahashi
MPhil Marine Biology
Griffith University

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Griffith School of Environment
Griffith University
Australia
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Declaration of Originality

The work presented in this thesis, and the research to which it pertains is, to the best of my knowledge and belief, original. Specific contributions made by others are referred to in the Acknowledgement and in the text. This material has not been submitted either in whole or in part, for a degree at this or any other university.

Signed.....

Eri Takahashi

Signed.....

Des Connell (Principal Supervisor)

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Publications by the Candidate Relevant to the Thesis

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Abstract

Algal toxins can have a significant impact on human and ecological health as the toxins accumulate in the food chain and are consumed by both humans and marine organisms. This study focussed on the following marine algal toxins that were present at the study sites: okadaic acid (OA), domoic acid (DA), gymnodimine (GD), pectenotoxin-e (PTX-2) and PTX-2 seco acid (PTX-2SA).

The study sites investigated for potential algal toxin exposure were selected from the waters around North Stradbroke Island, Queensland, Australia, where shellfish are harvested by the local population, and where dugongs are known to feed on seagrass. Samples were collected monthly for two consecutive years. The species of toxin-producing algae present at the sites studied were *Pseudo-nitzschia* sp., *Dinophysis caudata*, *D. acuminata* and *Prorocentrum lima*. The occurrence of *Dinophysis* species was observed to be dependent on the season while *Pseudo-nitzschia* sp was present both in colder and warmer months.

Data on the dose-response analysis were extracted from published literature. This data was categorised into whole organisms, human and animal cell lines, and compared to one another. For further toxicodynamic studies, human cell lines were dosed with known concentrations of the toxins: OA, DA and GD. These cytotoxicity and microarray analyses were performed to observe the effects of toxins on gene regulation. A more extensive analysis was performed using GD alone. Expression of numerous genes was affected, and real time polymerase chain reaction reactions were performed to confirm the regulation of those genes. Gymnodimine was demonstrated to affect genes within pathways relating to oxidative phosphorylation, apoptosis, MAPK and Wnt signalling pathways.

The cytotoxicity and microarray data and the data accumulated from the published literature were combined to form a comprehensive database of both chronic and acute effects. The database was then referred to for the dose-response analysis for the risk assessment. The exposure data attained from field sampling in the current study was analysed against the doses for any shown effects. Total daily intake for humans and dugongs sourcing food from around the island were calculated and health risks were

estimated by incorporating tolerable daily intake, guideline values and total daily intake.

The risk characteristics of algal toxins on the health of humans (consuming shellfish) and dugongs (consuming seagrass) indicated that acute health risks were unlikely, unless an outbreak of toxic algae (algal bloom) were to occur. Since there were no occurrences of algal blooms during the study period, high levels of toxins were not detected in any of the collected shellfish, phytoplankton or seagrass samples. However, if such blooms were to occur around the island, the phytoplankton could potentially produce algal toxins at high enough concentrations to cause acute toxic effects in the consumers.

The current study has also demonstrated that there is a potential for chronic, long term health effects from consuming shellfish and seagrass around the island. The presence of low-level concentrations of algal toxins in the food sources can lead to chronic effects. Toxins such as OA are known tumour promoters. For dugongs, which feed on seagrass on daily basis, the potential for chronic effects is high. It was demonstrated that GD also possesses toxicological characteristics that may enhance the possibility of tumour promotion because of its effect of down-regulating parts of the apoptosis pathway, which may prevent cell death and as a consequence, lead to uncontrolled cell growth.

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Abbreviations and Symbols

ASP	amnesic shellfish poisoning
DA	domoic acid
DSP	diarrhetic shellfish poisoning
GD	gymnodimine
OA	okadaic acid
PTX-2	pectenotoxin-2
PTX-2SA	pectenotoxin-2 seco acid
LD ₅₀	lethal dose (50% of individuals affected)
IC ₃₀	inhibition concentration (30% of individuals affected)
i.v.	intravenously injected
i.p.	intraperitoneally injected
SPATT	solid phase adsorption toxin tracking

AT	average time
BW	body weight
CF	contamination concentration
DE	daily exposure
ED	exposure duration
EF	exposure frequency
FI	fraction ingested
GV	guideline value
IR	ingestion rate
LOAEL	lowest observed adverse effect level
MAC	maximum allowable concentration
Max	maximum
Min	minimum
NOALE	no observed adverse effect level
RfD	reference dose
RQ	risk quotient
TDI	tolerable daily intake
TRV	toxicity reference value

K_{ow}	octanol-water partition coefficient
K_{sw}	SPATT-water partition coefficient
SG	seagrass
Wwt	wet weight

nM	nano-mole
μ M	micro-mole
μ L	micro litre
ng kg ⁻¹	nano gram per kilogram
μ g kg ⁻¹	microgram per kilogram
μ g kg ⁻¹ day ⁻¹	micro gram per kilogram per day
MU	mouse unit
°C	degree Celcius

Chapter 1 Introduction

1.1 Algal toxins

Human knowledge and avoidance of toxic seafood was recognised and practiced in pre-Columbus America (Gestal-Otero 2000). In Europe, there are descriptions of paralytic shellfish poisoning (PSP) outbreaks dating back to 1689 (Gestal-Otero 2000). Since then, various incidences of shellfish poisoning have been studied in different parts of the world (Hallegraeff 1993a). The increased awareness of these toxins among environmental monitoring groups and the increased usage of coastal areas may be responsible for an increase in the reporting of algal toxin-related outbreaks (Hallegraeff 1993a, Sellner & Doucette 2003).

Toxic algae, together with parasites, viruses and bacteria, are known agents for seafood poisoning. On a worldwide basis, marine algal toxins are estimated to be responsible for more than 60,000 intoxication incidents per year (Van Dolah 2000a). Although there are an estimated 3,400 to 4,000 known species of phytoplankton, only 60 to 80, (~2%), of the species are harmful (Smayda 1990). Diatoms and dinoflagellates can produce toxins that impact on human and ecological health; the persistent toxins they produce accumulate in organisms that consume the algae, such as shellfish, which are in turn consumed by organisms of a higher trophic level. Although a variety of marine taxa are known to accumulate biotoxins, approximately 90% of all known poisoning incidents from seafood are associated with molluscs (Soames-Mraci 1995) and the best known examples of such toxin-vector organisms are filter feeding bivalves.

Safety guideline levels for different algal toxins have been established by government regulators of various countries. These guidelines are based on acute effects established from laboratory experiments. However, studies on the effects of chronic exposure to algal toxins are limited (Van Dolah et al. 2001) and further studies are required to further understand the effects of long-term, low-level exposure to algal toxins.

Shellfish toxins may exceed government set safety levels in the water when toxic dinoflagellates are present at a density as low as 200 cells L⁻¹ (Yasumoto et al. 1985). The unpredictable incidence of toxicity has resulted in severe economic consequences for shellfish producers (Carmody 1996). Toxic marine phytoplankton blooms can

cause massive economic impacts and threaten both public health and shellfish industries (Lee et al. 1989, Pillet et al. 1995). In 1997 a *Pfiesteria piscicida* bloom in Chesapeake Bay caused a \$40 million loss for the seafood industry (Morris 1999). Between 2002 and 2003 there was a forced cessation to razor clam harvesting due to high levels of domoic acid (DA) that resulted in a \$10.4 million loss in Washington state (WDFW 1998). Some shellfish toxins, such as DA has also caused deaths in both humans and marine organisms(Perl et al. 1990).

1.1.1 Environmental aspects

Although harmful algal blooms are a natural phenomenon that have been reported in recorded history, in the past two decades, the public health and economic impacts of such events appear to have increased in frequency and intensity. There also appears to be a biogeographical expansion of toxin yielding alga species (Morris 1999). For example, between 1978 and 1982, paralytic shellfish poisoning-affected areas in Japan increased from two to ten sites (Anraku 1984). These global reports of increase in harmful algal blooms may reflect real increase in incidence, or simply better recognition and reporting (Morris 1999). There are four explanations given for such increases: increased scientific awareness, increased utilization of coastal waters, stimulation of plankton blooms by cultural eutrophication and climatological conditions, and transport by ballast water (Morris 1999, Van Dolah 2000a).

Nutrient enrichment is an international concern. Globally, between 7-35 million metric tons of nitrogen and 1-4 million metric tons of phosphorous are added to the environment each year (Tibbetts 1998, Glibert et al. 2004). Inorganic inputs such as phosphorous stimulate algal growths (Jones et al. 1995) and organic inputs from the food industry, such as carbohydrates, lipids and proteins increase biochemical oxygen demand, and produce ammonia and hydrogen sulphide (Jones et al. 1995). With increasing nutrient input into the water, it is likely that there will be an increasing trend in algal bloom outbreak frequencies and intensities. Intensity and occurrence of algal blooms are difficult to predict due to the nature of complex environmental and ecological influences that affect the growth and production of the toxins (Morton & Norris 1990, Sellner & Doucette 2003).

1.1.2 Types of toxins

Historically, incidences of poisoning related to dinoflagellate and diatom toxins were classified under the main categories of diarrhoetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP) and ciguatera fish poisoning or ciguatera toxins (CTX) (Table 1.1). However at the FAO/IOC/WHO workshop on biotoxins in bivalve molluscs in Dublin March 2004, it was decided to classify the toxins according to chemical structures (Toyofuku 2006). The toxins are now grouped into: Azaspiracid, brevetoxin, cyclic immines, domoic acid, okadaic acid, pectenotoxin, saxitoxin and yessotoxin (Table 1.2)

The domoic acid (DA) group, consisting of DA and its isomers are produced by diatoms of the genus *Pseudo-nitzschia* (Todd 1993, Van Dolah et al. 1997). As outlined in the recent review by Jeffery et al (2004), DA is a neurotoxin, causing neuronal damage in the hippocampal regions leading to permanent loss of short term memory, and formally classified in the ASP group. Recent studies have found eight new isomers of DA, most of which are found in, and isolated from New Zealand mussels (Seki et al. 1995, Jeffery et al. 2004).

The okadaic acid (OA) group, consisting of OA and Dinophysis toxins (DTXs) is produced by dinoflagellates *Dinophysis* and *Prorocentrum*. They are characterised by rapid onset of gastrointestinal symptoms such as vomiting and diarrhoea, generally resolving within 2-3 days, and formally belonged DSP (Van Dolah et al. 2003). Although there has not been any recorded incidences of death due to OA poisoning, OA is a known tumour promoter (Suganuma et al. 1988).

Toxins in the pectenotoxin (PTX) group were formally classified as DSP, along with OA group, but are now considered as a group of its own as they do not cause diarrhoea (Yasumoto 2000). They are produced by dinoflagellates from the genus *Dinophysis* (Draisci et al. 1996, Miles et al. 2004b).

Table 1.1 Summary of types of shellfish poisoning and the toxins causing the symptoms.

Types of shellfish poisoning	Main genus producing toxin	Toxin	Main effects	Main references
DSP diarrhoeatic shellfish poisoning	<i>Dinophysis</i> , <i>Prorocentrum</i>	Okadaic acid Dinophysis toxin	Diarrhoea, tumour promoter	Fujiki & Suganami 1991
ASP amnesic shellfish poisoning	<i>Pseudo-nitzschia</i>	Domoic acid	Dizziness, memory loss, seizure, death	Van Dolah 2000
PSP paralytic shellfish poisoning	<i>Alexandrium</i> , <i>Gonyaulax</i>	Saxitoxins	Respiratory distress, death	Yasumoto et al. 1985
NSP neurotoxic shellfish poisoning	<i>Karenia</i>	Brevetoxin	Nausea, neurosensory, neuromuscular effects	Van Dolah 2000
Ciguatera poisoning from fish	<i>Gambierdiscus</i>	Ciguatera toxin	Sensory disorder, diarrhoea	Hamilton et al. 2002
(No category)	<i>Karenia</i>	Gymnodimine	Very low oral toxicity but high potency when injected (ip)	Miles et al. 2000

Table 1.2 New classification of marine algal toxins found in molluscs.

Toxin Group	Genus producing toxins
Azaspiracid (AZA)	<i>Protoperidium</i>
Brevetoxin (BTX)	<i>Karenia</i>
Cyclic immines	<i>Karenia</i>
Domoic acid (DA)	<i>Pseudo-nitzschia</i>
Okadaic acid (OA)	<i>Dinophysis, Prorocentrum</i>
Pectenotoxin (PTX)	<i>Dinophysis</i>
Saxitoxin (STX)	<i>Alexandrium, Gonyaulax</i>
Yessotoxin (YTX)	<i>Protoceraium</i>

The cyclic immines group are comprised of gymnodimine (GD), spirolides, pinnatoxins, prorocentrolide and spirocentrimine (Toyofuku 2006). This group has a high acute toxicity in mice upon intraperitoneally (i.p.) injections, however, have low toxicity when administered orally (Munday et al. 2004). Gymnodimine is produced by dinoflagellate *Karenia selliformis* (Mackenzie & Beuzenberg 2003, Miles et al. 2003).

The azaspiracid (AZA) group of toxins is a relatively new group of toxins, reported for the first time in 1995 in Netherlands (Toyofuku 2006). The AZA group of toxins has similar symptoms to the OA group, as they cause diarrhoea. Azaspiracid is produced by dinoflagellate *Protoperidium* and have only been detected in Europe to date (James et al. 2003, Toyofuku 2006).

The brevetoxin (BTX) group is known to be responsible for NSP. This group of toxin is produced by the unarmoured dinoflagellate *Karenia breve*, that has been linked to

red tide outbreaks in the Gulf of Mexico, New Zealand and Japan (Baden & Adams 2000, Van Dolah 2000a). Due to the lack of armour in *K. breve*, its cells can easily lyse open, releasing toxin into the water column. Free toxin can be absorbed by fish through their gills and can lead to fish kills (Van Dolah 2000a).

The saxitoxin (STX) group of toxins are a family of water-soluble neurotoxins (Holmes & Teo 2002), produced by both dinoflagellates such as *Alexandrium* spp, *Gonyaulax* spp and *Pyrodinium* spp and cyanobacteria (Yasumoto et al. 1985, Van Dolah 2000a, Prati et al. 2002). STX is responsible for PSP and is the most widespread algae-derived toxin on a worldwide basis. It has been detected in both the North and the South American continents, Europe, Asia and Australasia (Van Dolah 2000a). On a global basis, 2000 cases of STX poisoning are reported each year with a 15% mortality rate (Hallegraeff 1993).

The yessotoxin (YTX) group, comprised of YTX and its analogues is produced by *Protoceratium reticulatum* (de la Rosa et al. 2001, Ciminiello et al. 2003). This group of toxins was also formally classified as DSP, however was removed from the DSP group as they do not cause diarrhoea (de la Rosa et al. 2001).

Ciguatera toxins (CTXs) have not been included in the eight categories addressed by the workshop, probably due to the fact that ciguatera poisoning occurs in fish rather than in molluscs. Several toxins can be responsible for CTX (Guzman-Perez & Park 2000). Like many other shellfish toxins, CTX presents with gastrointestinal and neurological symptoms. It causes sensory disorders in relation to temperature that may last for several months (Yasumoto 2000). These toxins are produced by the dinoflagellate *Gambierdiscus toxicus*, known to bloom in the Indian Ocean, Pacific Ocean and Caribbean Sea (Hamilton et al. 2002a, Hamilton et al. 2002b, Yasumoto 2000).

1.2 Risk Assessment

As marine phytoplankton toxins are frequently involved in human poisoning events through the consumption of seafood products, the increase of contamination of seafood by marine biotoxins imposes a specific risk factor (Sierra-Beltran et al. 1996).

A risk assessment can be used to assess the human health risk and natural ecological health risk from such toxins.

“Risk” is defined as the chance or probability of damage and a quantitative measure of probability for certain harmful effects to occur as a result of exposure (Burmester 1996, WHO 1999). The risk assessment could then be defined as the scientific characterisation of potential adverse health effects resulting from the probability of exposures to toxins (Faustman & Omenn 1995). Risk assessment has four major components: hazard identification, exposure assessment, dose-response assessment and risk characterisation (Dalefield et al. 2001).

Hazard identification is a process which conducts if the compound of concern can be related to occurrence of an adverse health effect. It is defined as the set of inherent properties of a substance or a process involving substances that is capable of causing adverse effects (WHO 1999). It answers questions such as what are the substances of concern and what are their adverse effects.

Exposure assessment involves determining the routes of the exposure and the magnitude or the frequency and the duration of the exposure (Dalefield et al. 2001). It is the dosage of a particular agent that reaches the target (WHO 1999). This is usually expressed in numerical values to measure the intensity, duration or frequency of the exposure to the agent (WHO 1999).

Dose-response assessment defines the relationship between the dose of the chemical and the potential to development adverse health effects (Dalefield et al. 2001). With the dose-response relationship, a variety of approaches are used: human observation, animal toxicology studies and assessment of structure-activity relationship (WHO 1999). In regards to human-based dose-response studies, data is mostly derived from either accidental exposures, or limits are established from conducting human equivalent calculations from data derived from animal studies. Therefore, animal testing is usually essential but there are several disadvantages.

There is uncertainty associated with the extrapolation of results from one species to another. Additionally, chronic and long-term effects are difficult to study perform and

typically, bioassays are typically limited to a small number of test animal and consequently, high doses are used, doses which far exceed typical levels of accidental human exposure (WHO 1999). It is therefore important to acquire and compile information from published material when conducting a risk assessment.

Risk characterisation examines the probability of adverse effects in humans that may arise from actual or hypothetical exposure conditions. This step involves the quantification of the risk following consideration of the exposure and dose-response relationships (WHO 1999).

Risk assessment, especially probabilistic risk assessment has been successfully used as a tool to study toxin-associated risk in different situations. For example, the probability of toxins affecting the breeding success in Ardeids in Hong Kong has been examined. The average concentration of heavy metals were obtained and transformed into a probabilistic form and then plotted as a cumulative probabilistic distribution (Connell 2003). Furthermore, probabilistic risk assessment has also been used to assess the risk posed by benzylchloride in household goods to humans (Djohan 2002).

1.3 Thesis overview

In the current project, the risks associated with the consumption of algal toxin affected shellfish and seagrasses (by humans and dugongs, respectively) were investigated. Waters around North Stradbroke Island that forms the eastern perimeter of Moreton Bay (Queensland, Australia) were chosen as the study site. Indigenous groups and the local community on the island consume a variety of shellfish found around the island. Moreton Bay is also known to accommodate dugongs and turtles, making it a suitable site for risk assessment study. The algal toxins included in the study were OA, DA, GD and some aspects of PTX-2s. A flow chart is presented that outlines the risk assessment framework used for this study (Figure 1.1). Discrete chapters will address each of these steps.

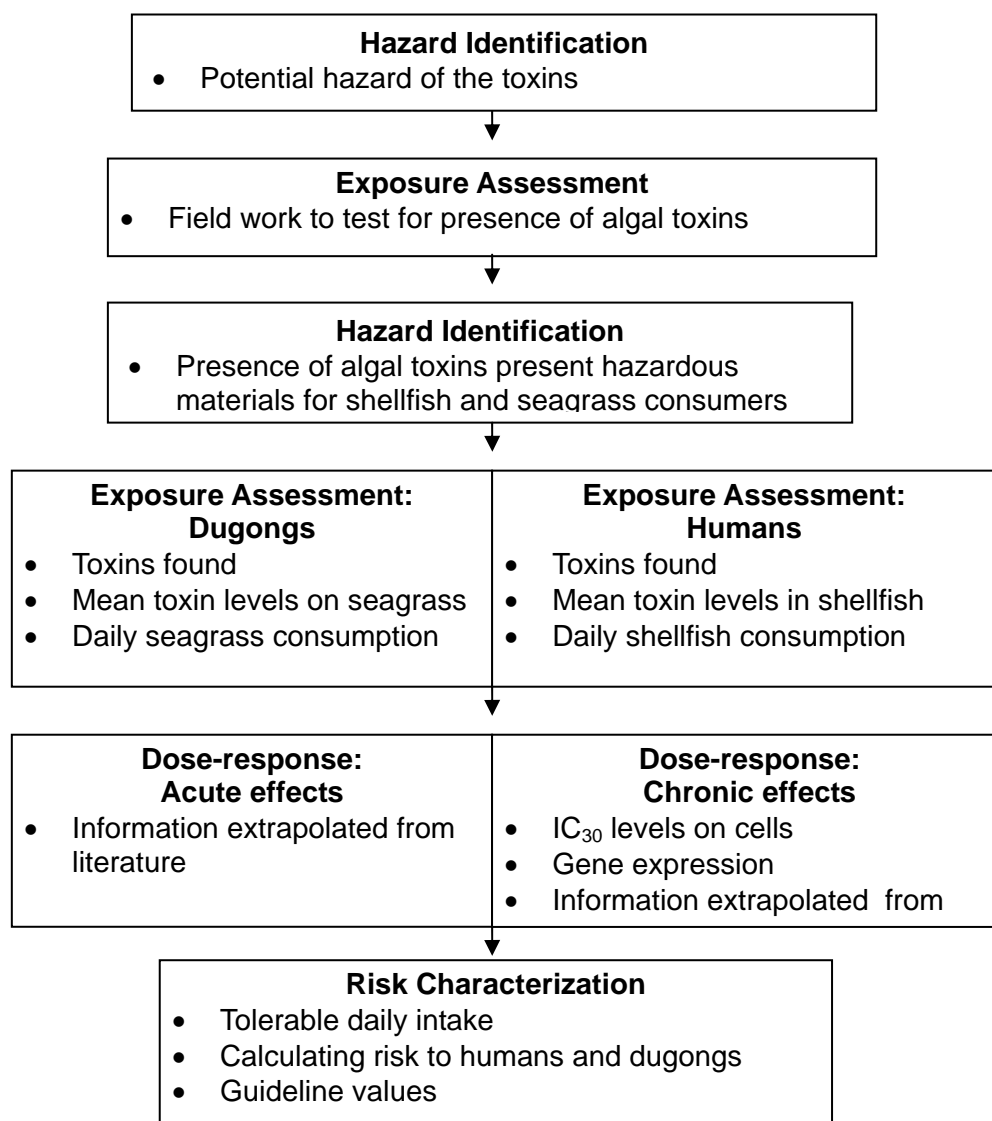


Figure 1.1 Strategy of risk assessment of algal toxins in this study.

The literature reviewed for the four toxins is discussed in Chapter 2. Those literature pertaining to PTX-2SA and GD was scarce compared to OA and DA because the toxicity of PTX-2SA is not as acute (and is therefore considered a lower risk and consequently less researched), and GD is a relatively new toxin and the known effects are not as severe as those for PTX-2SA and GD.

The third and the fourth chapters address the exposure data collected from the study sites around North Stradbroke Island. Chapter 3 discusses the exposure assessment of

algal toxins on humans consuming shellfish. In this chapter, the sampling sites, methods of sample collection, toxin extraction are discussed. The species of toxic algae and the class of toxins detected are presented. In Chapter 4, the risks posed on dugongs from consumption of seagrass with toxic epiphytes are assessed. The daily intakes are calculated and compared to the tolerable daily intakes. The same dose-response data as Chapter 2, obtained from the literature, were used.

Chapter 5 addresses the dose-response analysis. Data incorporated into the dose-response analysis are accumulated from the published literature. In Chapter 6, additional dose-response data were obtained for the cytotoxicity and mechanistic toxicity study from the microarray analysis conducted on human cell lines.

The seventh chapter presents work on the risk assessment and characterisation. For human risk assessment, the dose-response analysis from the previous chapter was used to compare the amount of toxins present in the exposure assessment, from the field data. Similarly, health risk assessment was conducted for dugongs from the seagrass data and the associated dose-response information.

Overall conclusions are presented in Chapter 8. This chapter summarises each of the other chapters of the thesis and draws conclusions on the results.

1.4 Aims and Objectives

The aim of this project was to investigate the health risks involved for both humans and dugongs, in regards to the consumption of foods contaminated with OA, DA, GD and PTX-2s. For the exposure aspect of the risk assessment, North Stradbroke Island was chosen as the study site.

To assess the risk, following objectives were considered:

- To evaluate the types, levels and distribution patterns of marine algal toxins in shellfish, seagrass and phytoplankton;
- To evaluate the potential relationships between the presence of toxins in shellfish and the abundance of toxin producing phytoplankton in the study sites;

- To evaluate the exposure assessment of humans and dugongs to marine toxins;
- To determine the dose-response relationships for algal toxins in humans and other animals from data in published literature;
- To determine the cytotoxicity levels;
- To obtain data relevant to mechanistic toxicity that may assist in investigating the long term chronic effects of the toxins;
- To conduct a risk assessment for the human health and that of dugongs.

Chapter 2 Occurrences and effects of algal toxins

2.1 Characteristics and occurrences

2.1.1 Okadaic acid group

This group of toxins contain okadaic acid (OA), Dinophysis toxin-1 (DTX-1), DTX-2 and their esters, such as DTX-3, DTX-4 and DTX-5 (Murata et al. 1982). These toxins are known to be responsible for causing diarrhetic shellfish poisoning (DSP) in humans. Toxins in this group are differentiated by their unique chemical characteristics (Lee et al. 1989, Yasumoto & Murata 1990, Draisci et al. 1996, Draisci et al. 1998, James et al. 1999a). Toxins related to OA and Dinophysis toxins (DTXs) are acidic polyether toxins. Algae that produce these toxins are dinoflagellates of the genres *Dinophysis* and *Prorocentrum*.

Toxins in the OA group are accumulated in the digestive tracts of filter feeding shellfish (Amzil et al. 1992, James et al. 1999a, Souto et al. 2001) and are a serious threat to aquaculture industries (Fernandez et al. 2000). The consumption of contaminated shellfish can cause gastroenteritis in humans (Carmody 1996). The gastrointestinal symptoms normally begin within 30 minutes to a few hours after consumption of toxic shellfish (Holmes et al. 1999a, Klopper et al. 2003). Symptoms include abdominal pain and cramps, diarrhoea, nausea and vomiting (Tubaro et al. 1996). Generally the ailment clears up within three days with or without medical treatment and there have been no reported deaths caused by DSPs (Yasumoto et al. 1978, Hallegraeff 1993a).

Although incidences of diarrhoea from consuming mussels have been reported in Norway since the 1960's (Aase & Rogstad 1997), DSP was formally recognised by Yasumoto and his colleagues in Japan in 1976 when they noticed frequent occurrence of gastroenteritis that coincided with the ingestion of shellfish during spring and summer. The presence of acetone-soluble toxins in the digestive glands of the implicated shellfish, together with the negative results for known pathogenic bacteria confirmed that it was caused by poisons in shellfish (Yasumoto et al. 1985). The toxin causing diarrhoea in Japan and Norway was later confirmed to have come from the dinoflagellates species *Dinophysis fortii* (Yasumoto et al. 1980). The toxin first detected in the hepatopancreas of toxic mussels was named Dinophysis toxin-1 (DTX-1) after its biogenetic origin (Yasumoto 2000). In 1982, Murakami and his colleagues

used the dinoflagellates species *Prorocentrum lima* to isolate the toxin thought to have caused diarrhetic effects. This toxin was found to be identical to OA isolated from sponges *Halichondria okadaic* and *H. melandocia* by Tachibana in 1981 (Yasumoto et al. 1985). There are numerous OA related incidences in various parts of the world, some of which are listed in Table 2.1.

Table 2.1 Recorded incidences of breakouts and poisoning of OA group

Location	Year	Notes	References
Japan	1976	DTX-1 first identified as cause of DSP	Yasumoto et al. 1985
Japan	1977-1982	More than 1300 human poisoning cases	Hallegraeff 1993
Spain	1981	More than 5000 human poisoning cases	Hallegraeff 1993
France	1983	More than 3300 human poisoning cases	Van Edmond et al 1983
Sweden	1984	Algal bloom causes mussel industry to close for 1 year	Hallegraeff 1993
N. America	1990	First incidence of DSP in this continent	(Quilliam et al. 1990)
Canada	1992	OA group found in <i>P. lima</i>	Marr and Jackson 1992
Australia	1997	50 cases of human poisoning by OA acyl esters - pipis	Burgess 2003
Portugal	1998	19 cases of human poisoning - clams	Sampayo et al. 2000
SE Asia	1999	First incidence of DSP in SE Asia – green mussels	Holmes et al. 1999a

Another group of toxins with similar structures to the OA group, formally associated with DSP is the pectenotoxins (PTXs). PTXs are polyether-lactones (Yasumoto et al. 1985), formally placed in the DSPs. Although no longer classified in the same group, because PTXs do not cause diarrhoea, they resembles OA in molecular weight and in having cyclic ethers and carboxyl groups, and are produced by *Dinophysis* spp. (Yasumoto 2000, Miles et al. 2004c) and for this purpose, PTXs will be included in the OA section in this review. Again, there are several PTXs, the most toxic one being PTX-2, which is converted into a less toxic form, pectenotoxin-2 seco acid (PTX-2SA) within the shellfish (Miles et al. 2004b).

The toxin PTX2-SA was first identified in New Zealand shellfish in 1997, when it was categorised as a new toxin present in shellfish foods (Daiguji et al. 1998). A shellfish-

related outbreak of diarrhoea in Australia occurred in December 1997, where 50 cases of illness were reported from the consumption of pipis (*Donax deltooides*) taken from Ballina, New South Wales (Zantiotis-Linton 1998). After several other reports of shellfish poisoning along the nearby beaches, state-wide closure of beaches for pipi harvesters was instituted until a food safety plan for biotoxins for bivalve molluscs was developed (Zantiotis-Linton 1998). The toxin responsible for this outbreak was suspected to be PTX2-SA but was later confirmed to be OA acyl esters (Burgess 2003).

2.1.2 Domoic acid

Domoic acid (DA) was originally isolated from the macroalgae *Chondria armata*, locally known as “domoi” in Japan where it was commonly used as an anthelmintic for children and also as a pesticide (Iverson et al. 1990). It was not until a toxic outbreak in Canada, in 1989, that this toxin was studied and identified as the source of amnesic shellfish poisoning (ASP) (Perl et al. 1990). During this incident, 107 people were affected including three people in their 70’s and 80’s that died after eating contaminated blue mussels harvested from Prince Edward Island (Perl et al. 1990, Lefebvre et al. 1999, Kerr et al. 2002). Between 1987 and 1991, detection of DA was reported in eastern North America. Domoic acid has also been detected in Scottish crabs in Europe (Hess 2001).

Known effects of domoic acid in animals include abdominal cramps, vomiting, diarrhoea, disorientation, short term memory loss, amnesia and even death (Perl et al. 1990, Cendes et al. 1995, Peng et al. 1997). According to the results of a survey performed on the exposed individuals, the first symptoms appeared between 15 minutes to 38 hours post-ingestion (Perl et al. 1990). Eighteen of these patients experienced a range of severe symptoms including agitation, seizures and coma (Teitelbaum et al. 1990). From these observations, it was apparent that young people were more likely to be afflicted with diarrhoea while older patients were more likely to suffer from memory loss (Perl et al. 1990).

There have been numerous DA-related events recorded since 1990, causing mass mortalities of marine mammals and birds (Table 2.2). For example, the next major

incident occurred in 1991 when more than a hundred brown pelicans (*Pelicanus occidentalis*) and Brand's cormorants (*Phalacrocorax penicillatus*) died after eating northern anchovies (*Engralis mordax*) contaminated with DA off the coasts of Monterey Bay and Santa Cruz, California (Todd 1993, Work et al. 1993, Gulland et al. 2002). Effects on seabirds included head swinging, scratching, vomiting, loss of righting reflex, clenching toes, loss of awareness, decreased responsiveness to stimuli and death (Gulland et al. 2002). Analysis of the toxin in whole anchovies showed DA concentrations ranging between $45 \mu\text{g g}^{-1}$ and $100 \mu\text{g g}^{-1}$ with the source of the toxin found to be the marine diatom *Pseudo-nitzschia australis* (Todd 1993). For other incidences in Baja California, it was suspected that stranded pelicans were eating mackerel (*Scomberomus japonicus*) containing toxic *P. australis* and *P. pungens* (Sierra-Beltran et al. 1997, Fladmark et al. 1998).

Table 2.2 Recorded incidences of outbreaks and poisoning by domoic acid.

Location	Year	Notes	References
Canada	1989	107 human victims: 3 deaths	Perl et al. 1990
California, USA	1991	More than 100 seabirds died	Todd 1993
Baja California	1996	More than 10 pelicans died	Sierra-Beltran et al. 1997
California, USA	1998	81 sea lions stranded	Scholin et al. 2000
California, USA	2000	184 sea lions stranded	Gulland 2002

In 1998, 81 Californian sea lions (*Zalophus californianus*) became stranded along the coast of California (Scholin et al. 2000, Gulland et al. 2002). The intoxicated sea lions showed similar signs to those observed previously, such as head weaving, scratching, ataxia, seizures, decreased responsiveness to stimuli and death (Lefebvre et al. 1999). This event coincided with blooms of *P. australis* (Gulland et al. 2002). A similar incident occurred again in 2000 where 184 sea lions were stranded off the Californian coast with similar clinical signs (Gulland et al. 2002).

Symptoms observed in laboratory rats exposed to DA were similar to those in the field, and include hind-limb scratching, wet dog shakes, head jerks and seizures (Todd 1993, Sobotka et al. 1996). In primates, symptoms also include vomiting and gagging (Tryphonas et al. 1990). These symptoms can occur within 30 minutes to 24 hours

post- consumption of toxic seafood. Domoic acid mostly affect the CA3 regions in the rodent hippocampus while it affects regions CA1-4 in primates (Tryphonas et al. 1990, Jeffery et al. 2004). No significant genotoxic effects have been found (Rogers & Boyes 1989).

2.1.3 Gymnodimine

Gymnodimine (GD) was first isolated in 1994, from *Tiostrea chilensis* (oyster), found in New Zealand (Seki et al. 1995). To date, reports on the presence of GD in shellfish has only been reported in New Zealand and Tunisia (Bire et al. 2002). The relatively recent discovery of GD in Tunisia may have resulted from ballast water exchanges, climate change and the increase in abundance of toxic algae, or an increased in awareness of this toxin (Bire et al. 2002).

There has been no recorded human intoxication from GD, as it has a very low toxicity when introduced orally, however it is a fast acting toxin when introduced intraperitoneally (i.p.) into mice (Stirling 2001). This causes problems during animal bioassays as the presence of GD can give false positive results when monitoring for other algal toxins.

2.2 Sources of toxins

Different types of algal toxins can be produced by several genera of algae. Table 2.3 summarises the toxic marine microalgae and their related toxins.

2.2.1 Okadaic acid group

Pyrrophyta (Dinoflagellates) are flagellated unicellular phytoplankton, ranging in size from 20 to 200 μm in diameter. Of the 60-80 classes of toxic algae, 75% are dinoflagellate species (Tibbetts 1998). The OA group of toxins are produced by the dinoflagellate genera *Dinophysis* and *Prorocentrum*, whilst the PTX group is exclusive to *Dinophysis* (Yasumoto & Murata 1990, Carmody 1996, James et al. 1999a, Souto et al. 2001) (Table 2.3). Although most of the OA poisoning incidents have been from temperate regions, *Dinophysis* genera are found in waters of many different climatic zones around the world (Holmes et al. 1999b). Outbreaks of the OA

group of toxins caused by *Dinophysis* have been reported from many regions of the world (Table 2.3).

Determining the involvement of *Dinophysis* species in toxic events can cause problems since they are very difficult to culture. Lee et al. (1989) tested seven species of dinoflagellates from the wild for OA group production and found that they all produce toxins (Table 2.3). In Australia, at least four species of this genera are known to produce toxins: *D. acuminata*, *D. acuta*, *D. fortii* and *D. rotundata* (Table 2.3) (Hallegraeff 2002). *D. caudata*, also found in Australia, are known to produce pectenotoxins (PTXs) but not OA or DTXs (Burgess 2003). A cell concentration as low as 200 cells L⁻¹ can produce enough toxins to cause intoxication for shellfish consumers (Yasumoto et al. 1985).

Table 2.3 List of algae and toxins produced in various geographical areas. (OA=okadaic acid, DA=domoic acid, DTX=Dino toxins, GD=gymnodimine).

<i>Genus</i>	<i>Species</i>	Known toxin produced	Distribution and toxicity	References
<i>Dinophysis</i>	<i>acuminata</i>	OA, DTX1, PTXs	Australia, France, Holland, Ireland, NZ, Spain - toxic	(Hallegraeff 2002)
	<i>acuta</i>	OA, DTX1, PTXs	Australia, Chile, NZ - rare in Australia	(Hallegraeff 2002)
	<i>caudata</i>	PTXs	Australia, Japan	(Burgess 2003).
	<i>Mitra</i>	OA, DTX1		(Lee et al. 1989, Smithsonian-Institution 2003)
	<i>norvegica</i>	OA, DTX1		(Lee et al. 1989, Smithsonian-Institution 2003)
	<i>rotundata</i>	DXT1	Australia, US - not toxic, Japan - toxic	(Lee et al. 1989, Smithsonian-Institution 2003)
<i>Prorocentrum</i>	<i>tripos</i>	DTX1	Australia	(Lee et al. 1989, Smithsonian-Institution 2003)
	<i>cordatum</i>	OA	world wide	(Hallegraeff 2002)
	<i>faustiae</i>		Australia	(Smithsonian-Institution 2003)
	<i>hoffmannianum</i>	OA	Australia	(Hallegraeff 2002)
	<i>Lima</i>	OA, DTX1	Australia , Canada, Japan, France, Norway	(Hallegraeff 2002)
	<i>maculosum</i>	OA	Australia	(Smithsonian-Institution 2003)
	<i>rhathymum</i>		Australia	(Hallegraeff 2002)

Table 2.3. (cont.)

Genus	Species	Known toxin produced	Distribution and toxicity	References
<i>Pseudo-nitzschia</i>	<i>australis</i>	DA	US, Australia	(Lapworth 2000)
	<i>americana</i>	Non toxic in US	Australia, Peru, Chile, Uruguay, US	(Hallegraeff 1994)
	<i>calliantha</i>	DA	Canada	(Lundholm et al. 2003)
	<i>cuspidata</i>		May be confused with <i>Pseudodelicatissima</i>	(Lundholm et al. 2003)
	<i>delicatissima</i>	DA - weak	NZ, Canada	(Hallegraeff 2002)
	<i>fraudulenta</i>	DA - weak	NZ - toxic, Denmark, Norwegian, Chile - not toxic	(Hallegraeff 2002)
	<i>galaxiae</i>	DA	Mediterranean Sea	(Cerino et al. 2005)
	<i>lineola</i>	Unknown	Denmark, Indian Ocean, Gulf Stream, Australia	(Hallegraeff 1994)
	<i>multiseriata</i>	DA	NZ - not toxic, US, Canada, Japan, Mexico, Korea- toxic	(Hallegraeff 2002)
	<i>multiseriata</i>	DA	Canada, Australia - not toxic	(Hallegraeff 2002)
	<i>pseudodelicatissima</i>	DA	Chile, Norwegian, Japan, Australia, Gulf of Mexico	(Lapworth 2000)
	<i>pungens</i>	DA	Africa, Chile, Japan, US, NZ - toxic, Australia- not toxic	(Hallegraeff 2002)
	<i>seriata</i>	DA	Europe, Canada	(Bates in press)
	<i>subpaeifica</i>		Australia, NZ, US, Africa, Portugal, Indian Ocean	(Hallegraeff 1994, Bates in press)
<i>Pseudo-nitzschia</i>	<i>turgidula</i>	DA - weak	NZ - toxic, Australia - not toxic	(Hallegraeff 2002)
<i>Karenia</i>	<i>selliformis</i>	GD	NZ, Tunisia	(Seki et al. 1995, Bire et al. 2002)

Prorocentrum are benthic dinoflagellates found growing on the surface of seagrass and macroalgae (Koike et al. 1998). *Prorocentrum lima* can be found in both cold and warm waters (McLachlan et al. 1994) as different strains have evolved and adapted to survive in different temperatures (Koike et al. 1998). For example, the Florida strain has been reported not to grow in conditions where the temperature drop below 19°C (Morton 1990), while the strains in Canada can grow in environments with varying temperature between 5°C and 25°C (Jackson et al. 1993 in Koike et al. 1998). Growth of *P. lima* has been shown to be directly related to nitrate concentrations and inversely related to phosphate concentrations (Sierra-Beltran et al. 1996).

Although *P. lima* is the main species of this genus to produce OA group, other species such as *P. maculosum*, *P. concavum* and *P. hoffmanianum* are also known to produce OA and DTXs (Morton & Tindall 1995, Van Dolah 2000a). Toxin production by these phytoplankton may vary with seasons. In addition, the presence of nutrients in seawater had a significant correlation to the observed variations in toxin production by *P. lima* isolates (Boland et al. 1993). In south west Ireland, the toxicity level was seen to vary not only between seasons but also vertically and horizontally within the water column (Boland et al. 1993, Carmody 1996). Additionally, DTX-2 levels in mussels sampled from Banty Bay in Ireland increased with depth, while OA levels decreased with depth (Carmody 1996).

Toxins in dinoflagellates have been reported to vary intraspecifically that may be related to geographical differences (Lee et al. 1989). For example, *P. lima* found in the Sanriku coast, Japan, was reported to produce DTX-1 but not OA, while those in Nova Scotia, Canada, were found to produce both toxins (Marr et al. 1992, Koike et al. 1998). Production levels may vary even within the same country, for example, *D. fortii* in northern Japan were found to produce DTX-1 while those in southern areas did not (Lee et al. 1989).

The correlation between the dinoflagellate concentration in the water and toxin concentration in shellfish has been a controversial point of discussion (Klopper et al. 2003). In both France and Norway, there was no correlation between the abundance of algae and the presence of toxins (Lassus & Bardouil 1991). However, the DSP

concentration in blue mussels in Norwegian waters, and in Germany showed a positive correlation with the abundance of *Dinophysis* spp. (Klopper et al. 2003).

Toxin production is also related to growth of the dinoflagellates (McLachlan et al. 1994). The ratio of OA:DTX-1 in mussels found in Europe was low when the cells were growing and increased as the cells entered the stationary growth phase (McLachlan et al. 1994). Imai and his colleagues (2003) have suggested that dinoflagellate species are originally non-toxic and may only become toxic seasonally through the ingestion of toxic small-sized phytoplankton. This may explain the inconsistency of toxicity within dinoflagellates (Imai et al. 2003).

The biological function of OA production in dinoflagellates is not understood, but OA, produced by *P. lima* has been observed to inhibit the growth of other dinoflagellates, which may help some this species compete with other species of dinoflagellates (Aguilera et al. 1997) for a common resource. In contrast, dinoflagellates can tolerate high levels of protein phosphatase inhibitory toxins due to up-regulation of protein phosphatase expression (Boland et al. 1993). With other toxins, it has also been observed that copepods avoid feeding on dinoflagellates which produce saxitoxin (Holmes & Teo 2002), suggesting that the purpose of toxins is to assist in avoiding predation.

2.2.2 Domoic acid

Bacillariophyta (diatoms) are unicellular organisms with unique shells that serve as their cell wall and consist of silica skeletons called frustules (Smithsonian-Institution 2003). They can be found in both fresh and marine waters. This group is divided into two main classes; centric diatoms, which are radially symmetrical, and pennate, or elongated diatoms.

Domoic acid is produced by the marine pennate diatoms belonging to the genus *Pseudo-nitzschia*, within which there are over 20 species found in both estuarine and oceanic habitats around the world (Hasle 2002). This genus was initially split from *Nitzschia* in the 1994 by Hasle (Bates 2000). Species such as *P. australis*, *P. calliantha*, *P. cuspidate*, *P. galaxiae*, *P. multiseriata*, *P. multistriata*, *P. pseudodelicatissima*, *P.*

pungens, and *P. seriata* are known DA producers (Table 2.3) (Hallegraff 1993, Hasle 2002, Lundholm et al. 2003, Cerino et al. 2005, Bates in press). Some other species, such as *P. delicatissima*, *P. fraudulenta*, and *P. turgidula*, have been reported to be weakly toxic (Hallegraff 2002).

Although the outbreak of ASP on humans has been limited to Canada, the reporting of *Pseudo-nitzschia*, the main causative agent, is more widely spread, and includes Australasia, Europe and America. The species of this genus may be toxic in one area while not in others (Hallegraff 2002).

At least eight species of this genus has been detected in Australian waters: *P. australis*, *P. americana*, *P. lineola*, *P. multiseries*, *P. pseudodelicatissima*, *P. subpacifica*, *P. turgidula* but DA has only been detected in low concentration in *P. pungens*, *P. pseudodelicatissima* and *P. australis* within Australia (Lapworth et al. 2000, Hallegraff 2002, Takahashi et al. 2003).

The distribution of these diatoms may change as it has been hypothesised that the shift in dominance in diatom species may be related to physiochemical conditions, such as decrease in phosphate and warmer water temperature (Ajani et al. 2000). This shift may explain the first record of this potentially toxic diatom *P. australis* in Australian waters (Ajani et al. 2000).

Two other genera of diatom have been reported to produce DA. *Amphora coffeaeiformis* was reported to have produced DA during the 1989 outbreak in Canada. However, since it was the only reported incidence of this species to produce the toxins, and this species has often being misidentified in the past, *A. coffeaeiformis* is not used for DA monitoring (Maranda et al. 1990, Bates 2000). Recently a new benthic species of diatom, *Nitzschia navis-varingica* was reported to produce DA in shrimp which are being cultured in Vietnam (Bates 2000). Since then, this species has been observed in brackish water in other Asian countries, but so far has not been a health threat (Kotaki et al. 2000).

2.2.3 Gymnodimine

Dinoflagellates *Karenia selliforme* have been associated with the presence of GD in shellfish (Mackenzie & Beuzenberg 2003). The species was founded when the genus *Gymnodinium* was separated into four genus: *Gymnodinium*, *Akashiwo*, *Karlodinium* and *Karenia* (de Salas et al. 2003). Other species from the genus, such as *K. brevis*, are known to produce brevetoxins, which are more potent than gymnodimine, and causes NSPs (Leblond & Chapman 2002).

2.3 Structure and properties

2.3.1 Okadaic acid group

Among the OA group of toxins, some contain a monocarboxylic acid and belong to the class of compounds known as ionophoric polyether (Shibata et al. 1982), which makes them lipid soluble. OA, DTX-1 and 3 share the same basic polyether structure (Figure 2.1). DTX-1 is a predominant DSP in Japan. Its structure was determined by Murata and his colleagues (1982) and was later found to be a methyl derivative of OA (Yasumoto 2000). DTX-2 is an isomer of OA isolated from Irish mussels and it is the predominant toxin along the Irish coast (Carmody 1996, Pavela et al. 2001) while DTX3 is a complex mixture of 7-O-acyl derivatives of OA and DTX-1 (Pavela et al. 2001). Okadaic acid, DTX-1,2 and 3 are lipophilic while DTX-4 and 5 are water-soluble sulphate derivatives of OA. While the latter two are weakly toxic, they are readily hydrolysed to form OA (Vieytes et al. 2000), hence they still pose a threat to consumers. PTXs also share a similar chemical make up to OA (Figure 2.2).

2.3.2 Domoic acid

Domoic acid is an amino acid which is a water soluble white powder resembling kainic acid, a neurotoxic excitatory amino acid (EAA), in both structure and behaviour (Figure 2.3) (Iverson et al. 1990, Kerr et al. 2002). It is a potent agonist of AMPA (2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and kainic acid sensitive glutamate receptors (Kerr et al. 2002) and this is responsible for epileptic symptoms. There are ten naturally occurring isomers of DA: seven iso-DA, two domoilactones and a 5-epi DA (Quilliam 2003).

In live shellfish, the rates of accumulation and elimination of DA also tend to differ between the shellfish types. The rate of elimination varies with season, water temperature and toxin storage within the shellfish (Van Apeldoorn et al. 1999). As the water temperature decreases, so does the toxin loss from the animal. Additionally, toxin in the gastrointestinal tract eliminates rapidly, in matter of days, as compared to toxins bound in tissues, which may take months (Van Apeldoorn et al. 1999).

2.3.3 Gymnodimine

Gymnodimine is a cyclic immine (Figure 2.4), functionally related to spirolides, pinnatoxins and prorocentrolides, all of which are known as “fast-acting toxins” as they are highly toxic when injected into mice (Seki et al. 1995, Stewart et al. 1997, Bire et al. 2002). There are five isomers of and analogue of gymnodimine isolated from the algae *Karenia selliformis* (Miles et al. 2000, 2003)

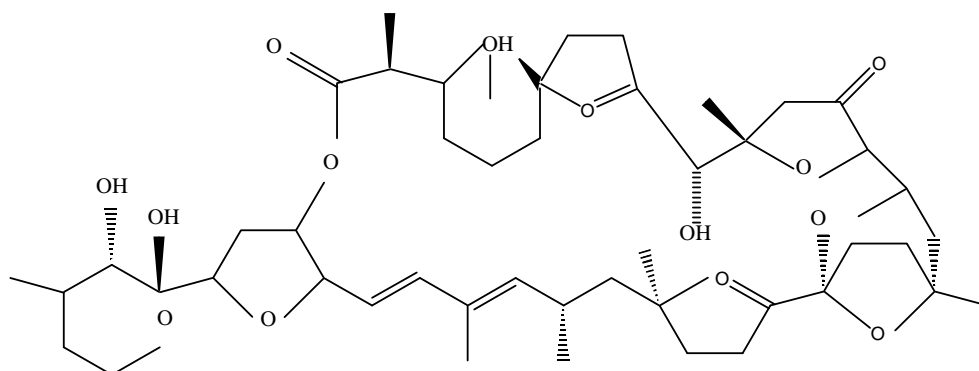


Figure 2.2 Structure of pectenotoxin-2

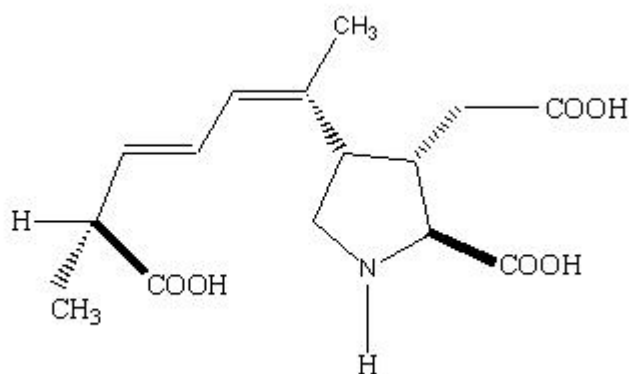


Figure 2.3 Structure of domoic acid

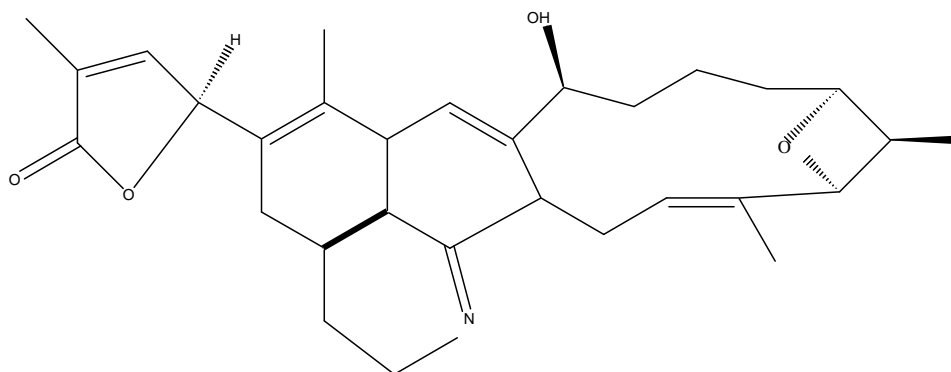


Figure 2.4 Structure of gymnodimine

2.4 Toxicity and toxic mechanisms

2.4.1 Health effects and toxicity of okadaic acid group

Okadaic acid group can cause severe gastrointestinal symptoms in victims. Although OA have been detected in the lung, liver, heart, kidney and the large intestine of laboratory mice, the major effects are seen in the small intestine (Edebo et al. 1988, Ito et al. 2002). The livers accumulate OA the most, as it is the first route of organs connected to the small intestine. The first recorded DSP incidence was in Japan where a dose of 48 μg per person of OA was enough to cause mild poisoning in humans (Holmes et al. 1999b). Reports on effect of OA contaminated shellfish, consumed orally by people have been documented. In most cases, the effects were mild poisoning or mild diarrhoea (Yasumoto et al. 1978, Lee et al. 1987, Holmes et al. 1999a, Ramstad 2001) and the concentration ranged from 40 μg to 48 μg per 60 kg person (Scoging & Bahl 1998). There is one report on OA where concentration, as high as 32 μg 100 g^{-1} was detected, using the HPLC-MS technique. This was found in the remnants of food consumed by victims who suffered from profuse diarrhoea (Vale & Sampayo 2002). The maximum tolerable level for OA is 24 μg 100 g^{-1} shellfish meat (Stabell et al. 1992). The doses and effects of OA group on humans and other animals are summarised in Table 2.4.

The LD_{50} in mice injected intravenously (iv) has been reported to be 192 μg kg^{-1} (Matias & Creppy 1996b). The time lag from injection to death was directly dependant on weight of the animal (Stabell et al. 1992). OA has also been reported to cause epileptic seizure at a concentration of 525 μg kg^{-1} (150 μM per 230g rat) when injected into hippocampus of rats (Ramirez-Munguia et al. 2003). In another study, effects of OA on rats were compared to that on salmon. Surprisingly, salmon hepatocytes were 10-20 fold more sensitive to OA and DTX-1 than rat hepatocytes (Fladmark et al. 1998). This suggests that salmon, a marine organism which would have been expected to be exposed to these toxins have not developed resistance to these toxins.

The OA and DTX-1 and DTX-2 are potent and selective inhibitors of three of the four protein serine/threonine phosphatases; type 1, 2A and 2B (PP1, PP2A, PP2B)

(Bialojan & Takai 1988, Arias et al. 1993). They inhibit PP2A completely at 1nM while inhibiting PP1 at 10-15nM. The sensitivity of PP1A and PP2A to OA is similar in many organisms ranging from mammals to fruit flies (Orgad 1989). Their relative toxicity is related to their affinity for protein phosphatases (Holmes & Teo 2002). It has been reported that the structure of type 1 protein phosphatase has been evolutionary conserved as it is biochemically indistinguishable in *P. lima* and in rabbit skeletal muscle (Cohen 1988).

Although very different in their chemical structures, OA and microcystins have the same targets as they inhibit protein phosphatases type 1 and 2A (Puisseux-Dao 1995). The binding site for OA is on the catalytic subunit of protein phosphatase at the active site of the enzyme. Serine/threonine protein phosphatases are critical components of signalling in eukaryotic cells that regulate a diverse array of cellular processes involved in metabolism (Werra 1995).

Table 2.4 List of okadaic acid dose-response studies. Doses are in $\mu\text{g kg}^{-1}$ of body weight ($\mu\text{g kg}^{-1}$) unless stated otherwise

Dose	Organism	Method of dosing	Effects	References
Mammals/birds				
48 $\mu\text{g 60kg}^{-1}$	Human	orally	mild diarrhea	Lee et al 1987
12MU=48 $\mu\text{g 60kg}^{-1}$	Human	orally	mild diarrhea	Yasumoto et al 1978
40 $\mu\text{g 60kg}^{-1}$	Human adult	orally	diarrhoea	Ramstad et al 2001
20-70MU = 80-280 $\mu\text{g kg}^{-1}$	Human	orally	severe diarrhea	Yasumoto et al 1978
320	Human	orally	profused diarrhea	Vale & Sampayo et al 2002
50	Mice	orally	mild diarrhea, OA in organs	Matias & Creppy 1996
160	Mice	ip	lethal dose	Stabell et al 1992
192	Mice	ip	LD ₅₀	Cohen et al 1990
0.5-5 $\mu\text{g 20g}^{-1}$	Rat	ip	small intestine liquid	Edebo et al 1989
4 $\mu\text{g 20g}^{-1}$	Mice	ip	lethal dose per 20g mouse	Yasumoto et al 1978
13MU= 52 $\mu\text{g 100g}^{-1}$	Chick	ip	lethal dose per 100g chick	Yasumoto et al 1978
Human Cells				
3ng ml ⁻¹	KB cells	3	IC ₃₀	Amzil et al 1992
0.01-0.03uM	Human bronchus	0.4	no effect	Naline et al 1994
0.1-10uM	Human bronchus	0.4	series of contraction and relaxation	Naline et al 1994
1ng ml ⁻¹	Human myeloma		no effect	Kang et al 1996
20ng ml ⁻¹	Human myeloma		OA showed cellular toxicity	Kang et al 1996
Animal Cells				
20nM	Mouse keratinocyte cells	12	detachment from substratum	Elegbede et al 2002
10nM	Mouse fibroblasts		stimulated DNA synthesis	Setkov & Epifanova 1997
long incubation	Mouse oocyte		arrested oocyte maturation	Gavin et al 1992
10nM	Mouse neuroblastoma cells	24-48	significantly altered cell morphology	Pahud et al 2001
20nM	Mouse neuroblastoma cells	24	detachment and cell deaths	Pahud et al 2001
10 - 100nM	Mouse neuroblastoma cells	48	cell death	Solovyan et al 1999
5 ng ml ⁻¹	BGM cells	5	moderate toxicity	Croci et al 1997

The PTX-2 and PTX-2SA concentrations of 5000 $\mu\text{g kg}^{-1}$, dosed via gavage, showed no effect on mice (Miles et al. 2004b). The LD_{50} of PTX-2 when injected (i.p.) into mice was 219 $\mu\text{g kg}^{-1}$, however, PTX-2SA had no effect at a level of 5000 $\mu\text{g kg}^{-1}$ (Burgess 2003, Miles et al. 2004b).

The current monitoring for algal toxins, adopted internationally, only screens for high levels of toxins which cause acute intoxication. This method of screening does not screen for low levels of toxins, which have the potential to cause chronic effects such as carcinogenesis (Puisseux-Dao 1995). It remains to be determined if there are any long term effects on humans caused by chronic exposure to persistent low concentrations (Holmes et al. 1999a). Carcinogenesis is a multiple step process which may involve a genotoxic event, followed by tumour promotion which is usually reversible and proceeds slowly. Cells then start to express a new phenotype to the advantage of highly proliferating cells, and the last stage is irreversible tumour progression with chromosome abnormalities (Puisseux-Dao 1995).

First marine natural tumour promoter was isolated from a cyanobacterium, *Lyngbya majuscula* (Fujiki 1984). Since then a number of other marine toxins have been shown to be tumour promoters, with OA being one of the most common promoters (Suganuma et al. 1988). After treating mice with 100 μg of DMBA (7, 12-dimethylbenzanthracene), OA was applied to the skin and within 16 weeks, 93% of the mice developed tumours (Suganuma et al. 1988). This toxin is a non TPA-type tumour promoter, which means it does not bind to phorbol ester receptors in cell membranes (Suganuma et al. 1988).

Along the French coast, 59 coastal areas were studied for a correlation between shellfish consumers and digestive tract cancer rates. After allowing for possible confounding by alcohol consumption, the only statistically significant results that remained involved colon cancer (Cordier et al. 2000). This study was based on population surveys, and further investigation involving carcinogenesis research will be required for establishing the relationship between OA and cancer.

Okadaic acid induces DNA adducts and is therefore, suggested to be a carcinogen (Creppy et al. 2002). Even without changing or mutating DNA base sequences epigenetic alterations in DNA methylation can occur (Creppy et al. 2002). Such changes in DNA methylation patterns can strongly affect the regulation of expression of many genes (Cerdeira 1997). Low concentrations of OA increase DNA methylation (Matias & Creppy 1996b), hence OA may interfere with gene regulation expression and cellular proliferation and participate in epigenetically-induced tumours by gap junction communication (GJIC) inhibition (Creppy et al. 2002). This inhibition of GJIC is believed to be important in the promotion stage of carcinogenesis (Budunova 1996).

Other studies have shown the OA group to be an apoptosis inducer in several cell systems (Leira et al. 2001). Mitochondria membrane potential, DNA content and phospholipid asymmetry in plasma membranes have been evaluated (Leira et al. 2001). OA is also able to transfer Na^+ , K^+ , Mg^{2+} and Ca^{2+} from aqueous solution to an organic medium and to facilitate the transport of these cations through the chloroform liquid membrane model (Blaghen et al. 1997). Puiseux-Dao (1995) has shown that OA inhibits the phosphatase involved in the dephosphorylation of gene *cdc25* product which is itself a phosphatase crucial for entry into mitosis. It also inhibits phosphatase coded by *nim2*, which is important for inter control between S phase and mitosis (Puiseux-Dao 1995).

2.4.2 Health effects and toxicity of domoic acid

Victims of the 1989 ASP outbreak in Prince Edward Island, Canada, suffered from nausea and vomiting from consuming up to 1.1mg kg^{-1} DA (Table 2.5). In severe cases, DA has been known to cause anterograde memory deficit (Peng et al. 1997). This lasting neurological deficit could be due to the lesions in the hippocampus, amygdala and limbic pathways (Kerr et al. 2002). The poor memory may result from biochemical changes or structural level effects at synaptic contacts (Clayton et al. 1999). One of the victims of ASP in the 1989 incidence was an 84-year old male (Cendes et al. 1995). Seizure occurred after the second day, but he improved slowly after the third week and was released from the hospital on the fourth month (Cendes et al. 1995). Although he was seizure free, he had a severe memory anterograde. After a

year, he had partial seizure. This recurrence of seizure is similar to those suffering from temporal lobe epilepsy (TLE) (Cendes et al. 1995). He died from pneumonia after three and a half years and autopsy showed that there was total neuronal loss in CA1 and CA3 regions of the hippocampus (Cendes et al. 1995).

Although ASP may cause long term memory defects, repeated exposure does not enhance toxicity beyond the effects observed with a single dose (Peng et al. 1997). This suggests that low doses over prolong periods will not necessarily produce more harmful effects than a single dose. This is in contrast to OA which is suspected of having chronic effects after exposure to persistent low concentrations (Holmes et al. 1999a).

The LD₅₀, injected intraperitoneally (i.p) for mouse is 3.6mg kg⁻¹, and the LD₅₀ (i.p) for both rats and primates is 4mg kg⁻¹ (Iverson et al. 1990, Todd 1993). The lethal dose of DA is similar in mice and primates, but the rate of neuron degeneration differs between the two groups (Schmued et al. 1995). Research with DA has also been performed on pregnant mice, which has shown it to be more toxic to newborns since it could penetrate through the incomplete blood-brain barriers of the offspring (Mayer 2000).

Glutamate receptors in the hippocampus is a region responsible for functional memory, and these receptors have been determined to be damaged by DA (Cendes et al. 1995, Sari & Kerr 2001). This may explain the head jerking and seizures seen in the patients and laboratory animals (Truelove et al. 1997). As the glutamate receptors open the sodium channels in the postsynaptic membrane, the cells depolarises (Sierra-Beltran et al. 1997). This in turn increases the calcium permeability in the brain where it causes nerve cells to transmit impulses continuously until cells die (Sierra-Beltran et al. 1997). Ross and colleagues (2000) showed that DA, at a concentration of 10μM elevated glutamate uptake by 64%, and then decreased the uptake to 48% after 60 minutes when tested on newborn rats. This suggests that DA induced neurotoxicity may be related to failure of astrocytes to remove extracellular glutamate, which then leads to excitotoxicity injury (Ross et al. 2000).

The histological study on the brains from three patients who died from ASP, revealed that neuronal necrosis and cell loss of astrocytes were most prominent in the hippocampus (Perl et al. 1990). In laboratory animals where rats were treated with DA via intraperitoneal (i.p.) injection, degeneration of neurons in the hippocampal CA1 and CA3 regions was seen (Sobotka et al. 1996). The study conducted on the brains of the sea lions which died from ASP also showed hippocampal neuropile and high activities of serum creatine kinase (Scholin et al. 2000, Gulland et al. 2002). As Scholin and colleagues (2000) pointed out, this was similar to other studies on mice and primates (Smayda 1990, Strain 1991, Dakshinamurti et al. 1993).

Although DA is known to cause damage in the hippocampus, it also produces degeneration in the limbic structure including the cortex, lateral septum and dorsal nucleus of the thalamus (Schmued et al. 1995).

2.4.3 Health effects and toxicity of gymnodimine

Although GD showed no oral toxicity on mice (Stirling 2001, Munday et al. 2004), it is neurotoxic to mice when injected, and it has been suspected to be affiliated with fish kill (Okaichi 1988). Another concerning factor is that GD has been shown to be highly toxic when orally dosed to mice via gavage (Table 2.6) (Munday et al. 2004). Munday concludes that when GD is administered with food such as shellfish it loses its toxicity. Since for humans, GD will normally be consumed in seafood, there will be little health risk involved with the consumption.

Table 2.5 List of domoic acid dose-response studies. All doses are in $\mu\text{g kg}^{-1}$ unless stated otherwise

Dose ($\mu\text{g kg}^{-1}$)	Organisms	Method	Effect	References
48 Hours	Oysters	exposed to diatoms	haemocytes activity increased	Jones et al 1995
2000	Mice	ip	no effect	Calyton et al 1999
120	Mice	ip	no effect	Iverson et al 1990
	Mice	ip	no symptoms	Beltran et al 1997
23	Mice	ip	lowest effect detected	Beltran et al 1997
600	Mice pregnant	injected tail	offspring affected	Dakshinamurti et al 1993
500	Mice	ip	highest nonlethal dose	Peng et al 1997
3600	Mice	ip	LD50	Todd 1993
5000	Mice	ip	lethal	Iverson et al 1990
2400	Mice pregnant	injected tail	lethal 100% death	Dakshinamurti et al 1993
35000	Mice	orally	scratching	Iverson et al 1990
2250	Rats	ip	scratching, convulsions	Appel et al 1997
1320	Rats	ip	weight loss, neurons	Sobotka et al 1996
1000	Rats	ip	hippocampal seizure, recovery	Fujita et al 1996
5000	Rats	ip	limbic seizure, fatal	Fujita et al 1996
4000	Rats	ip	lethal	Iverson et al 1990
500	Rats	iv	slight seizure, one had no effect	Truelove & Iverson 1994
70000	Rats	orally	sign of toxicity	Iverson et al 1990
70000	Rats	orally	not lethal	Iverson et al 1990

NB: kg per body weight; * = in shellfish tissue

Table 2.5. (cont).

Dose ($\mu\text{g kg}^{-1}$)	Organisms	Method	Effect	References
4000	Monkeys	ip	lethal	Iverson et al 1990
4000	Monkeys	ip	lethal	Tryphonas et al 1990
4000	Monkeys	ip	neuronal structure -	Schmued et al 1995
50	Monkeys	iv	vomiting, gagging	Truelove & Iverson
50	Monkeys	iv	lip smacking, vomiting,	Tryphonas et al 1990
500	Monkeys	orally	no effect	Iverson et al 1990
5000	Monkeys	orally	vomiting	Iverson et al 1990
5600	Monkeys	orally	neurotoxic symptoms	Todd 1993
1100	Whale	estimated daily intake		Lefebvre et al 2002
7000	Sea lions	orally	lethal, brain lesions in	Scholin et al 2000
45000	Pelicans	orally	lethal	Works et al 1993
	Pelicans	orally	lethal	Fritz et al 1992
58	Rainbow trout	orally	no effect, no accumulation	Hardy et al 1995
1 to 14	Anchovies	orally	severe neurotoxic	Iverson et al 1990
113	Anchovies		found within whole anchovies	Works et al 1993
1100	Human	orally	nausea, vomiting	Perl 1990, Todd 1993
2900	Human	orally	Confusion/disorientation	Todd 1993
4200	Human	orally	Permanent neuronal damage	Todd 1993
40	Human	*	level of action	Beltran et al 1997
20	Human	*	upper limit for human	Beltran et al 1997
30000	Human	*	Tolerable daily in take	Marien 1996
30	Human	orally	Allowable level	Vale & Sampayo 2002

NB: kg per body weight; * = in shellfish tissue

Table 2.6 List of gymnodimine dose-response studies. All animal study data are performed on mice.

Dose of GD $\mu\text{g kg}^{-1}$	Methods of dosing	Organism	Effects	References
7500	oral no effect	mice	not toxic. In this study toxic effects via blockage of nicotinic receptors at neuromuscular junction	Munday <i>et al</i> 2004
96	ip LD ₅₀	mice	LD ₅₀	Munday <i>et al</i> 2004
450	ip lethal	mice	Lethal	Seki <i>et al</i> 1995
700	ip lethal	mice	Lethal	Stewart <i>et al</i> 1997
755	gavage LD ₅₀	mice	LD ₅₀ , uptake with food - blocks toxicity, toxic effects via blockage of nicotinic receptors at neuromuscular junction	Munday <i>et al</i> 2004
10 μM	Cell: Neuro2a	Neuroblastoma	significant cell reduction MTT assay	Dragunow <i>et al</i> 2005
10 μM	Cell: Neuro2a	Neuroblastoma	pre-exposure to GD sensitised cells to okadaic acid. This shows mixed toxins in the wild may be more toxic than determined in the lab where only single toxin is tested.	Dragunow <i>et al</i> 2005

All doses are in $\mu\text{g kg}^{-1}$ unless stated otherwise.

2.5 Uptake of toxins in shellfish and mammals

2.5.1 OA group of toxins

Shellfish accumulate toxins, which are then transformed by hydrolysis, oxidation or acylation and eliminated (Morono et al. 2003). The accumulation and depuration rates vary according to the shellfish and the toxins (Shumway et al. 1995). In one study, when mussels (*Mytilus galloprovincialis*) were fed with toxic *Dinophysis acuminata*, a maximum OA level of $10\mu\text{g g}^{-1}$ was reached in 16 days and this fell to below $2\mu\text{g g}^{-1}$ in 45 days (Morono et al. 2003). There has been an indication that shellfish, such as Pacific oysters (*Crassostrea gigas*) can be affected by *Prorocentrum rhathymum*, which shares the same genus as a DSP- producing *P. lima* (Pearce et al. 2005). Although, the authors do not specify which toxin these oysters were affected by, oyster spat mortalities increased up to 40% as the cell numbers increased (Pearce et al. 2005).

Crabs have previously been known to pose the threat of paralytic shellfish poisoning (PSP) but it was not until 2002, with the outbreak of DSP poisoning in Aveiro, that crabs (*Carcinus maenas*) were associated with DSP (Vale & Sampayo 2002). It was suspected that crabs became contaminated from consuming DSP containing shellfish. In larger crabs, only the muscle tissue is consumed by people, hence it is unlikely for the toxins to be consumed since they are accumulated in the digestive glands. However in smaller, soft shelled crabs, the whole crab is consumed (Vale & Sampayo 2002), thus seafood which are higher up in the food chain could potentially be a source for DSPs.

When $25\mu\text{g OA kg}^{-1}\text{ body weight}$ was injected into mice, OA was found in bile and intestine after 1 hour (Matias & Creppy 1996a). This elimination patterns showed biliary excretion and enterohepatic circulation. Not much of OA is metabolised as it was found mostly in intestinal tissue (49%) and urine (11.6%) after 24hours when $50\text{--}90\text{mg OA kg}^{-1}\text{ body weight}$ was administered by gavage.

Derivatives of OA and DTX-1, such as DTX-3, has been detected in shellfish but not in dinoflagellates (Quilliam et al. 1996). It has therefore been proposed that such toxins are produced within the digestive glands of shellfish (Yasumoto et al. 1989).

Okadaic acid, DTX-1 and PTX-2 are found in *D. fortii* but acyl esters of OA, DTX and PTX-2 are not, hence other analogues found in scallop has been assumed to be results of acylation of DTX-1 and oxidation of PTX-2 in scallop hepatopancreas (Lee et al. 1989, Draisci et al. 1996, James et al. 1999b). PTX-2SA and 7-epi-PTX-2SA are commonly found in *Perna canalicus* (green mussels) in NZ coasts, and are thought to be converted from PTX-2, within the mussels. When non-toxic mussels were fed DTX-1 containing *D. fortii*, both DTX-1 and 7-O-acyl DTX-1 (DTX-3) were also found in the mussels (Suzuki et al. 2000). This showed that OA diol-esters, observed in *P. lima*, may not phosphatase inhibitors but have the potential to be hydrolysed in the digestive tract of shellfish to yield active parent OA and pose a threat to shellfish consumers (Quilliam et al. 1996).

2.5.2 Domoic acid

The depuration of DA has been reviewed by Appeldoorn et al. (1999). It was mentioned that the rate and the amount of toxin accumulation by the shellfish are dependent on the number of diatom cells available. The depuration of toxin within the shellfish is highly variable and may vary with season and water temperature, as low water temperature delays the depuration rate (Van Apeldoorn et al. 1999). Mussels tend to take up and depurate toxins rapidly while razor clams have slow depuration rates (Todd 1993). When scallop (*Pecten maximus*) samples were frozen, overall toxin level decreased, but, DA was observed to seep out of the hepatopancreas, into the rest of the body tissues, therefore increasing the DA level in the edible tissues (Van Apeldoorn et al. 1999). Stewart et al. (1998) showed that the DA level remained stable for up to 19 months in the digestive gland of sea scallops (*Placopecten magellanicus*) and red mussel (*Modiolus modiolus*) (Apeldoorn 1999, Van Apeldoorn et al. 1999). This implies that evaluating the safety level of consumption of shellfish on the abundance of *Pseudo-nitzschia* alone may not provide a satisfactory result.

Jones and his colleagues (1995) studied the effects of DA on Pacific oysters (*Crassostrea gigas*) which are known to accumulate toxins. After being exposed to the toxic algae for four hours, animals showed an increase in haemocyte activity. The toxin increased in the oysters in the 48 hour exposure, but the activity of haemocytes declined after 24 hour clearance, and after 48 hour clearance, the concentration of DA

declined to a trace level.

Lefebvre and colleagues (2001) tested the affects of DA on anchovies (Lefebvre KA 2001). Fish showed severe neurotoxic behaviour at doses ranging between 1-4 $\mu\text{g g}^{-1}$ of body weight. This finding is different from the effects on rainbow trout (Hardy et al. 1995). Rainbow trout were fed with 58 $\mu\text{g g}^{-1}$ DA for 15 weeks and there were no signs of intoxication nor were any traces of DA found in the fillet of the fish (Hardy et al. 1995). Results from these two studies suggest that the uptake of toxin by marine fish may be species dependant.

In mammals, DA does not seem to be as persistent as in fish. In previous studies, the average half life of DA in laboratory animals such as rats was 0.5 hours, and in primates, it was 2 hours (Truelove & Iverson 1994, Truelove et al. 1997). Thus with fish, DA persists long enough to affect the predations which feed on the fish, while it seems to have short half lives in mammals.

2.5.3 Gymnodimine

Gymnodimine has shown to have slow depuration rate in oysters, which has the potential to remain in the ecosystem for a prolong time (Stirling 2001). There have not been much studies performed on uptake and metabolism related to GD.

2.6 Guidelines and monitoring for human health

2.6.1 Okadaic acid group

Anderson et al. (2001) has summarised the guidelines levels for OA group from different countries, as shown in Table 2.7. The guidelines for OA vary between countries but most do not exceed 200 ng g^{-1} edible tissue (Quilliam & Wright 1995). For example in Japan, Norway and Korea, value of 5MU 100g^{-1} of tissue is used (Yasumoto & Murata 1990, Fernandez 2000, Anderson et al. 2001), where “MU” stands for mouse unit which is the minimal amount required to kill a mouse of 20g body weight within 24 hours (Yasumoto et al. 1980).

Some countries have adopted monitoring methods to avoid intoxication. For example,

along the Norwegian coast, 24 sites are monitored for OA group producing dinoflagellates (Dahl & Tangen 1999). In Norway, the accepted levels of *Dinophysis* species in seawater (cells L⁻¹) are as follows: 900 *D. acuminata*, 900 *D. acuta*, 1200 *D. norvegica* and 1200 total *Dinophysis* species (Dahl & Tangen 1999).

In Yasumoto et al. (1978), the mouse bioassay was used to calculate the concentration of OA. It is said that 12 MU (15-20 g mouse) OA causes mild diarrhoea in adult human while 20 to 70 MU causes severe diarrhoea (Yasumoto et al. 1978). This can be calculated as 0.8 µg kg⁻¹ (48 µg 60 kg⁻¹ person) OA for mild diarrhoea and 1.3 and 4.6 µg kg⁻¹ (78 – 276 µg 60 kg⁻¹ person) OA for severe diarrhoea. This study was conducted after poisoning incidences and the samples were taken from actual shellfish which were consumed. The concentration of OA determined by Yasumoto and his colleagues in 1978 may not be as precise as the later work, since they had used mouse bioassay to determine the toxin concentration, which does not provide readings of the toxins as some of more recent techniques, such as HPLC-MS. Another problem with these data is that the toxin was not purified, hence it is difficult to be certain if OA was the sole toxin responsible for the diarrhetic effect.

These allowable levels of OA may be low enough for preventing DSP outbreaks, but they may be enough for tumour promotion or other chronic health effects over a longer time frame. As previously mentioned, OA is a known tumour promoter and there have not been long term studies on the effects of OA which would allow the development of a safe level of consumption.

2.6.2 Domoic acid

Studies on humans after accidental consumption of shellfish, contaminated with DA, have been recorded in several papers (Perl et al. 1990, Todd 1993, Marien 1996). In 1959 Daigo reported that DA has been used on children as an anthelmintic in Japan, without any ill effects, at a concentration of 0.5 mg kg⁻¹ (Iverson et al. 1990). This study cannot be considered to be accurate for exposure analysis since it dates back to the 1950s and the method used to measure the toxin concentration is not clear.

Concentrations of DA from shellfish consumed by patients suffering from ASP were

measured by HPLC (Todd 1993). At an average of 1.1 mg kg^{-1} body weight, patients suffered from nausea and vomiting, while those consumed up to 4.2 mg kg^{-1} suffered from memory loss, disorientation and confusion (Todd 1993). The level allowed for human consumption is $20 \text{ } \mu\text{g g}^{-1}$ shellfish (Sierra-Beltran et al. 1997, WDFW 1998, Leira et al. 1999). In crabs, the allowable DA level was changed from $20 \text{ } \mu\text{g g}^{-1}$ in 1992, to $30 \text{ } \mu\text{g g}^{-1}$ in 1993 (Hatfield et al. 1995). The tolerable daily intake of DA has been calculated by Marien (1996) using the highest no-effect level on cynomolgus monkeys, lowered an order of a magnitude to 0.075 mg kg^{-1} for safety factor. This was then used to estimate the tolerable DA concentrations; $30 \text{ } \mu\text{g g}^{-1}$ in crabs (Marien 1996), which supports the guidelines.

2.6.3 Gymnodimine

Although gymnodimine has an acute toxicity when injected into mice, it has not shown to be toxic when orally dosed (Munday et al. 2004), hence there are no guidelines for human consumption at this stage.

Table 2.7. Guidelines for levels of okadaic acid (OA) in various countries. The concentrations are per shellfish tissue.

Concentration (OA)	Organism	Countries	References
0.8µg g ⁻¹	Human	Limit in Ireland	Camody et al. 1996
5MU	Human	Limit in most countries	Fernandez 2000
24µg 100g ⁻¹	Human	Max. tolerable level	Stabell et al. 1992
20ug 100g ⁻¹	Human	Canada	Anderson et al. 2001
2-3MU	Mice	Denmark	Anderson et al. 2001
2-3MU	Mice	France	Anderson et al. 2001
2-3MU	Mice	Italy	Anderson et al. 2001
20µg 100g ⁻¹	Mice	Japan (= 5MU 100g ⁻¹)	Anderson et al. 2001
20µg 100g ⁻¹	Mice	Korea	Anderson et al. 2001
20µg 100g ⁻¹	Mice	NZ	Anderson et al. 2001
20-30µg 100g ⁻¹	Mice	Norway	Anderson et al. 2001
20µg 100g ⁻¹	Mice	Portugal	Anderson et al. 2001
Presence	Mice	Spain	Anderson et al. 2001
16µg 100g ⁻¹	HPLC	Sweden	Anderson et al. 2001
24H mortality	Mice	Uruguay	Anderson et al. 2001
20µg 100g ⁻¹	Rat	UK	Anderson et al. 2001

MU = mouse unit

2.7 Effects on marine organisms

Dispersal of toxic algae and occurrences in toxic blooms can affect marine organisms in many adverse ways. Domoic acid from *Pseudo-nitzschia* has been known to cause mass mortalities of marine mammals and birds as they are passed down in the food chain through pelagic fish (Work et al. 1993). The mass stranding of birds in 1991 and 1992 in California and between 1994 and 1996 in Baja California were both found to be caused by DA (Sierra-Beltran et al. 1997). These birds were thought to have been eating contaminated anchovies and mackerels containing *P. australis* and *P. pungus*, as empty *Pseudo-nitzschia* frustules were found in both birds and fish (Sierra-Beltran et al. 1997). This was the first time *P. australis* was associated with the production of DA (Walz et al. 1994).

In the case of poisonings of sea lions in 1998, the animals showed clinical signs of head weaving, scratching and seizures, which are typical of ASP (Gulland et al. 2002). Although some of the animals recovered and were released, 11 out of 129 released re-stranded again within four months of release (Gulland et al. 2002). Again, the source of toxins were anchovies contaminated with DA from *P. australis* (Lefebvre et al. 1999). DA was detected from both sea lion faeces and tissues of anchovies as well as from *P. australis* (Lefebvre et al. 1999). In the 1998 outbreak, humans may not have been threatened for two reasons. During this bloom, very little DA was found in the blue mussels (*Mytilus edulus*) (Scholin et al. 2000). Also, in a study conducted by Lefebvre and colleagues (2002), DA was found in viscera of sardines and anchovies, but very little were found in edible tissues which are normally consumed by humans.

Domoic acid has been suggested to cause disoriented behaviour for some sea mammals, perhaps leading to beaching phenomenon of some whale species (Fritz et al. 1992), and it should be considered as a potential cause of marine vertebrate deaths of unknown origin (Work et al. 1993). In the wild, between 10 and 207 $\mu\text{g g}^{-1}$ DA was found in whale faecal samples, while it ranged between 136 and 152 $\mu\text{g g}^{-1}$ in faeces of intoxicated sea lions (Lefebvre et al. 1999, Lefebvre et al. 2002b). Domoic acid can accumulate in food sources, such as krill and fish consumed by marine mammals (Bargu

et al. 2002) which could explain how traces of DA were reported to be found in the tissue samples from mass stranding of humpback whales off Georges Banks, Maine, in July 2003 (Fraser 2003). Although it has not been proven that DA was the main cause of their deaths, the possibility cannot be ruled out. As Work et al. (1993) suggests, this may be difficult to prove especially when DA causes vomiting which makes it difficult to analyse the stomach content of possibly intoxicated animals (Work et al. 1993). It may, however, be possible to observe the neuronal degeneration in the CA1 and CA3 region of the hippocampus regions, where it is known to cause the most irreversible damage (Tryphonas et al., Appel et al. 1997, Sari & Kerr 2001).

Fish such as anchovies are used for human consumption and are an essential component of the marine food web (Work et al. 1993). Atlantic sardines (*Saedinia pilchards*) are known to accumulate up to $128.5 \mu\text{g g}^{-1}$ DA in their guts (Costa & Garrido 2004). Other toxins such as brevetoxins have been known to cause deaths of manatees in the southwest coast of Florida (Bossart et al. 1998) and bottle nose dolphins (Geraci et al. 1989). Saxitoxin was also found in stranded humpback whales (Geraci et al. 1989) and in monk seals (Costas 1998). In some animals, such as sea otters and sea gulls, it is believed that they can test and sense for the presence of toxins, such as saxitoxin, and avoid being poisoned (Kvitek 1991a, Kvitek & Degange 1991b). When it comes to consuming fish however, the predators do not have the chance to sense for toxins prior to consumption, as it is in the case of bivalves, where animals can open the shells and check prior to consumption.

During the 1988 bloom of *Pseudo-nitzschia pseudodelicatissima* and the 2000 bloom of *P. australis* in Monterey Bay, krill were reported to be feeding on these toxic algae (Bargu & Silver 2003). Although it is still not known if krill are affected by algal toxins, they act as potential vectors to transfer DA to fish, squids, birds and baleen whales, which prey on them (Bargu & Silver 2003).

Prorocentrum lima, an epiphytic dinoflagellate, known to produce OA, have been found on seagrass blades (Bouaicha et al. 2001). Although no study has looked at the possible affects of DSPs on dugongs and turtles, they feed on seagrass such as *Halophila*, *Halodule*, *Sytingodium* and *Zostera* (Lanyon 1991, Bryden et al. 1998), hence there is a

chance for dugongs to be affected by algal toxins.

Being a relatively new toxin, GD has so far not been reported to have any effects on the marine organisms in the wild. This may be due to the fact that it is not toxic when orally consumed with food (Munday et al. 2004).

2.8 Summary/ Conclusions

Toxins related to OA and DTX's are produced by dinoflagellates from the genus *Dinophysis* and *Prorocentrum*, and they are responsible for diarrhetic shellfish poisoning. PTXs have similar chemical structures to the OA group, but do not cause diarrhoea. The OA group causes gastrointestinal symptoms at a concentration of 40 μg per person, and the LD_{50} for mice (i.v.) is 192 $\mu\text{g kg}^{-1}$. It is also known to inhibit protein phosphatase (PP1A, PP2A) and promote tumours. LD_{50} for mice for PTX-2 and PTX-2SA are 219 $\mu\text{g kg}^{-1}$ and 875 $\mu\text{g kg}^{-1}$ respectively.

Domoic acid is produced by diatoms *Pseudo-nitzschia*. It is responsible for amnesic shellfish poisoning. It causes nausea and vomiting at a concentration of 1.1 mg kg^{-1} . The LD_{50} for mice (i.p.) is 3.6 mg kg^{-1} . This poisoning can result in memory loss as the neurons in the CA1 and CA3 regions of hippocampus are irreversibly damaged.

Gymnodimine is produced by dinoflagellate *Karenia selliformis*. It is highly toxic when injected into mice (i.p.) with LD_{50} of 96 $\mu\text{g kg}^{-1}$ and when it is dosed via gavage with LD_{50} of 750 $\mu\text{g kg}^{-1}$. It is, however, not toxic when orally dosed.

Guidelines for OA group for human consumption of shellfish, are stated as not to exceed 200 ng g^{-1} tissue, but this value is based on the acute toxicity level and not the long term chronic toxicity level. The allowable level of DA in shellfish determined by US Federal Food and Drug Administration is 20 mg kg^{-1} . There are no guidelines for GD at this stage.

Accumulation and depuration rates of toxins seem to vary between different types of shellfish. Temperature and toxic cell availability may also play a role.

Chapter 2

Algal toxins have dramatic effect on human health and have lead to mass beaching of marine organisms. There is also a rise in ecological concern as the occurrence of toxic algal blooms seems to be increasing. For these reasons, health risks posed by such algal toxins will be addressed in this thesis.

Chapter 3

Human Exposure Assessment: Occurrence and seasonal variations of algal toxins in water, phytoplankton and shellfish from North Stradbroke Island

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Takahashi, Eri, Yu, Qiming, Eaglesham, Geoff, Connell, Des W., McBroom, James, Costanzo, Simon, Shaw, Glen R. Occurrence and seasonal variations of algal toxins in water, phytoplankton and shellfish from North Stradbroke Island, Queensland, Australia.

Abstract

A number of marine microalgae are known to produce toxins which causes shellfish poisonings in humans. In this paper, the occurrence and seasonal variations of algal toxins were studied in the water column, phytoplankton and shellfish from south-east Queensland, Australia. Okadaic acid (OA), domoic acid (DA), gymnodimine (GD), pectenotoxin-2 (PTX-2) and pectenotoxin-2-seco acid (PTX-2-SA) were detected in shellfish using HPLC-MS/MS. Okadaic acid, PTX and GD were also detected in the water column. This was the first occasion when DA and GD were detected in shellfish, phytoplankton and the water column in Queensland waters. Phytoplankton tows contained both the toxic *Dinophysis* and *Pseudo-nitzschia* species, most likely producers of OA and DA found in shellfish in this area. The number of cells, however, did not correlate with the amount of toxins present in either shellfish or phytoplankton, indicating that toxin production by algae varies with time and the species present. Okadaic acid and PTX-2s were detected more frequently in the summer, while DA and GD were detected throughout the year, without any obvious seasonal patterns.

3.1 Introduction

Toxic shellfish poisonings occur when toxins are transferred through the food chain to higher trophic levels, including humans. Diarrhetic shellfish poisoning (DSP), named after the human symptom of gastroenteritis, is one such example (Yasumoto et al. 1987, Carmody 1996). This group of toxins formally included okadaic acid (OA), *Dinophysis* toxins (DTX's) and its derivatives, pectenotoxins (PTX's) and yessotoxins (YTX) (Yasumoto et al. 1985). Yessotoxin was then removed from the group under EU classification in 2002 (Fernandez et al. 2006). Recently a FAO/ICO/WHO Expert Consultation group separated this group into three groups of lipophilic toxins (Toyofuku 2006) and Fernandez et al (2006) refers to the OA group and PTX group as lipophilic shellfish toxins (LST). The LST group is harmful to humans and a serious threat to aquaculture industries (Fernandez 2000). The toxins classified within LSTs are produced by dinoflagellates and contain okadaic acid (OA), *Dinophysis* toxins (DTXs) and derivatives (Yasumoto et al. 1987, Yasumoto & Murata 1990, Draisci et al. 1996, James et al. 1999a). The OA group is responsible for causing diarrhea, inhibiting protein-phosphatase enzymes and has the potential to be a tumor promoter (Fujiki 1990,

Creppy et al. 2002). Although pectenotoxins (PTXs) do not cause such diarrhoea related illnesses, they were formally placed into this group since they are commonly found with the OA (Fernandez 2000, Creppy et al. 2002, Miles et al. 2004b). To date, no records of OA or DTXs have been recorded in the waters around south-eastern Queensland.

Domoic acid (DA) is a toxin produced by diatoms from the genus *Pseudo-nitzschia*, and is known to cause amnesic shellfish poisoning (ASP) (Van Dolah 2000a, Jeffery et al. 2004). It causes abdominal cramps, vomiting, diarrhoea, disorientation, short term memory loss and even death (Perl et al. 1990). It has been documented to affect not only humans but also marine organisms such as marine mammals and sea birds (Lefebvre et al. 1999, Gulland et al. 2002, Shumway et al. 2003). Although domoic acid has been detected in Australia, there has not been any reported incidences of DA in Australia (Lapworth et al. 2000).

Gymnodimine (GD) was first detected in New Zealand in 1994 (Seki et al. 1995, Stirling 2001). It is a compound produced by dinoflagellates from the genus *Karenia*, with *Karenia selliforme* being associated with the toxin in New Zealand (Seki et al. 1995, Mackenzie & Beuzenberg 2003). Although this compound has low toxicity when introduced orally, it is a fast acting toxin when it is injected intraperitoneally (ip) into mice (Stirling 2001). Due to its high potency when injected into mice, GD can cause false positive result during mouse bioassays designed to test for paralytic and neurotoxic shellfish poisoning (Seki et al. 1995). This toxin can also be extracted with the methanol extraction used for OA and DA (Miles et al. 2003), hence it was detected in the preliminary study conducted for the current study.

This study was conducted to assess the occurrence and seasonal variations of marine algal toxins in shellfish and phytoplankton collected from waters around North Stradbroke Island. North Stradbroke Island is located in Moreton Bay, off the coast of south-eastern Queensland. Waters around the island are inhabited by shellfish, some of which are consumed by the local people. Although there have been no reported incidences of algal toxin poisoning within the study sites, there have been algal blooms seen around the island (Osborne et al. 2001). The dissolved lipophilic toxins in water column were also measured by deploying a passive sampling device.

3.2 Material and Methods

3.2.1 Sample collection

Total of six sampling sites were selected for shellfish and phytoplankton tows, three within the bay and three along the beach (Figure 1). The first site within the bay was an oyster lease in Palmer Passage (S27° 26' 49.14" E153° 23' 49.74"), the second site was a bank in between the Myora lights and the island (S27° 28' 21.09", E 153° 24' 27.24"), and the third site was Amity Jetty (S27° 24' 5.53" E 153° 26' 12.97"). The three sites on the open ocean beach side included two sites along Main Beach (S 27° 26' E 153° 32'), facing the Pacific Ocean, and one site on Flinders Beach (S27°25' 15.15" E 153° 30' 17.67"), facing north. Monthly sampling was conducted at these sites, for 24 consecutive months. Pipis (*Donax deltoides*) were collected from three separate sites along the ocean beaches. Mussels (*Modiolus proclivis*) and oysters (*Saccostrea glomerata*) were collected from Palmer Passage and Myora, and oysters were also collected from Amity Jetty.

At each of the six sites, a plankton net (diameter 30 cm, mesh size 30 µm) was towed behind the boat for 50 m at the depth of up to 1 m. A flow meter was attached to the net to enable quantification of the water flow through the net, and was later used to calculate the amount of cells per volume of water. A 15 mL aliquot of the captured organisms was preserved immediately in Lugol's iodine for microscopic observation. The rest of the towed samples were on ice and brought back to the laboratory.

Solid phase adsorption toxin tracking (SPATT) bags (Cawthron Institute) containing porous synthetic resin (Mackenzie & Beuzenberg 2003) are designed to accumulate dissolved lipophilic toxins from the water column. To determine if any algal toxins were present in the water column, SPATT bags were deployed at two sites within the bay, Palmer Passage and Rous Channel (S27° 24' 47.72", E153°22' 20.32") for a duration of one to two week periods (Figure 3.1).

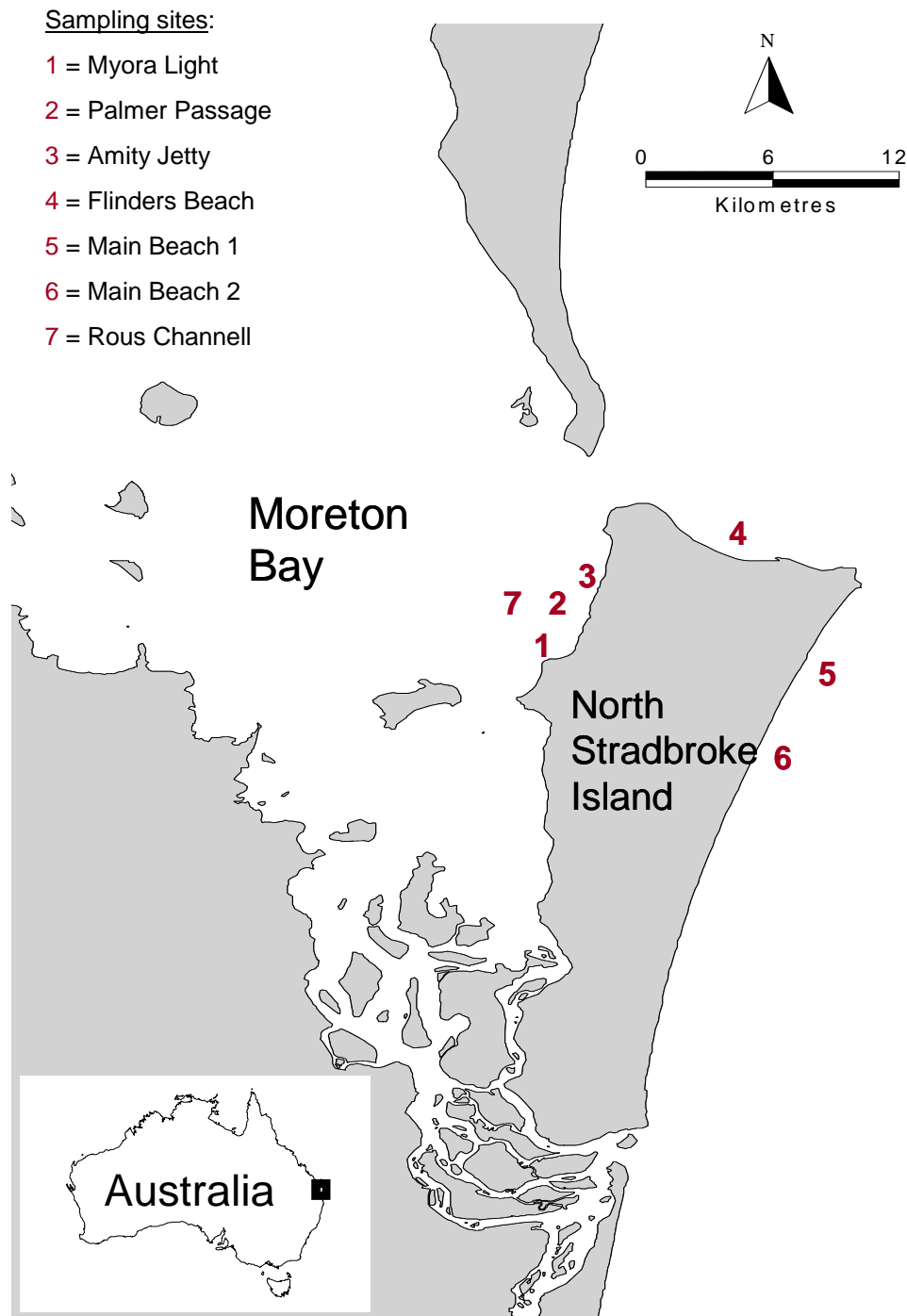


Figure 3.1 Map of sampling sites around North Stradbroke Island, off the southeastern coast of Australia

3.2.2 Microscopic observation of phytoplankton

Plankton net samples were examined by phase contrast microscopes (Nikon Labphot; Graticules, UK) at 400x magnification. Prior to microscopy, the 15 mL aliquot of phytoplankton tow sample was inverted 20 times to thoroughly mix the samples. One mL of the 15 mL aliquot was placed on Sedgewick Slide. The number of OA, PTX-2 and DA-producing species from the genera *Dinophysis* and *Pseudo-nitzschia* were identified and recorded per one mL for each of the samples. This was then multiplied by 1000 to obtain the estimated cell number per L.

For speciation of the diatom of *Pseudo-nitzschia*, transmission electron microscope (TEM) (JEOL model 100SX) was used for microscopic investigation (Fehling et al. 2004). Samples were acid cleaned (Villac & Fryxell 1998, Lundholm et al. 2002, Fehling et al. 2004). Fifteen mL of each of the concentrates from the tows was left overnight in 1 mL 10% HCL, 2 mL 30% H₂SO₄, 10 mL saturated KMnO₄. Saturated oxalic acid was added to remove the colour. Samples were centrifuged (1512 g) and washed three times with distilled water. Acid cleaned samples were then mounted on 200 mesh copper grid (ProSciTech, Thuringowa, Australia) with polyethylenimine (PEI). Specific taxonomic characters described in previously study (Skov et al. 1999) were used for species identification.

For the samples in which GD were detected, 15 mL aliquot of the phytoplankton samples were sent to Cawthorne Institute for DNA probe analysis for *Karenia selliformis* (Rhodes et al. 2001).

3.2.3 Toxin extraction

3.2.3.1 Shellfish

Toxins were extracted from macerated shellfish, using the methods described in previously studies (Lee et al. 1987, Marr et al. 1994). Shellfish flesh (4 g) were homogenised with 16 ml 80% methanol. After separation by centrifugation (2690 g for ten minutes) the methanol extract was washed with an equal volume of hexane, and filtered (0.45 µm) for analysis. Esters of okadaic acid (DTX-3) were hydrolysed to okadaic acid, DTX1 and/or DTX2 by using base hydrolysis (Mountfort et al. 2001). A 200 µL aliquot of methanol extract was hydrolysed in 2M NaOH at 75°C for 45 minutes

and neutralised using HCl. These samples were then filtered (0.45 µm) and analysed using HPLC-MS/MS (3.2.3.4).

3.2.3.2 Plankton

For the extraction of toxins from plankton net tows, the protocol by Pan (Pan et al. 1999) was incorporated. Each sample was centrifuged at 2690 g for ten minutes. The pellets were boiled in distilled water at 100°C for five minutes to release the toxins from phytoplankton cells and to stop the enzymes from degrading the toxins. The water was extracted in 80% methanol, evaporated under N₂, re-dissolved in 200 µL 80% methanol, filtered (0.45 µm) and analysed as detailed below (3.2.3.4).

3.2.3.3 SPATT Bags

Dissolved toxins in the water column were extracted from SPATT bags following the methods described in previous study (Mackenzie et al. 2004). Each bag was rinsed twice in fresh water for five minutes to remove any surface materials attached on the mesh. The resin was emptied into 100% methanol for two hours. The solvent was then filtered through glass wool and an anhydrous NaSO₄ column to remove any water. The sample was rotary evaporated, re-suspended in 5 mL 80% methanol and dried under N₂. This was then re-suspended into 2 mL 80% methanol and filtered (0.45 µm) and analysed as detailed below (3.2.3.4).

3.2.3.4 HPLC-MS/MS Analysis

All extracts from shellfish, phytoplankton tows and SPATT bags were analysed by using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord, Canada) coupled to a Perkin Elmer 200 series HPLC system (Perkin Elmer, Norwalk, USA) by a high flow Electrospray interface (Turbo-Ionspray). Analytes were separated on an Alltima C18 column (150 x 4.6 mm, Alltech Associates, Deerfield, IL.) with a mobile phase of acetonitrile, 2 mM ammonium formate and 0.1% formic acid gradient at a flow rate of 0.8 mL min⁻¹. The mass spectrometer was operated in the positive ion mode for PTX, gymnodimine and domoic acid and negative ion mode for okadaic acid, DTX1, 2 and 3.

3.2.4 Statistics

Data collected from the beach and the bay was pooled separately. Field data on the abundance of phytoplankton and the concentration of the toxins in phytoplankton tows and in shellfish, collected between July 2003 and June 2005, were organized in chronological order for analysis. Two analyses were conducted; one on the toxins extracted from the phytoplankton and the other from the shellfish. Two models were run for each of the analyses: logistic regression and linear mixed models. The logistic regression model allowed the probability of presence of the toxins to be calculated. The linear mixed model was used to incorporate time series correlation as all samples were collected from specific sites, every month, for two consecutive years. These models also partition variation, created by the hierarchical nature of the experimental design of having three sites within the two separate locations, beach and bay.

For the first set of logistic regression models on phytoplankton data, the effects of temperature, seasons, sampling sites within locations and phytoplankton genus on presence or absence of toxins were investigated. This was then repeated for each of the toxins separately. Linear mixed models were conducted on combined toxin data, and then on each of the toxins individually.

3.2.5 Dissolved toxins in water column

For quantification of SPATT bag data, the n-octanol-water partition coefficient (K_{ow}) was estimated by graphing log HPLC-MS/MS retention times of similar chemicals with known log K_{ow} . The SPATT bags were spiked with a known amount of OA in 500 mL beaker for duration of one week. Toxin concentration in the water was extrapolated using the information on toxins extracted from the SPATT bags and the calculated spat-water partition coefficient (K_{sw}) (Formula 1). To obtain K_{sw} for GD and PTX-2, the relation between K_{ow} and K_{sw} for OA was used (Formula 2).

$$\text{Formula 3.1: } K_{sw} = \frac{\text{Toxin in SPATT}}{\text{Toxin in water}}$$

$$\text{Formula 3.2: } K_{sw} \text{ toxin} = \left(\frac{K_{ow} \text{ toxin}}{K_{ow} \text{ OA}} \right) \times K_{sw} \text{ OA}$$

3.2.6 Exposure assessment

Estimates of potential exposure to algal toxins were based on average daily shellfish intake of 250 g day⁻¹ and the calculated abundance of toxin shellfish. A survey conducted on shellfish consumption for residents of North Stradbroke Island showed a different daily consumption of 12 g day⁻¹ (Mathews pers. Comm.). Estimated human daily exposure (DE) to toxin was calculated by multiplying the toxin concentration per weight of shellfish tissue by the estimated daily consumption of shellfish (Formula 3). For the toxins in shellfish around North Stradbroke Island, both mean concentrations of toxins and maximum concentrations of the toxins from monthly field collection were used. Additionally, information on abundance of dinoflagellates and levels of okadaic acid were obtained from pipi harvesters in South Australia and was also incorporated into this study.

These data were used to calculate the estimated toxin level in tissue by dividing the value by the body weight (BW; Formula 4). For mean body weight of humans 70 kg was estimated.

Formula 3.3: $DE(\mu\text{g}.\text{day}^{-1}) = \text{Toxin in food}(\mu\text{g}.\text{g}^{-1}) \times \text{daily consumption}(\text{g}.\text{day}^{-1})$

Formula 3.4: $\text{Toxin} = \frac{DE(\mu\text{g}.\text{day}^{-1}.\text{kg}^{-1})}{\text{mean BW}(\text{kg})}$

3.3 Results

3.3.1 Species identification

The abundance of *Dinophysis* and *Pseudo-nitzschia* are plotted (Figure 3.2a, b). This indicates that *Dinophysis* genus was mostly seen in the open beaches compared to the bay, and present during the warmer months (Figure 3.2a). *D. caudata* was most abundant in November and January 2004, with the highest concentration of 15 cells L⁻¹ recorded at Main Beach. *D. acuminata* was also present between November 2004 and January 2005, and only seen on the open beaches.

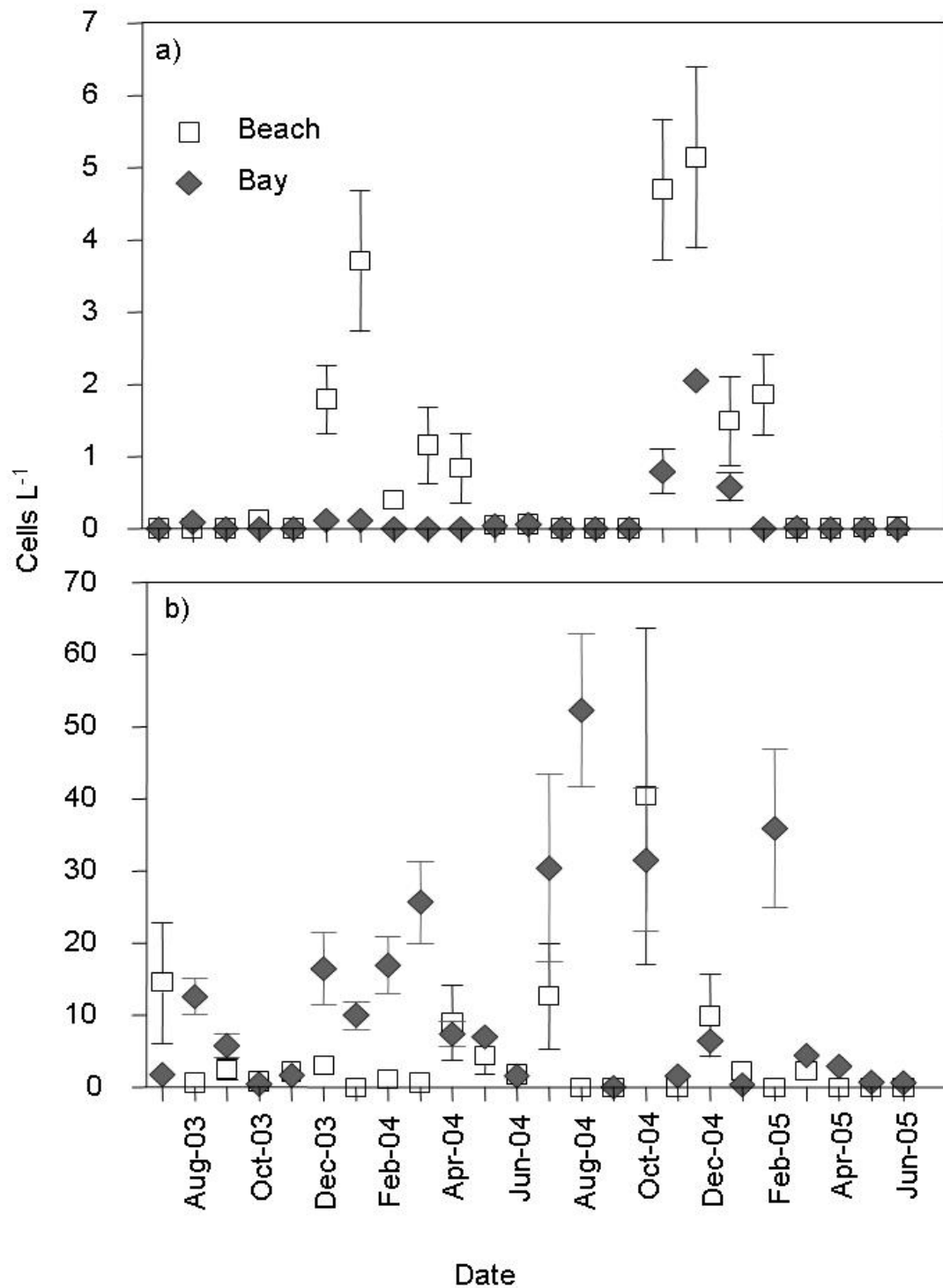


Figure 3.2. Number of a) *Dinophysis* and b) *Pseudo-nitzschia* cells in the phytoplankton samples collected from the beach and the bay of North Stradbroke Island between July 2003 and June 2005. Bars represent standard errors.

Table 3.1. Transition Electron Microscope analysis on *Pseudo-nitzschia* collected from Site 3: Amity Jetty, in February 2005

Length (μm)	Width (μm)	Central Interspace	Rows	Poroids (μm^{-1})	Interstriae (μm^{-1})	Fibulae (μm^{-1})	Species
63	2.3	Yes	1	5 to 6	27	12	<i>P. lineola</i>
90	1.7	No	2	3 to 4	13	15	<i>P. pungens</i>
123	4.2	Yes	2	5 to 6	24	16	<i>P. fraudulenta</i>
87.5	2.3	Yes	1	4	39	15	<i>P. lineola</i> & <i>P. cuspidata</i>

Pseudo-nitzschia, was observed throughout the course of the investigation, with the highest concentration of 340 cells L^{-1} in February 2005 in the bay (Figure 2b). From the TEM analysis (Table 3.1), several species of *Pseudo-nitzschia*; *P. pungens*, *P. pseudodelicatissima*, *P. lineola* and *P. fraudulenta* were identified as potential producers of DA during February 2005, when DA was present in the phytoplankton samples. The DNA analysis showed the presence of *Karenia selliformis* from the field collected samples containing GD.

3.3.2 Toxins from phytoplankton

All the toxins detected from both phytoplankton and shellfish are summarised (Table 3.2). Total algal toxin concentration was analysed using logistic regression model indicated that spring and summer were not different from one another while both were significantly different to autumn ($P < 0.05$). As for the sampling sites, none were significantly different from one another in terms of the probability of observing toxins. When using linear mixed models, combined toxin concentrations were positively related to the rise in temperature ($P < 0.05$). There was more variation between the two locations, beach and the bay, than between the sites within the locations.

Overall, PTX-2 was most abundant toxin detected from phytoplankton in this area, with maximum of $0.26 \mu\text{g L}^{-1}$ (Figure 3.3a). *Dinophysis. caudata* was significantly related to the presence of PTX-2 and PTX-2SA ($P < 0.05$), and it was 2.8 and 1.3 times more likely to see the toxins than not when this species was present, respectively. With the linear mixed models, there were significantly more PTX-2s found in summer and spring than in autumn ($P < 0.05$) (Figure 3.3b).

Table 3.2. Mean, maximum and sample size of toxins extracted from a) shellfish and b) phytoplankton are divided into those collected from the bay side and the beach side of the island.

a) Shellfish

Bay					
	OA	DA	GD	PTX-2	PTX-2SA
Mean ($\mu\text{g kg}^{-1}$)	0	4	15	2.7	0
Max ($\mu\text{g kg}^{-1}$)	0	147	137	270	0
Sample size	120	120	120	120	120
Beach					
Mean ($\mu\text{g kg}^{-1}$)	2.3	10	8	0.7	59
Max ($\mu\text{g kg}^{-1}$)	44.5	256	220	18	1940
Sample size	72	72	72	72	72
Detection limit	15	20	0.05	1	1

b) Phytoplankton

Bay					
	OA	DA	GD	PTX-2	PTX-2SA
Mean ($\mu\text{g L}^{-1}$)	0	0.004	0.0003	0.0003	0.008
Max ($\mu\text{g L}^{-1}$)	0	0.120	0.0060	0.0350	0.170
Sample size	69	69	69	69	69
Beach					
Mean ($\mu\text{g L}^{-1}$)	0.0003	0.003	0.00003	0.03	0.0035
Max ($\mu\text{g L}^{-1}$)	0.0020	0.105	0.00040	0.26	0.0400

OA = okadaic acid; DA domoic acid; GD = gymnodimine; PTX-2 = pectenotoxin-2; PTX-2A = pectenotoxin-2 seco acid.

Analyses with seasons as an explanatory variable in the logistic regression model showed that *Pseudo-nitzschia* had significant effects on the presence of DA ($P < 0.05$). Although the relationship between DA and *Pseudo-nitzschia* was significant, it was only 1.06 times more likely for DA to be present than not when *Pseudo-nitzschia* were detected. For example, in some samples, no DA was detected when more than 150 cells L^{-1} of *Pseudo-nitzschia* were present (Figure 3.3c). The high abundance in February 2005 did, however, correlate with the high levels of DA in the phytoplankton tows. Overall, the occurrence of DA in the beach and the bay were low (sample size of four and five per site), and on average the concentrations were higher in the beach.

GD was detected for the first time in Australia with a maximum of $0.006 \mu\text{g L}^{-1}$ from plankton net samples (Table 3.2; Figure 3.3d). GD was present throughout the year with higher amount of GD found in summer than in winter ($P < 0.05$).

3.3.3 Toxins in shellfish

Using the logistic regression model, the presence of PTX-2SA was significantly higher in summer and spring compared to autumn ($P < 0.05$), and was correlated with the rise in temperature ($P < 0.05$) (Figure 3.3b). Linear mix model for PTX-2SA showed that there was significant variation between two locations whilst not much variation between the sites within the beach. The frequency of PTX-2, detected in shellfish, was too low for any statistical analysis.

Domoic acid was found in all three types of shellfish, with maximum of $255 \mu\text{g kg}^{-1}$, in pipis in December 2004 (Table 3.2). While DA was related to decrease in temperature ($P < 0.05$), using the logistic regression model, there were no seasonal patterns in the presence of the toxins (Figure 3.3c). Linear mix model for DA showed opposite of PTX-2SA, as there was little variation between the beach and the bay, whilst high variation between the three sites within each of the two locations.

Presence of GD was also significantly higher in summer and spring, as well as in winter, when compared to autumn ($P < 0.05$) (Figure 3.3d). Highest amounts of GD were found in mussels with the maximum of $220 \mu\text{g kg}^{-1}$ and lowest in oysters ($P < 0.05$). The linear mixed model for GD showed high variation between both locations and sites. It was also positively related to the temperature of the water ($P < 0.05$).

Detectable amounts of OA (maximum: $44.5 \mu\text{g kg}^{-1}$) were found from pipis collected from the open ocean side of the island, between November 2004 and February 2005 (Figure 3.3e), however, frequency of occurrence of OA were too low for statistical analysis. Despite the presence of *Dinophysis*, no DTX's were found in shellfish tows from the sampled sites.

3.3.4 Dissolved toxins in water column

Lipophilic toxins PTX-2, OA and GD were extracted from SPATT bags deployed within the bay. PTX-2 was the most abundant toxin, with a maximum amount in November 2004 (Figure 3.4). The maximum OA was in February 2005 and maximum GD was in August 2004. From plotting the log retention times obtained from HPLC column, log of K_{ow} for OA (3.4), PTX-2 (3.7) and GD (2.2) were calculated. From the laboratory experiment with spiked SPATT bags, SPATT-water partition coefficient (K_{sw}) for OA was determined and information K_{sw} for the other toxins was estimated. Using these K_{sw} , the mean and maximum concentration in the water for PTX-2, GD and OA were calculated (Table 3.3).

Table 3.3. Calculated mean and maximum dissolved toxin concentration in water (derived from SPATT bags).

Toxin	PTX-2	GD	OA
Mean ($\mu\text{g L}^{-1}$)	0.3	0.06	0.04
Max ($\mu\text{g L}^{-1}$)	1.1	0.3	0.2
Sample size	16	16	16

PTX-2 = pectenotoxin-2; GD = gymnodimine; OA = okadaic acid.

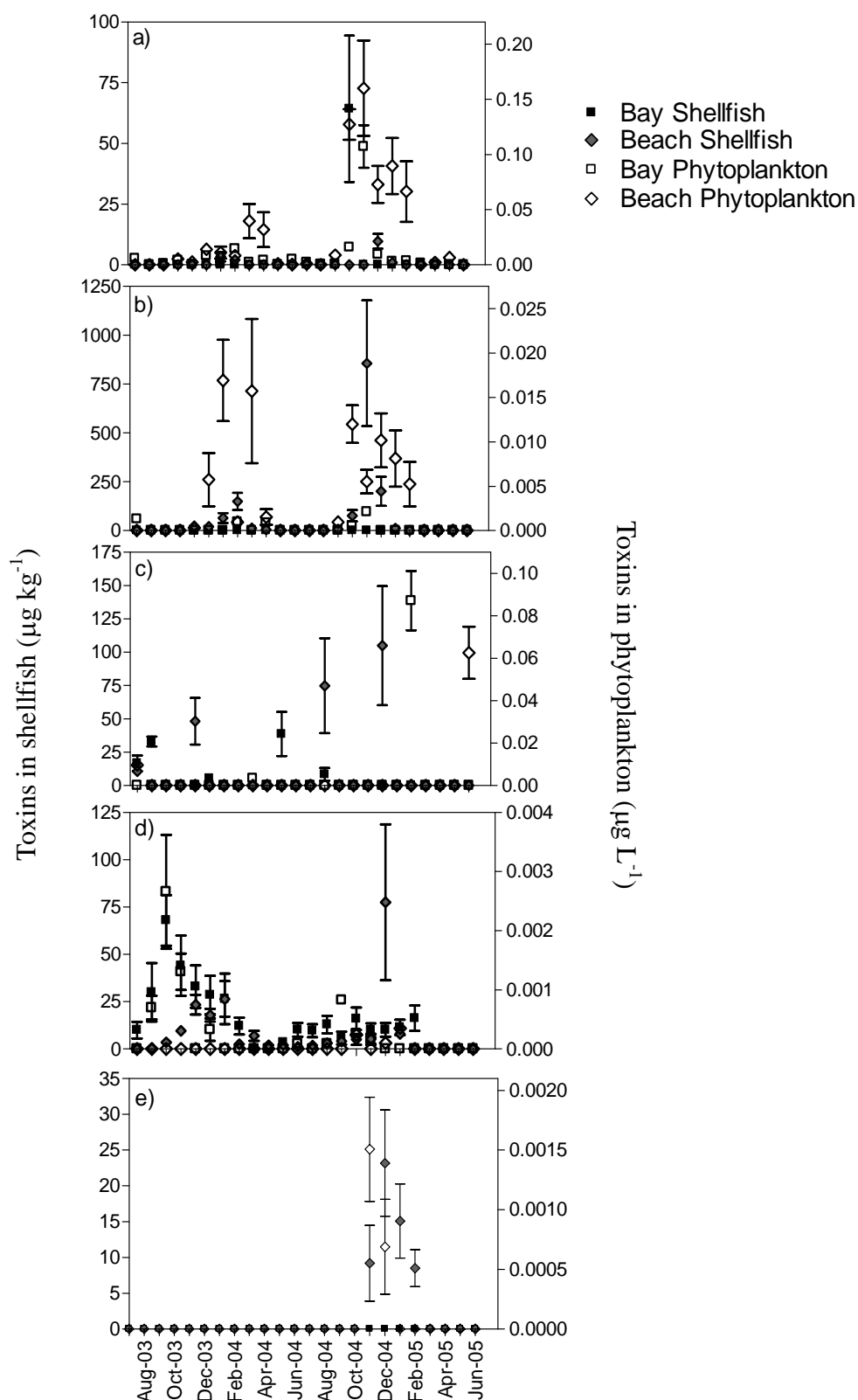


Figure 3.3. Average algal toxins extracted from phytoplankton and shellfish samples collected monthly from the bay and the beach of North Stradbroke Island between July 2003 and June 2005. a) Pectenotoxin-2; b) Pectenotoxin -2 seco acid; c) domoic acid; d) gymnodimine; e) okadaic acid. Bars represent standard errors.

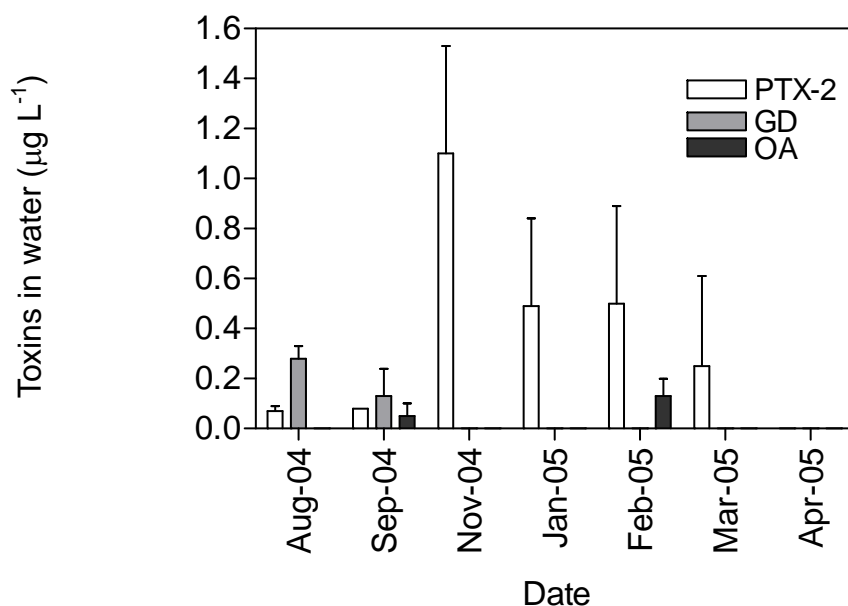


Figure 3.4. Estimated dissolved algal toxins in water, extracted from SPATT bags deployed within the bay side of North Stradbroke Island. N/A represents months when samples were not available. Standard errors are shown with error bars. PTX-2 = pectenotoxin-2; GD = gymnodimine OA = okadaic acid.

3.3.5 Exposure assessment

The daily exposure (DE) of OA, DA, GD, PTXs to humans were calculated for each of the toxins (Table 3.4). Both mean and maximum DE for each of the toxins from Queensland and South Australia were calculated. The only data available from South Australia were on OA and PTX-2, whilst OA, DA, GD and PTX2 were measured from Queensland shellfish. Toxin levels were higher in South Australia than Queensland samples. Toxins recorded from South Australia were up to 100 fold higher than those of Queensland.

Table 3.4. Estimated daily exposure (DE; $\mu\text{g kg}^{-1} \text{ day}^{-1}$) values were calculated using mean and maximum toxin levels in shellfish ($\mu\text{g kg}^{-1}$) from Queensland (QLD) and South Australia (SA), and average shellfish consumption*. 70kg was used as the mean body weight for the calculation of DE. OA = okadaic acid; DA domoic acid; GD = gymnodimine; PTX-2 = pectenotoxin-2; PTX-2A = pectenotoxin-2 seco acid.

	OA	DA	GD	PTX-2	PTX-2SA
Mean toxin in shellfish QLD ($\mu\text{g kg}^{-1}$)	6.2	6.2	12.4	1.9	22
Max toxin in shellfish QLD ($\mu\text{g kg}^{-1}$)	45	256	220	270	1940
Mean toxin in shellfish SA ($\mu\text{g kg}^{-1}$)	31	N/A	N/A	188	N/A
Max toxin in shellfish SA ($\mu\text{g kg}^{-1}$)	280	N/A	N/A	9600	N/A
*Daily intake using 250 g day ⁻¹					
Mean DE QLD ($\mu\text{g kg}^{-1} \text{ day}^{-1}$)	0.02	0.02	0.04	0.01	0.02
Max DE QLD ($\mu\text{g kg}^{-1} \text{ day}^{-1}$)	0.2	0.9	0.8	1.0	7
Mean DE SA ($\mu\text{g kg}^{-1} \text{ day}^{-1}$)	0.1	N/A	N/A	0.7	N/A
Max DE SA ($\mu\text{g kg}^{-1} \text{ day}^{-1}$)	1	N/A	N/A	34.2	N/A
*Daily intake using 12 g day ⁻¹					
Mean DE QLD ($\mu\text{g kg}^{-1} \text{ day}^{-1}$)	0.001	0.001	0.002	0.000	0.001
Max DE QLD ($\mu\text{g kg}^{-1} \text{ day}^{-1}$)	0.008	0.044	0.038	0.046	0.083

* Two types of average shellfish consumption were used. Data obtained from FAO of 250 g day⁻¹ (Toyofuku 2006) and 12 g day obtained from North Stradbroke Island, QLD (Mathew pes. Com.)

3.4 Discussion

Dinophysis detected around the North Stradbroke Island were most abundant in the warmer months, showing similar trend to this genus, found in other geographical locations (Hallegraeff 2002). Although *D. acuminata* was present in the second year of sampling, they were seen in early spring to summer, similar to those in Portugal, which are assumed to be related to organic nutrients (Vale & Sampayo 2003). Studies conducted in Adriatic sea showed a difference in presence between *D. caudata* and *D. acuminata* (France & Mozetic 2006), however, in this study, seasonal distinction between the two species could not be made. Data on OA were limited, making it difficult to perform any statistical analysis. However, the occurrence of OA coincided with the presence of *D. acuminata*, making this species the most likely producer of OA in this area. This is supported by other studies associating *D. acuminata* with OA (Hallegraeff 1992, 2002, Madigan et al. 2005).

The maximum concentration of OA ($44.5 \mu\text{g kg}^{-1}$), found in pipis, is lower than the recommended safety limit of $160 \mu\text{g kg}^{-1}$ (FAO 2004), hence there are little risk of any acute effects for OA, from consumption of pipis in the waters around North Stradbroke Island. OA was not detected in mussels or oysters during the sampling period, indicating that the OA-producing *Dinophysis* are found in the open ocean side of the island, and not within the bay. It is possible that the East Australian Current (EAC) running along the eastern coast of the island may be responsible for this effect. The current runs all year around, but predominantly in summer, bringing south the warmer water, which assists in algal growth. Toxin production by *Dinophysis* have been documented to be related to water temperature, salinity, rainfall and nutrient levels (France & Mozetic 2006, Vale 2006). The current study compared the temperature to the presence of toxins, and future study on the relation between other ecological influences on abundance of toxic algae and the toxins will assist in understanding ecological effects on toxin production.

PTX-2s occurred in the warmer months, and was related to the presence of *D. caudata*, making them the most likely producer of these toxins. This species has recently been detected to produce PTX-2 in northwest Spain (Fernandez et al. 2006). Pectenotixin-2SA are metabolites of PTX-2 and not known to be produced by phytoplankton

directly (Miles et al. 2004b, Wilkins et al. 2006). The PTX-2SA seen in the phytoplankton samples most probably had broken down from PTX-2 after either being released into the water column or after the cells have died during the sampling or transportation processes (MacKenzie et al. 2002), hence the significant relationship may not be relevant in this case.

PTX-2 and PTX-2 SA were also detected in the summer months in shellfish samples, collected from the bay and the beach side of the island. In pipis, the less toxic form of PTX-2 SA was the most abundant. Both PTXs peaked in November 2004, and in general were less abundant in the bay, and PTX-2-SA was only seen in the pipis. This confirms that pipis are capable of converting PTX-2 into PTX-2-SA. As blue mussels (*Mytilus edulis*) have been known to convert PTX-2 to PTX-2SA in New Zealand and Ireland (MacKenzie et al. 2002, Wilkins et al. 2006), if PTX-2 were more abundant in this area, the seco acid form may have been present in the mussels and oysters in this area. Another explanation for less PTX-2SA seen in mussels and oysters may be that they are more capable of rapidly eliminating the less toxic seco acid. The mussels have been known to depurate 50% of OA within a month and the depuration rate may be affected by food availability for the shellfish (Svensson 2003).

Pseudo-nitzschia population detected in this study were made of mix species. Among which, *P. pseudodelicatissima* and *P. fraudulenta* have been known to produce DA in other parts of the world (Lapworth et al. 2000, Hallegraeff 2002). Although, *P. pungens* has been known to produce DA in Africa, Chile, Japan and NZ, this species has not been recorded to be toxic in Australia (Hallegraeff 2002). However, *P. pungens* cannot be ruled out as cause of DA present in Moreton Bay. Further studies using the RNA probe to screen toxic species, may help clarify the causes of DA in this area (Miller 1996, Scholin et al. 1997, Rhodes et al. 2001, Lundholm et al. 2002). In this study, *Pseudo-nitzschia* did not correlate with the season, but their presence was negatively related to the decreasing temperature, which is also seen in other studies where they are known to bloom in winter, when other species are at the lowest (Mos 2001).

Although presence of DA and abundance of *Pseudo-nitzschia* was significant, their relationship was low and there was no correlation between the presence of DA in

phytoplankton and shellfish. Since plankton nets were towed on the same day as shellfish collection, there may not have been enough exposure for the shellfish to have accumulated the toxins in their organs, causing a time lag between the two samples. This genus is known to produce toxins during the stationary phase of their population growths, after the bloom (Bates et al. 1991). Since there were no blooms of this genus during the study period, the population may never have reached this stationary phase to produce vast amount of toxins. Another reason for lack of correlation between the presence of the genus and DA could be related to the fact that all *Pseudo-nitzschia* species were pooled together, but not all species are known to produce the toxins, hence the samples without presence of DA may have been composed of non-toxic species. Furthermore, the low levels of DA and number of cells may have been insufficient for a concrete conclusion. For the duration of this study, DA posed no threat of acute effects as the maximum level detected was below the guideline value of 20 mg kg⁻¹ (FAO 2004).

Karenia selliformis is a known GD producer in New Zealand (Mackenzie & Beuzenberg 2003, Miles et al. 2003), and this species was also present in Moreton Bay when GD was detected in the phytoplankton samples. *K. selliformis* was not monitored during the monthly sampling, however, the occurrence of GD correlated with the presence of *D. caudata*. This implies that *K. selliformis* are most probably found when other species of phytoplankton are present, as they were also more abundant in summer than in winter.

Gymnodimine was reported for the first time in Australia. The levels detected in shellfish are relatively low compared to the lethal level of 700 µg kg⁻¹ when injected (i.p.) into mice (Stirling 2001). Since gymnodimine has very low toxicity when dosed orally, these levels found around the island most probably would not cause risks to the consumers. However, since GD is known to have a slow depuration rate in oysters (Stirling 2001), there is a potential for the toxin to remain in the ecosystem for a prolonged time.

Dissolved algal toxins were detected using the SPATT bags, indicating that algal toxins can be released by the dinoflagellates into the water column. It is interesting that OA was only detected in shellfish and phytoplankton tows from the beach and not

from the bay, while it is extracted from the passive samplers deployed in the bay. This may be due to the fact that there are other dinoflagellates, from the genus *Prorocentrum*, that are known to produce OA (Murakami et al. 1982, Marr et al. 1992, Koike et al. 1998, Ten Hage et al. 2000), and are present in Moreton Bay (Takahashi et al. submitted). Since *Prorocentrum* are benthic, they would not have been caught in the plankton net towed on the surface of the water. The chance of epiphytes contaminating the SPATT extraction is minimal as the bags are rinsed with water prior to extraction, and only the resins within the bags are used for toxin extraction. Work conducted in New Zealand using the same passive samplers have also found PTXs, OA, DTX-1 and YTX (Mackenzie et al. 2004). Domoic acid was not found in the SPATT bags as unlike other algal toxins detected, this toxin is water soluble and would not accumulate in the resin. Relationship between SPATT bags and the concentration of toxins in water was estimated using laboratory experiment. However this was assuming that the SPATT bags reached equilibrium within the 7 days of deployment, hence further work is required to establish the duration it takes for the SPATT bags to reach equilibrium.

Data obtained from South Australia were collected only when blooms of *Dinophysis* were detected, whilst those from Queensland were collected all year round for two years. Since there were no blooms seen during the two years of monthly collection in Queensland, it is understandable that both mean and maximum toxin levels in Queensland are up to 100 fold lower than that of South Australia. Data from South Australia, however, gives an insight into how much toxin could be present in case of a bloom in Queensland. With the global increase in harmful algae (Hallegraeff 1993b), and the global warming and nutrient increase which are known to increase algal blooms (Mos 2001), one cannot rule out the possibility of a sudden algal bloom in this area. There has not been any blooms of DSP's or ASP's around North Stradbroke Island, however toxic cyanobacteria, *Lyngbya majuscula* have been known to bloom in this area (Osborne et al. 2001). Future studies are required to monitor the levels of toxins in shellfish during any future blooms.

The daily consumption of shellfish varied between the FAO (250 g day⁻¹) data and the specific survey conducted for the sampling site (12 g day⁻¹). This proved to have significant difference in the daily exposure of the algal toxins. Since the exposure

study was site specific to North Stradbroke Island, it is important to use the consumption rate obtained from the survey.

Chapter 4

Exposure Assessment: Occurrence of okadaic acid in the feeding grounds of dugongs (*Dugong dugon*) and green turtles (*Chelonia mydas*) in Moreton Bay

This chapter has been submitted for publication in Harmful Algae.

Takahashi, Eri M., Arthur, Karen E., Shaw, Glen R. Occurrence of okadaic acid in the feeding grounds of dugongs (*Dugong dugon*) and green turtles (*Chelonia mydas*) in Moreton Bay, Australia.

Abstract

Okadaic acid (OA) is a diarrhetic shellfish poison (DSP) produced by a number of marine organisms including the benthic dinoflagellate *Prorocentrum lima*, which are often found on seagrass. As seagrass forms the basis of the diet of dugong (*Dugong dugon*) and green turtle (*Chelonia mydas*), these herbivores may potentially be exposed to OA through ingestion of *P. lima* found on the seagrass. In this study, the abundance of epiphytic *P. lima*, on seagrass, and the concentration of OA produced by these epiphytic dinoflagellates were measured in Moreton Bay, Queensland, Australia. *Prorocentrum lima* and OA were found on all four species of seagrass collected. OA was detected in epiphytic material collected from seagrass with a maximum of 460 ng OA kg_(wwtSG)⁻¹ found on *Halophila spinulosa*. From this information, the estimated maximum daily exposure of OA by an adult dugong consuming 40 kg_(wwtSG) day⁻¹ was 18,400 ng day⁻¹, and an adult turtle consuming 2 kg_(wwtSG) day⁻¹ was 920 ng day⁻¹. Analysis by HPLC/MS/MS of 54 stranded dugongs and 19 stranded turtles did not yield OA above the detection limit of 10,000 ng kg (animal tissue)⁻¹. OA was found on seagrass, however it was not detected in the tissue samples of dugongs and turtles.

4.1 Introduction

Okadaic acid (OA) is a lipophilic marine algal toxin, which was first extracted from sponges *Halichondria okadai* and *H. melanodocia* (Tachibana & Scheuer 1981) and was later found to be identical to the toxin isolated from the dinoflagellates genus *Prorocentrum* and *Dinophysis* (Murakami et al. 1982, Yasumoto et al. 1985, Yasumoto & Murata 1990, Carmody 1996). Okadaic acid is one of the main toxins responsible for diarrhetic shellfish poisoning (DSP) (Murata et al. 1982), a term used to describe the rapid onset of gastrointestinal symptoms such as vomiting and diarrhoea in people who have consumed toxic shellfish (Amzil et al. 1992, James et al. 1999a, Souto et al. 2001, Van Dolah et al. 2003). *Prorocentrum* spp. are single cell benthic (Takai et al. 1987, Bialojan & Takai 1988, Ishihara et al. 1989, Arias et al. 1993) epiphytes that are often found on seagrass and macroalgae (Koike et al. 1998, Bouaicha et al. 2001). Amongst the OA-producing *Prorocentrum* species, *P. lima* is the main species known to produce OA (Morton & Tindall 1995, Van Dolah 2000a) and, being a photosynthetic benthic organism, *P. lima* occurs on substrates such as seagrass and macroalgae where it has access to light (Morton et al. 1998).

Although OA has not been known to cause death when orally consumed, the LD₅₀ for OA in mice injected intravenously (i.v.) has been reported to be 192 µg/kg (Matias & Creppy 1996b). Chronic exposure to OA promotes tumor development on the skin of laboratory animals by initiating hyper-phosphorylation (Fujiki 1992). This is achieved by potently inhibiting three of the four protein serine/threonine phosphatases type 1, 2A and 2B (PP1, PP2A, PP2B) (Takai et al. 1987, Bialojan & Takai 1988, Ishihara et al. 1989, Arias et al. 1993). Suganuma (1992) demonstrated that when 10 µg/day of OA is administered in rat drinking water for nine weeks, it can cause neoplastic changes in the glandular stomach. Another way in which OA could potentially lead to tumour promotion is by DNA hypermethylation which may lead to silencing of tumour suppressor genes (Creppy et al. 2002). Additionally, OA has also been shown to induce ornithine decarboxylase (ODC) in mice, which is a significant step in tumour promotion (Suganuma et al. 1992).

As seagrass is the primary food source of dugongs (*Dugong dugon*) and green turtles (*Chelonia mydas*), it is hypothesised that these vertebrate herbivores may be exposed

to OA produced by *P. lima* found epiphytically on seagrass. In Moreton Bay, Australia, both green turtles and dugong are protected under the Australian Environmental Protection and Biodiversity Act 1999 (Bryden et al. 1998, Read & Limpus 2002). *Halophila ovalis* is the favoured species of seagrass by these herbivores, however, they also feed on *Halodule uninervis*, *Zostera muelleri* and *Halophila spinulosa* (Lanyon 1991, Read & Limpus 2002). The potential exposure of green turtles to OA was assessed in a previous study through determination of *P. lima* and other *Prorocentrum* species abundance on common macroalgal food sources in Hawaii (Landsberg et al. 1999). The exposure to OA was investigated using the protein phosphate inhibition assay and demonstrated that presumptive OA concentration in kidneys of turtles ranged from 26 to 670 $\mu\text{g g}^{-1}$ (26,000 to 670,000 $\mu\text{g kg}^{-1}$). The Hawaiian study examined the potential exposure of green turtles to OA in a region where turtles feed predominantly on a macroalgal diet.

To date, no studies have assessed the potential exposure of dugongs to OA. Since dugongs feed on seagrass, it is likely that dugongs are ingesting epiphytic microalgae that are known to occur on the seagrass and may consequently be exposed to the toxins they produce. *Prorocentrum lima*, *P. micans* and *P. minimum* have previously been observed in Moreton Bay (Heil et al. 1998), however their toxicity has not been tested. This study aims to assess the presence of *P. lima* on seagrass collected in Moreton Bay and quantify the production of OA by these microalgae. The potential exposure to OA will then be estimated in turtles and dugong based on known feeding rates and the actual exposure assessed by measuring the OA concentration in tissues from dead or moribund animals.

4.2 Materials and Methods

4.2.1 Seagrass collection

4.2.1.1 Field sites and seagrass collection

Four species of seagrass were collected from three separate sites within Moreton Bay (Figure 1). The sites were selected based on the known high occurrence of dugong and green turtle foraging in these areas (Limpus et al. 1994, Lanyon 2003). Site one (Myora: S 27°28'.21.09", E 153° 24'27.24") is approximately 1 km west of North

Stradbroke Island and receives terrigenous freshwater run-off from North Stradbroke Is., but is also flushed daily on the tidal cycle with oceanic water entering from Rainbow Channel. Site two (Palmer Passage: S27° 26'49.14"835, E153° 23'49.74") is approximately 4 km west of North Stradbroke Is. and characterized by typically clear water due to daily flushing with the tides. Site three (Rous Channel: S27° 24'47.72", E153° 22'20.32") is located on a bank adjacent to the Rous Channel and is characterized by clear water due to daily flushing with oceanic water. Dugongs have been seen to aggregate in large numbers at this last site (pers. obs.).

Seagrass sampling was conducted on a monthly basis between February 2004 and June 2005. Water temperature was recorded at each collection. Where available, approximately 50 g seagrass wet weight (_{wwtSG}) of each seagrass species were collected from depths of 1-1.5 m at low tide. To avoid dislodging the epiphytic microalgae, individual leaves were carefully collected by hand underwater and gently brought to the surface. Samples of each seagrass species were collected in separate resealable plastic bags with 50 – 200 mL of seawater. These were maintained on ice until further processing.

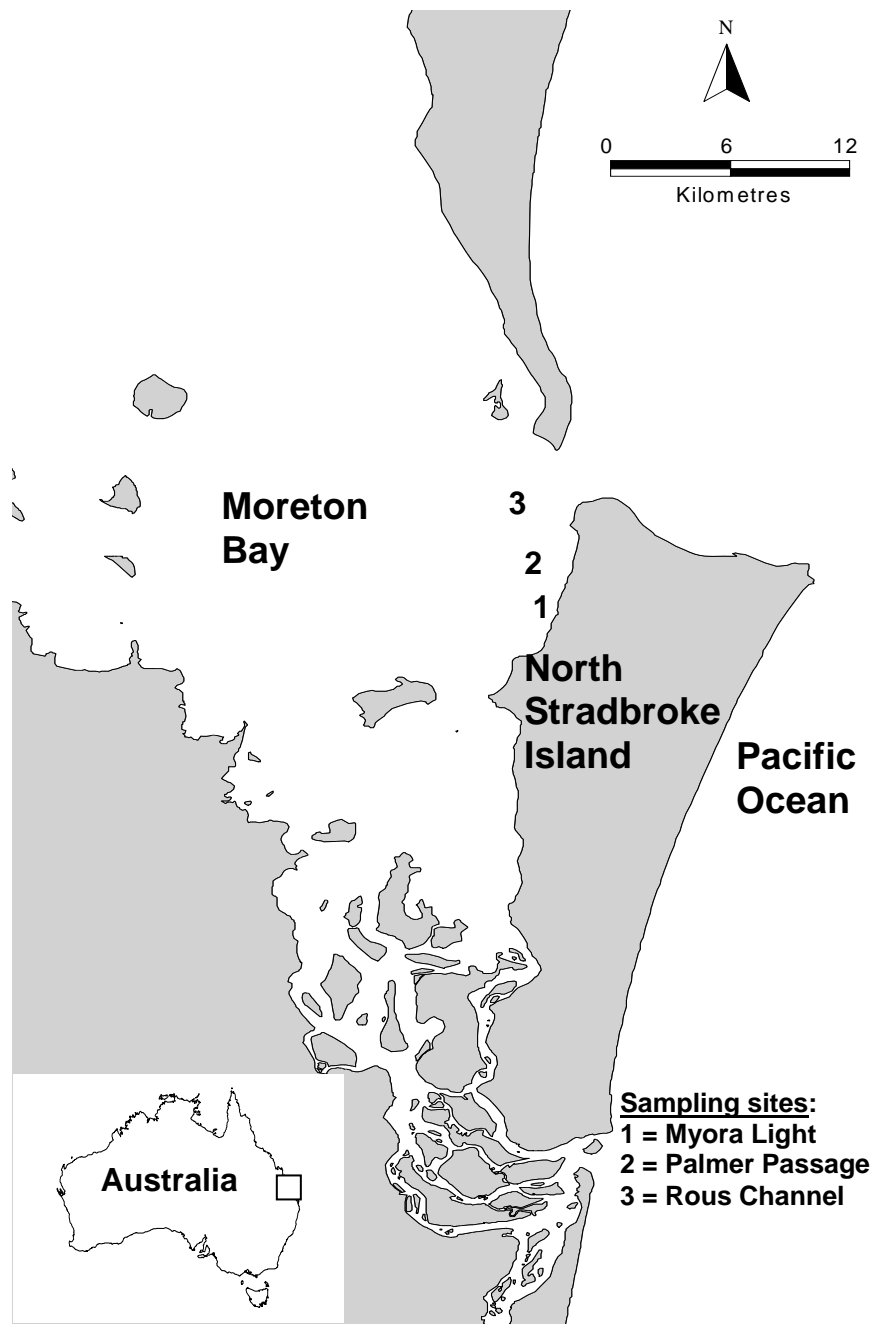


Figure 4.1. Map of seagrass sampling sites around North Stradbroke Island, off south-eastern coast of Australia: 1 = Myora light, 2 = Palmer Passage, 3= Rous Channel.

4.2.1.2 Sample processing

Seagrass samples were returned to the laboratory where they were vigorously shaken twenty times to dislodge epiphytic material (Morton & Tindall 1995, Landsberg et al. 1999). The water was carefully decanted from the seagrass and the volume of water and seagrass wet weight noted. Each water sample was gently shaken to resuspend particulate material and 15 mL aliquots were preserved in Lugol's iodine for microscopic observation. The remaining water was collected for OA extraction of epiphyte material dislodged from seagrass and collected in the water samples.

4.2.1.3 *Prorocentrum lima* abundance

The number of *P. lima* per gram_(wwtSG) was determined by examining three 1 mL aliquots of water with suspended epiphytic material from shaken seagrass, preserved in Lugol's iodine. Each aliquot was examined using phase contrast microscopy (Nikon Labphot; Graticules, UK) at 400x magnification. *Prorocentrum lima* were identified as per Faust (2002). The entire sample was scanned and counts made of all *P. lima* observed on the slide. The abundance of *P. lima* cells per volume of sea water was extrapolated to give number per wet weight of seagrass. *Prorocentrum* species other than *P. lima* were not recorded for the purpose of this study.

4.2.1.4 Okadaic acid extraction & analysis

Seawater in which seagrass had been shaken was centrifuged at 1500 g for ten minutes, and the supernatant was discarded. Pellets were boiled at 100° C for five minutes to lyse microalgal cells, releasing toxins, and to stop enzymes from degrading the toxins (Pan et al. 1999). The pellet was extracted in 2 mL 80 % methanol, evaporated under N₂, re-dissolved in 200 µL 80 % methanol and filtered for High Performance Liquid Chromatography-Mass Spectrometry/ Mass Spectrometry (HPLC-MS/MS) analysis (Lee et al. 1987, Quilliam & Wright 1995, Quilliam et al. 1996).

4.2.2 Dugongs and turtles

4.2.2.1 Sample collection

Tissue samples from dead stranded dugongs and turtles found in Moreton Bay were received by Queensland Environmental Protection Agency (QEPA), Queensland Health Pathology and Scientific Services (QHPSS) and The University of Queensland (UQ). Tissue samples were stored at -20°C prior to extraction in either plastic containers or aluminium foil. A total of 48 tissue samples from 41 individual dugongs were obtained (19 blubber samples, 19 muscle samples, two liver samples, six diet samples and two stool samples). In addition, pectoral muscle from 19 green turtles was collected. Tissue samples were collected at the time of necropsy by veterinary staff at the University of Queensland Veterinary School or the Department of Primary Industries, Brisbane. No mass mortality events occurred during the sampling periods and the cause of death of many of the animals was not established. All tissue samples were collected between August 2000 and August 2004 with most stranding occurring in the winter / spring months, and all from within Moreton Bay and Sunshine Coast area, Southeast Queensland.

4.2.2.2 Toxin extraction and analysis

The extraction of OA from turtle and dugong tissues followed the method previously developed with some alteration (Lee et al. 1987, Marr et al. 1994, Lefebvre et al. 2002a). Four grams of wet tissue was homogenised in 16 mL 80 % methanol. The homogenate was vortexed, sonicated and homogenised again to ensure complete breakdown of cells. It was then centrifuged for 20 minutes at 2700 g. The supernatant was filtered for HPLC-MS/MS analysis. To verify the extraction efficiency, two dugong muscle tissue samples were spiked with 185 µg kg⁻¹ OA prior to the centrifugation step.

All seagrass epiphyte, dugong and turtle extracts were analysed using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord, Canada) coupled to a Perkin Elmer 200 series HPLC system (Perkin Elmer, Norwalk, USA) by a high flow Electrospray interface (Turbo-Ionspray) (Burgess 2003). Analytes were separated on an Altima C18 column (150 x 4.6mm, Alltech Associates, Deerfield, IL.) using an acetonitrile 2 mM ammonium formate 0.1% formic acid gradient at a flow rate of

0.8 mL min⁻¹. The mass spectrometer was operated in the negative ion mode for OA. Samples were compared against certified reference material (NRC-CNRC, Halifax).

4.2.3 Statistics

Statistical analysis was undertaken using SigmaStat 3.11 (Systat Software, California, USA). The concentration of OA per wet weight of seagrass was calculated for each seagrass species by dividing the OA concentration by the volume of water used for extraction and then multiplying by the wet weight of seagrass collected. For this reason, all OA concentrations will be provided on a wet weight basis. Okadaic acid production per cell was determined by dividing the OA concentration by abundance of *P. lima* counted in each sample. Pearson product moment correlations were used to assess the relationships between OA concentration and the abundance of *P. lima* cells, water temperature and the concentration of OA in seagrass epiphytic material. Okadaic acid concentration data failed the test for homogeneity of variance and as such a Kruskal-Wallis One Way Analysis of Variance on Ranks was used to compare OA concentration on each species of seagrass and the site at which samples were collected. Where a significant result was obtained, a post-hoc pairwise multiple comparison procedure (Dunn's Method) was used to assess each pair.

Estimated daily exposure (DE) of OA for dugongs and turtles was calculated by multiplying the OA concentration measured for each seagrass species by the daily consumption of seagrass by the animals based on a 400 kg dugong consuming ~40 kg_(wwtSG) day⁻¹ (Lanyon 1991) and a 60 kg turtle consuming ~2 kg_(wwtSG) day⁻¹ (Formula 4.1) (Bjorndal 1980). The average daily exposure per kg body weight of animal was calculated by dividing the estimated daily exposure by the average body weight (Formula 4.2). This value was then used as the estimation of OA in tissue samples.

Formula 4.1: $DE (\mu\text{g day}^{-1}) = \text{Toxin in food } (\mu\text{g g}^{-1}) \times \text{daily consumption (g day}^{-1})$

Formula 4.2: $\text{Toxin } (\mu\text{g day}^{-1} \text{ kg}^{-1}) = \frac{DE (\mu\text{g day}^{-1})}{\text{mean BW (kg)}}$

4.3 Results

4.3.1 *Prorocentrum lima* abundance & algal toxins from seagrass

Prorocentrum lima was observed on all four species of seagrass and at all three sites examined in Moreton Bay between February 2004 and June 2005. Similarly, OA was detected in seagrass epiphyte extracts collected at all sites and from all seagrass species. There was a significant correlation between the *P. lima* cell abundance and the OA concentration (Pearson correlation: $r = 0.40$; $P < 0.001$; Figure 2) with the average OA production per cell being $0.24 (\pm 0.04) \text{ pg cell}^{-1}$.

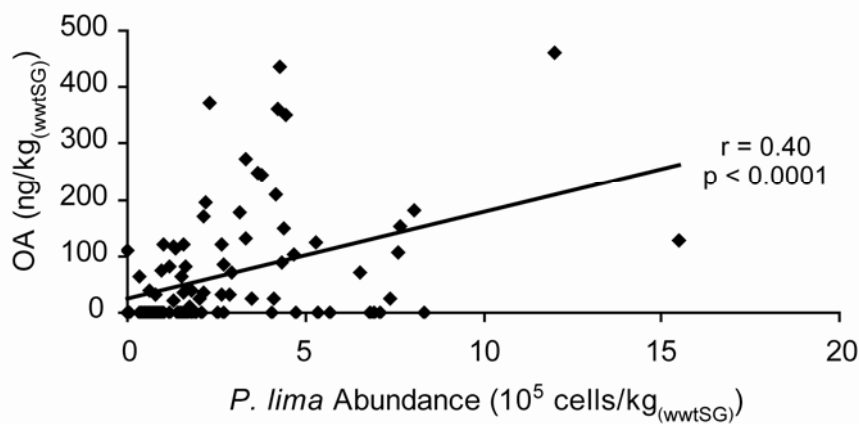


Figure 4.2. The relationship between *Prorocentrum lima* cell abundance and okadaic acid (OA) concentration in epiphyte material collected from seagrass samples collected in Moreton Bay, Australia ($n = 99$).

Two of the four seagrass species examined were found at all sites. These were *Zostera muelleri* and *Halophila ovalis*. In addition to these two species, *Cymodocea serrulata* was found at Myora and Pumicestone Passage, while *H. spinulosa* was only found at Rous Channel. *H. spinulosa* samples had significantly more OA present (than the other seagrass species (Kruskal-Wallis One Way Analysis of Variance on Ranks: $H = 13.875$, $df = 3$, $P = 0.003$: Figure 3.1), however, this species was only observed at Rous Channel where a significantly higher concentration of OA was observed in seagrass epiphytes (Kruskal-Wallis One Way Analysis of Variance on Ranks: $H = 19.505$, $df = 2$, $P < 0.001$: Figure 3.2). To elucidate whether these differences were driven by site or species, we examined the two species that were found at all sites (*Z. muelleri* and *H. ovalis*) and found that while there was not a

significant difference between the concentration of OA found on these two species (Kruskal-Wallis One Way Analysis of Variance on Ranks: $H = 0.336$, $df = 1$, $P = 0.562$; Figure 3.1), there was a significant effect of site when only these two species were considered (Kruskal-Wallis One Way Analysis of Variance on Ranks: $H = 8.826$, $df = 2$, $P = 0.012$; Figure 4.3).

During the sampling periods, temperature of the water varied between 16 and 27 °C. OA concentration was significantly correlated with the rise in temperature (Pearson correlation: $r = 0.24$, $p = 0.016$), however, the number of *P. lima* cells present in seagrass epiphyte material was not significantly correlated to water temperature (Pearson correlation: $r = 0.02$, $p = 0.813$). The peak cell abundance and OA concentration were seen in November 2004, during spring (Figure 4.4), at which time the water temperature was at 24°C. Both cell abundance and OA concentration also showed a slight increase during winter months (water temperature 16-17 °C).

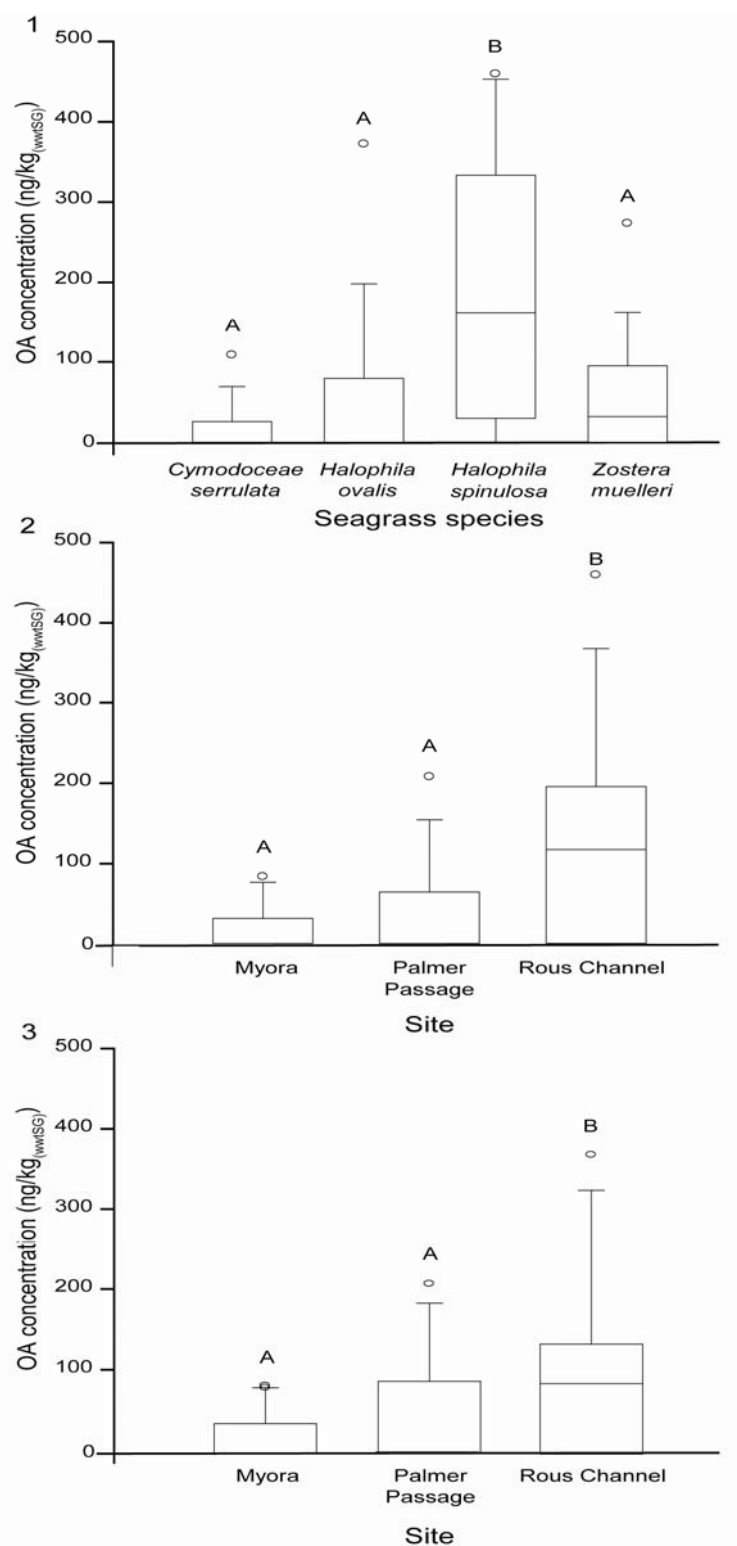


Figure 4.3. Box plots showing the concentration of okadaic acid found in epiphyte material collected from 1. four species of seagrass with all sites combined, 2. three sites in Moreton Bay, Australia combining all seagrass species and 3. combined data for *Z. muelleri* and *H. ovalis* at all sites.

*The bottom and top of the box represents the 25th and 75th percentile respectively, while the median is represented by the bar in the middle of the box. Error bars represent 90th percentile and dots the maximal value observed. Statistically significant groups (Dunn's post hoc multiple comparisons test) are denoted by the letter above the box.

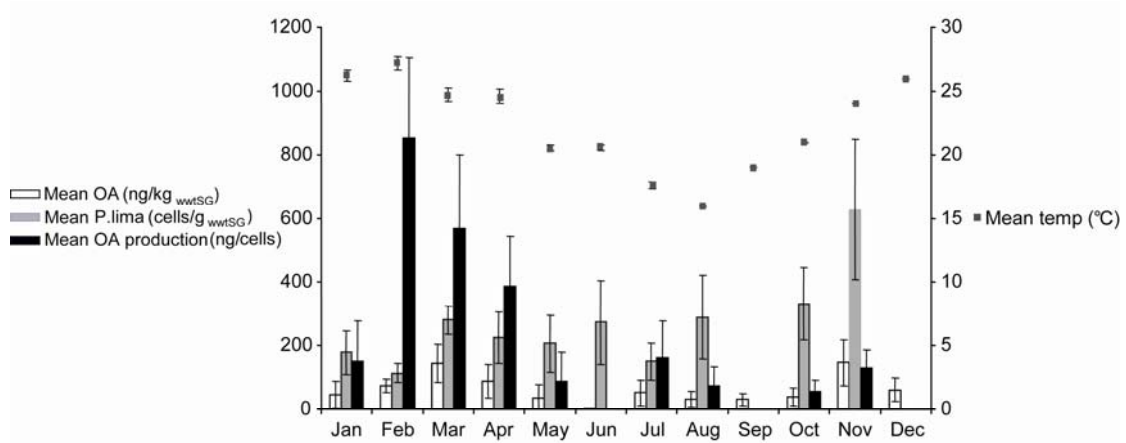


Figure 4.4. Mean OA concentration, abundance of *P. lima* and temperature over a twelve month period. Error bars represent standard error per month (n = 99).

4.3.2 Exposure of dugongs and turtles to OA

Halophila spinulosa was found to be associated with the highest concentration of OA, therefore, the maximum potential exposure of dugongs and turtles to OA were calculated using the maximum concentration of OA extracted from *H. spinulosa*. For estimating the mean OA exposure, the mean concentration of OA extracted from *H. ovalis* was used since *H. ovalis* is known to be the preferred species of seagrass for both dugongs and turtles in this area (Lanyon 1991, Read & Limpus 2002).

The maximum and mean daily exposures (DE) of OA were calculated for dugongs and turtles (Table 4.1). The estimated maximum DE by dugongs was $18,400 \text{ ng day}^{-1}$ and mean $2,440 \text{ ng day}^{-1}$. From the estimated DEs, the maximum daily exposure per body weight of a 400kg dugong per kilogram of tissue was estimated to be $46 \text{ ng day}^{-1} \text{ kg}_{(\text{dugong})}^{-1}$ and mean to of $6 \text{ ng day}^{-1} \text{ kg}_{(\text{dugong})}^{-1}$. Similarly, a 60 kg green turtle is known to consume, on average, $218 \text{ g/seagrass/day}_{(\text{dwtSG})}$ (Bjorndal 1980), which equates to approximately $2 \text{ kg/seagrass/day}_{(\text{wwtSG})}$ using a wet:dry conversion of 8.75 (Gaus 2002). From this, it can then be estimated that these green turtles are consuming a maximum of 920 ng day^{-1} and a mean of 120 ng day^{-1} . The maximum and mean OA concentrations per body weight are $15 \text{ ng day}^{-1} \text{ kg}_{(\text{turtle})}^{-1}$ and $2 \text{ ng day}^{-1} \text{ kg}_{(\text{turtle})}^{-1}$ respectively.

Table 4.1. Estimated maximum and mean okadaic acid (OA) daily exposure by dugongs and turtles was based on a maximum OA concentration observed on *H. spinulosa* ($460 \text{ ng kg}_{(\text{wwtSG})}^{-1}$), a mean concentration ($60 \text{ ng kg}_{(\text{wwtSG})}^{-1}$) detected in epiphyte material on *H. ovalis* and a daily seagrass intake of $40 \text{ kg}_{(\text{wwtSG})} \text{ day}^{-1}$ and $2 \text{ kg}_{(\text{wwtSG})} \text{ day}^{-1}$ for dugong and green turtle respectively.

	Dugongs	Turtles
Max OA daily exposure (ng day^{-1})	18,400	92
Mean OA daily exposure (ng day^{-1})	2,440	12
Max OA dose per day per body weight ($\text{ng day}^{-1} \text{ kg}^{-1}$)	46	15
Mean OA dose per day per body weight ($\text{ng day}^{-1} \text{ kg}^{-1}$)	6	2

4.3.2 Okadaic acid in tissue samples of dugongs and turtles

Okadaic acid was not detected from any of the tissue samples from dugongs or turtles using HPLC/MS/MS analysis which has a detection limit of $10,000 \text{ ng kg}_{(\text{animal tissue})}^{-1}$. Concurrent spike and recovery quality assurance samples demonstrated $96.0 (\pm 3.2) \%$ recovery rate from dugong muscle tissue.

4.4 Discussion

Prorocentrum lima and OA were detected on epiphyte material collected from seagrass in Moreton Bay. The significant correlation between *P. lima* cell abundance and OA concentration suggests that the *P. lima* observed on the seagrass was the dominant producer of OA.

The concentration of OA in seagrass epiphytic material varied significantly between the seagrass species from which they were collected, with *H. spinulosa* found to have significantly more OA present in epiphytic material per wet weight of seagrass (Figure 3.1). In addition, this species of seagrass was only found at Rous Channel, the site found to have significantly higher concentrations of OA when only *Z. mulleri* and *H. ovalis* (the two species found at all sites) were considered (Figure 3.3). This suggests that the environmental conditions at the Rous Channel site may promote *P. lima* growth and / or OA production.

The difference in OA production around North Stradbroke Island is similar to studies conducted around Heron Island, in which presence and toxicity of *P. lima* have been previously documented (Morton & Tindall 1995, Morton 1998). Morton and Tindall (1995) showed that toxin production by *P. lima* varied significantly from different sites around the island, implying there may be different strains of *P. lima* within the same area, which could lead to varying levels of OA production.

Environmental factors such as light, nutrient availability, salinity and temperature may effect the growth of *P. lima* and OA production (McLachlan et al. 1994, Morton et al. 1994, Souto et al. 2001). In the present study, OA concentration in seagrass epiphytes on a seagrass weight bases, was significantly correlated with water temperature, however, cell abundance was not. This suggests that temperature may be the driver for OA production, while cell abundance may be more greatly affected by nutrient or light availability.

In the current study, *P. lima* were observed in seagrass epiphyte samples throughout the year with very little fluctuation in abundance, however, OA production peaked in the warmer summer and autumn months with the highest concentrations observed at 24 °C. This is an indication of potential higher exposure of OA in summer for the dugongs and turtles.

Despite the presence of OA in seagrass epiphyte samples throughout the year, OA was not detected in dugong or turtle tissue samples from animals thought to have been feeding in the region where *P. lima* were observed. There may be several explanations for this. Firstly, it is possible that while *P. lima* was ingested, the OA was not absorbed and assimilated during digestion. In addition, we estimated that the likely average exposure to OA in a dugong or a turtle consuming *H. ovalis* from any of the sites we assessed was 2,440 ng day⁻¹ and 12 ng day⁻¹ respectively. Based on this estimated intake, and assuming 100% uptake through digestion with no metabolic breakdown or excretion and uniform distribution through tissue, the expected concentration in tissue after a twelve month average accumulated exposure would be 2,226 ng kg_(dugong)⁻¹ or 73 ng kg_(turtle)⁻¹. These estimates are well below the detection limit (10,000 ng kg_(tissue)⁻¹) available using HPLC/MS/MS. Also, as the animals that were available for analysis

were all stranded animals and hence potentially sick prior to stranding, it is possible that the animals were not foraging normally prior to stranding. In addition, these animals were collected from various areas around the bay, and, as location appears to influence the amount of OA present in samples (Figure 3), these animals may have been in residence in areas where OA exposure was less than estimated here. Furthermore, since samples used in this assessment were collected for other purposes, limited tissue types were available for analysis. Had more turtle gastro-intestine kidney or liver samples been available, results from these tissue types may have yielded higher concentration of OA (Toyofuku 2006).

A previous study in Hawaii estimated that the presence of OA in green turtle kidney ranged between 24 - 670 $\mu\text{g g}_{(\text{tissue})}^{-1}$ using a protein phosphatase inhibition assay (PPIA) (Landsberg et al. 1999). This estimate is much greater than the HPLC/MS/MS detection limit determined in the current study even though the mean cell abundances were similar (current study: 247.7 cells $\text{g}_{(\text{wwtSG})}^{-1}$ Kaneohe Bay: 239.1 cells $\text{g}_{(\text{wwtSG/algae})}^{-1}$). The major difference between these sites is the substrate on which the turtles forage. In Moreton Bay turtles predominantly feed on seagrass with small amounts of macroalgae (Read & Limpus 2002), while turtles in Kaneohe Bay feed predominantly on macroalgae supplemented with small amounts of seagrass (Arthur 2005). OA in seagrass epiphyte material was not measured in the Hawaiian study and since production of OA by *P. lima* is known to vary with environmental factors, *P. lima* in Hawaii may have been producing a greater concentration of OA than those in Moreton Bay. This difference in OA production per *P. lima* cells has also been observed between this study (0.24 pg cell^{-1}) and that conducted in waters around Heron Island by Morton and Tindall in 1995 (5.88 pg cell^{-1}).

Another major difference between the two studies is the method used to estimate the amount of OA in turtle tissue. The PPIA is an indirect measure of OA, as it is non-specific for the suite of protein phosphate inhibitors (Gehringer 2004). Since there are other natural toxins known to inhibit protein phosphatases, such as microcystins and nodularin, produced by freshwater and marine cyanobacteria respectively (Honkanen et al. 1994), using PPIA alone could provide a false positive result for OA. The common tropical marine cyanobacterium, *Trichodesmium erythraeum* has been shown to produce microcystin-like compounds which inhibit protein phosphatases (Shaw et

al. 2001) and as such, an organism other than *Prorocentrum* spp. may have been the source of the protein phosphatase inhibiting compound in the Hawaiian study.

The potential exposure of dugong and turtle to OA that we have identified here indicates that these large herbivores may be impacted by the deleterious effects of this toxin. It has been suggested that naturally produced tumour promoting compounds such as OA may play a role in green turtle fibropapilloma (Landsberg et al. 1999, Arthur et al. 2006), a disease in which benign tumours grow both internally and externally in marine turtles (Herbst 1994). Although there are no records of tumours in dugongs, these animals are likely to be consuming OA, and the presence of OA producing *P. lima* in Moreton Bay provides a potential route of exposure to OA for seagrass consumers. Hence long term risks for these animals are potentially an issue. Carcinogenic chemicals, including persistent organic pollutants have been determined in dugong tissue from Moreton Bay (Gaus 2002), and the presence of tumour promoters such as OA have the capacity to increase the risk of tumour formation to an undetermined degree.

The metabolism of OA in marine mammals and reptiles is unknown, and little is known in laboratory mammals. Matias et al (1999) found that when mice were orally dosed with 50 $\mu\text{g kg}^{-1}$ OA, traces of the toxin were found in all tissues. However, 37% were found in the intestine content (Matias et al. 1999). Other studies also support that the target organ for OA is the small intestine (Ito et al. 2002). Although dugongs are also mammals, the metabolism would be slower than rodents and they are also known to be a post-gastric fermenter (Lanyon 1991). Being a reptile, turtles may differ even further from laboratory rodents. For these reasons, it is difficult to extrapolate bioaccumulation and metabolism of OA in dugongs and turtles from laboratory studies on rodents.

This is the first time that OA has been detected in epiphytic material from seagrass collected in Moreton Bay. The presence of OA on seagrass suggests that large marine herbivores such as dugong and turtle may be exposed to these compounds throughout the year. However, no mass die-offs have been observed in the past (Haines & Limpus 2000, 2002) and although the presence of fibropapilloma in the turtle population is high in some areas of the Bay (Limpus & Miller 1994, Aguirre et al. 1999), there is at

this stage, no direct evidence to suggest that OA poses a threat to these endangered species. However, the high detection limit using HPLC/MS/MS may mean that other techniques such as the non-specific PPIA in association with cell counts and OA analysis may be required to monitor OA exposure in the future.

Chapter 5

Literature Base Dose-Response Analysis on Effects of Algal Toxins

5.1 Introduction

Dose-response studies provide quantitative information on the relationship between the toxin exposure and its effect on the organism. The effects of toxins may be divided into several categories: acute vs. chronic; local vs. systemic; reversible vs. irreversible (WHO 1999). Acute effects are defined as the effect which manifests after a single dose, while chronic effects are only observed after repeated exposure over a prolonged period of time to a substance. A local effect can be seen at the point of contact while a systemic effect takes place at a point, or multiple target tissues different from where it entered the body. A reversible effect allows the tissues to recover and return to normal when the exposure ceases while an irreversible effect consists of permanent damage.

Numerous studies have investigated the effects of algal toxins, using exposure to various toxin concentrations. There are at least three methods for administering the toxin to the subject. Toxins can be dosed orally through the mouth of the subject, normally with a food source. Another method of dosing is via gavage, in which toxins are delivered by a stomach tube. The third method is by injection, either intravenous (i.v.) or intraperitoneal (i.p.).

The dose-response studies on algal toxins have been advancing with time. One of the first dose-response study on okadaic acid, for example, was conducted on laboratory mice (Yasumoto et al. 1978). As more tissue and cell culturing became accessible, cytotoxicity study increased in the field (Amzil et al. 1992, Kang et al. 1996, Burgess 2003). Such dose-response studies describe the change in effects of an organism, both whole organism or at cellular levels, with differing levels of toxin concentrations.

The aim addressed in this chapter is to investigate the dose-response relationships for okadaic acid (OA), domoic acid (DA), gymnodimine (GD) and pectenotoxin-2s (PTX-2s) using published literature data. A review of the published literature is conducted for information on acute and chronic effects on both whole organism and on cellular levels. The extended information on dose-response information obtained through this chapter will be combined with exposure assessment data with toxins around North Stradbroke Island for humans and dugongs for risk characterisation. The

lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) will be used to calculate the tolerable daily intake (TDI) and the guideline values (GV). Such information is critical in assessing risks of toxins.

5.2 Methods – Data Collation Techniques

For each of the four toxins: OA, DA, GD and PTX-2s, dose-response data were assembled from the published literature, described in Chapter 2 (Tables 2.4, 2.5, 2.6). To establish a comprehensive summary of these data collated from the literature, results were divided into three groups; whole organisms, human cell lines and non-human animal cell lines according to subjects of the studies. When information was available on whole organisms, it was subdivided into humans, primates and rodents. When necessary, data were further divided into application of toxins, either orally or by injection. These data were then categorised into severity of effects (no effect, mild, severe and lethal). These categories were loosely classified as follows: no effect: no observed effect reported in the article; mild: mild diarrhoea and LOAEL, light seizure; severe: permanent neuronal damage, convulsion and hippocampal seizure; lethal: when the victim or the laboratory animals died. The severity of effect was plotted on graphs using logarithmic scales for the dose.

Dose-response data used in this chapter have been listed in Chapter 2, in Tables 2.4 for OA; Table 2.5 for DA and Table 2.6 for GD. Some studies have used the mouse unit “MU” (dose sufficient to kill a mouse weighing 20 g) for their dose-response study. In this study the MU was converted into weight of toxins per body weight of the animals been dosed. For example, 12MU is equivalent to $48 \mu\text{g kg}^{-1}$. All units were then standardised to $\mu\text{g kg}^{-1}$ body weight for all toxins. For studies conducted on cell lines, units of doses were also standardised to $\mu\text{g L}^{-1}$ which is equivalent to $\mu\text{g kg}^{-1}$.

5.3 Results- Data Evaluation

5.3.1 Okadaic acid

The comparison between severity of effects and dose of OA can be observed in Figures 5.1a and b. Information on human data comes from accidental consumption of shellfish contaminated with algal toxins, hence the “doses” for these data are from the amount obtained from the food source, which contain estimates of the consumed amounts. There have been no lethal levels recorded for humans, as the response with human data comprises only of differing severity of diarrhoea. High OA doses were used on laboratory tested animals, such as mice and chickens. Lethal doses per body weight for these two organisms are similar, ranging from 190 to 520 $\mu\text{g kg}^{-1}$. Doses causing mild diarrhoea in mice (50 $\mu\text{g kg}^{-1}$) were significantly higher than those observed in humans (0.8 $\mu\text{g kg}^{-1}$), highlighting the importance of using human data where available, or the use of appropriate interspecies safety factors in establishing the TDI for humans.

The range of doses used for animal cell lines was relatively low, ranging from 5 to 80 $\mu\text{g kg}^{-1}$ (Figure 5.1b). The severity of the effects showed a linear pattern related to the doses of the toxins. Doses on human cell lines showed that effects may depend on the types of cells used (Figure 5.2c). For example, concentrations corresponding to the NOAEL for myeloma cells were up to 10 fold less than that observed for bronchus cells (3 to 6 $\mu\text{g kg}^{-1}$). The IC_{30} and IC_{50} for KB cells ranged from 1 to 24 $\mu\text{g kg}^{-1}$.

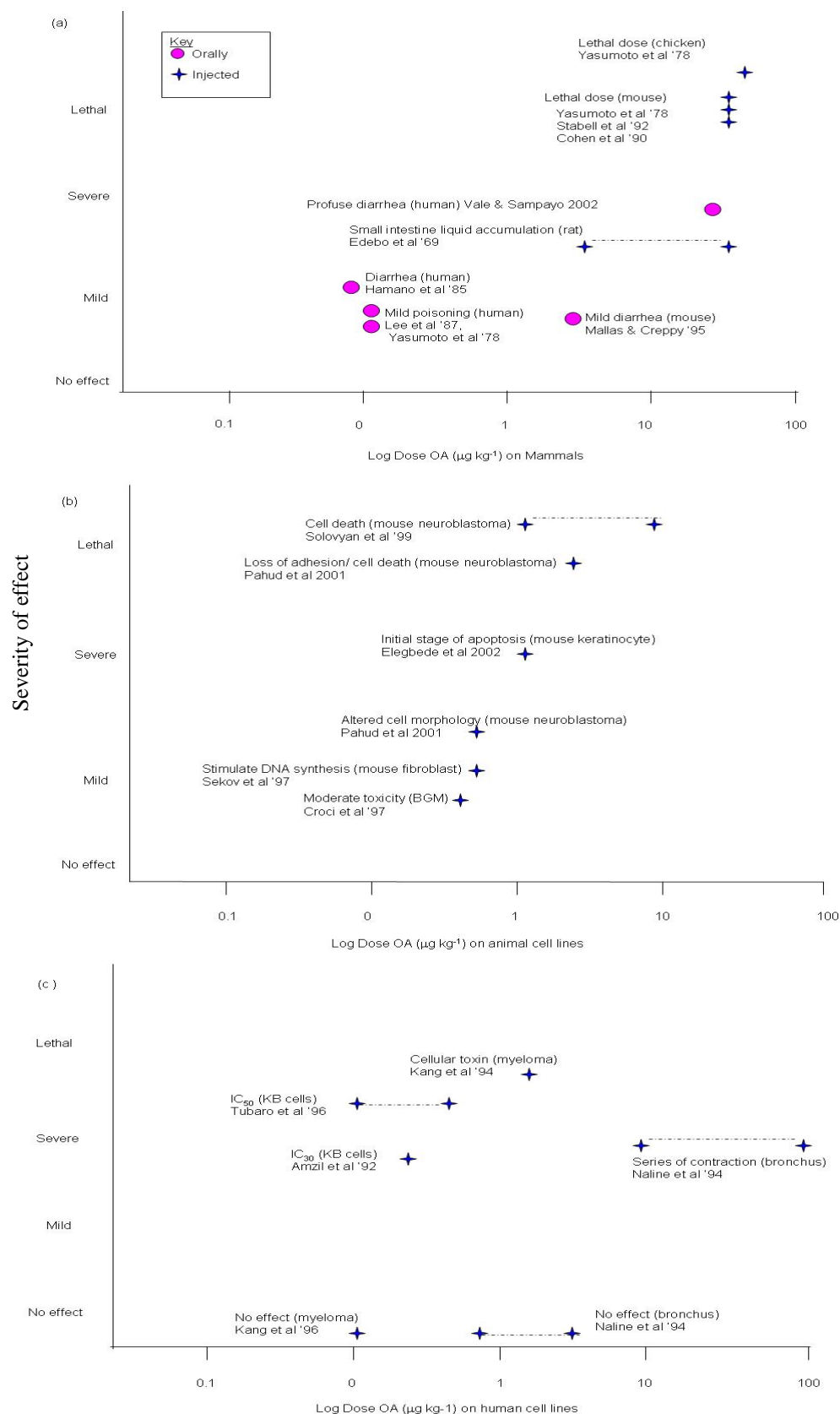


Figure 5.1. Dose –response (as severity of effects) for okadaic acid (OA) (a) dose-response on whole organisms; (b) dose-response on animal cells; (c) dose-response on human cells.

5.3.2 Domoic acid

A large toxicity data set on whole organisms was available, hence data for domoic acid were split into oral doses and injected doses, and plotted on separate graphs with a logarithmic dose scale. Accidental consumption by humans showed a linear dose-response relationship, from no effect ($20 \mu\text{g kg}^{-1}$) to severe and lethal effects ($4200 \mu\text{g kg}^{-1}$) (Figure 5.2a). Effects of DA appear to be species dependent. For example, the dose causing effect of vomiting was ten fold different between humans ($1,000 \mu\text{g kg}^{-1}$) and monkeys ($10,000 \mu\text{g kg}^{-1}$) (Iverson et al. 1990, Perl et al. 1990, Todd 1993). This again highlights the requirement for safety factors to account for interspecies differences in susceptibility. Even within the same class of organisms, for example in fish DA showed very different effects, to rainbow trout which showed no sign of toxicity at 58mg kg^{-1} whilst anchovies showed severe neurotoxic signs at 14mg kg^{-1} (Hardy et al. 1995, Lefebvre et al. 2001). Sea lions had the lowest lethal dose of 0.2mg kg^{-1} , indicating their sensitivity to DA, whilst rats had the highest lethal dose of 80mg kg^{-1} (Todd 1993, Scholin et al. 2000).

Despite the differences within the fish group, mammals were similar to one another in the severity of effects when DA was administered by injection (i.p. or i.v.). The dose-response relationships for humans, monkeys and rodents were roughly linear, ranging from no effect to lethal amounts (Figure 5.2b). Both the rodents and the primates species shared similar lethal doses between $4 - 5 \text{mg kg}^{-1}$ DA when injected (i.p.) (Tryphonas et al. 1990, Schmued et al. 1995, Fujita et al. 1996).

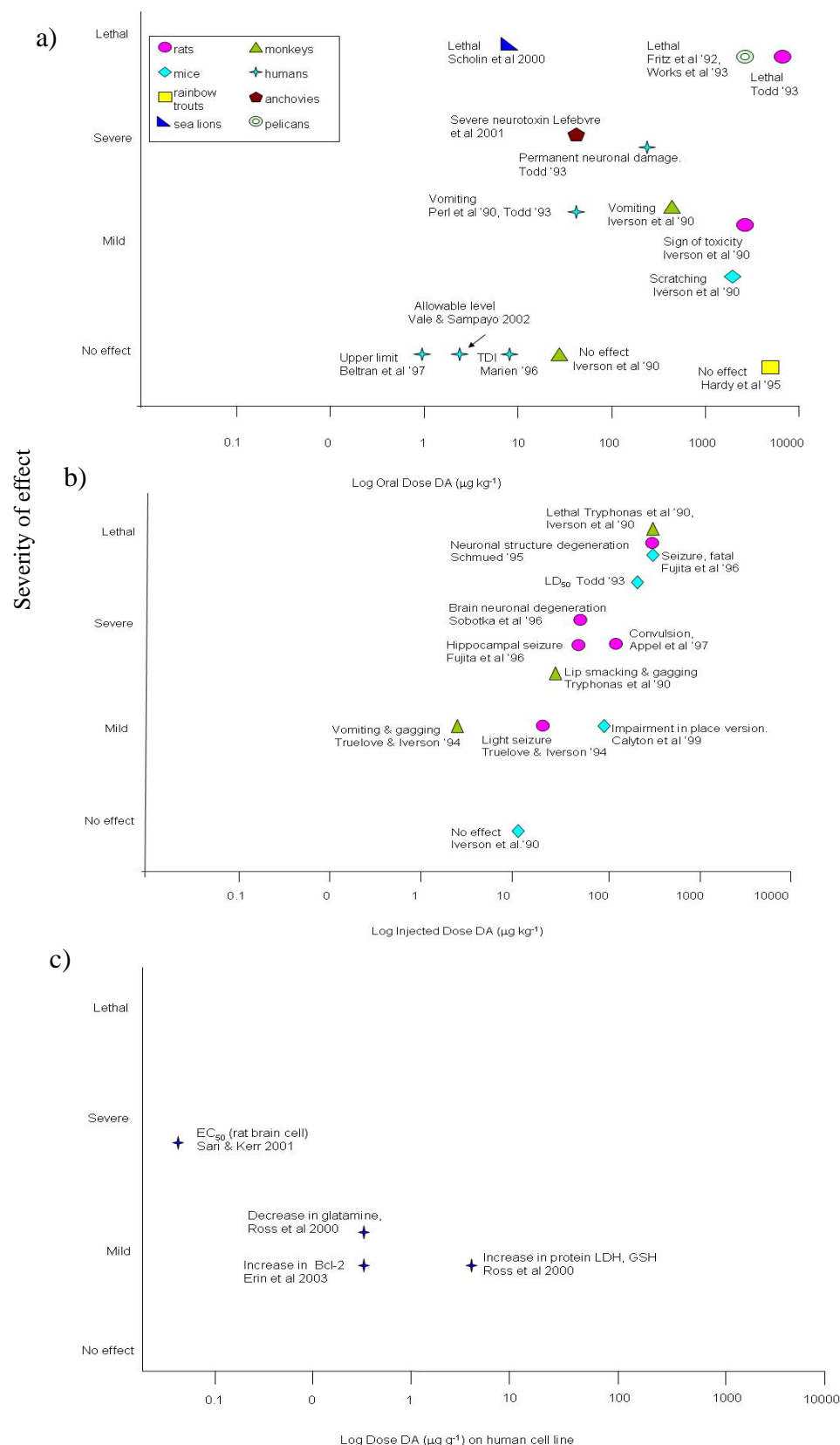


Figure 5.2. Dose-response (as severity of effects) of domoic acid (DA) compared to severity of effects. (a) dose-response when dosed orally to different whole organisms; (b) dose-response when dosed by injection. (c) dose-response for cell lines.

5.3.3 Gymnodimine

As a relatively newly described toxin, the whole organism studies found for this toxin were all performed on mice. There are no human data for this toxin, nor lethal oral dose data for laboratory animals. The LD₅₀ level when dosed by gavage was 755 µg kg⁻¹ (Munday et al. 2004) (Figure 5.3). The lethal level (LD₅₀) for i.p. injection ranged between 96 and 700 µg kg⁻¹ (Stewart et al. 1997, Munday et al. 2004). When animals are orally dosed, the toxins are normally taken up with food which may reduce its toxicity. The most potent method of dosing is normally by injection, as the toxin is directly placed into the peritoneal vein and has no chance of breaking down. Information on cell toxicity is limited as there was only one published article in the literature found at the time of the study. This was performed on neuroblastoma cells, showing significant cell reduction at the dose of 10 µM (Dragunow et al. 2005).

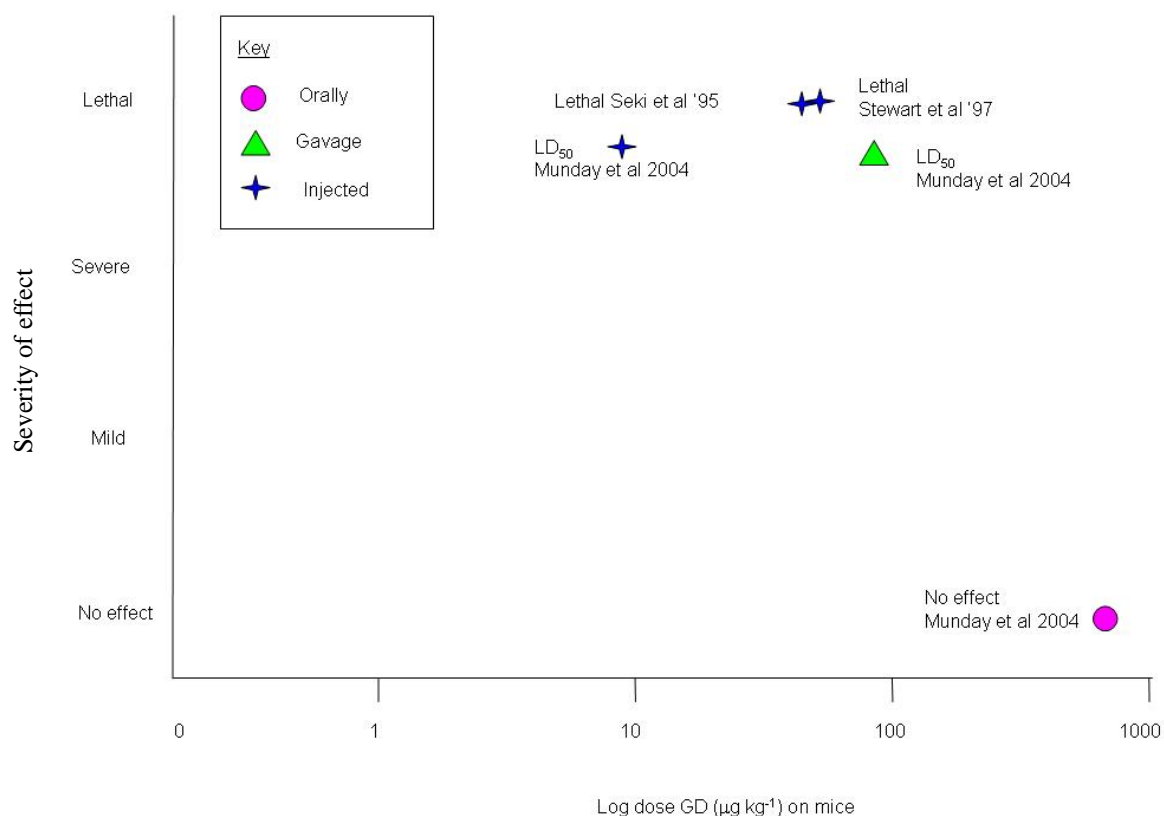


Figure 5.3. Dose-response (as severity of effects) of gymnoidimine (GD) compared to severity of effects on mice.

5.3.4 Pectenotoxin-2

Although PTX-2s are not as potent as OA, occurrence of PTX-2s is normally accompanied by OA. The toxicity of PTX-2 varied between and within species (Figure 5.4), depending on the study. The LOAEL for oral dose was lower than the NOAEL for gavage (EU/SANCO 2001, Miles et al. 2004a), probably due to the differences in the dosing method. The two acute intake data by Toyofuky (2006) are the estimated acute human intake levels, obtained by separate studies in two geographically different regions, which may explain the vast difference in the levels.

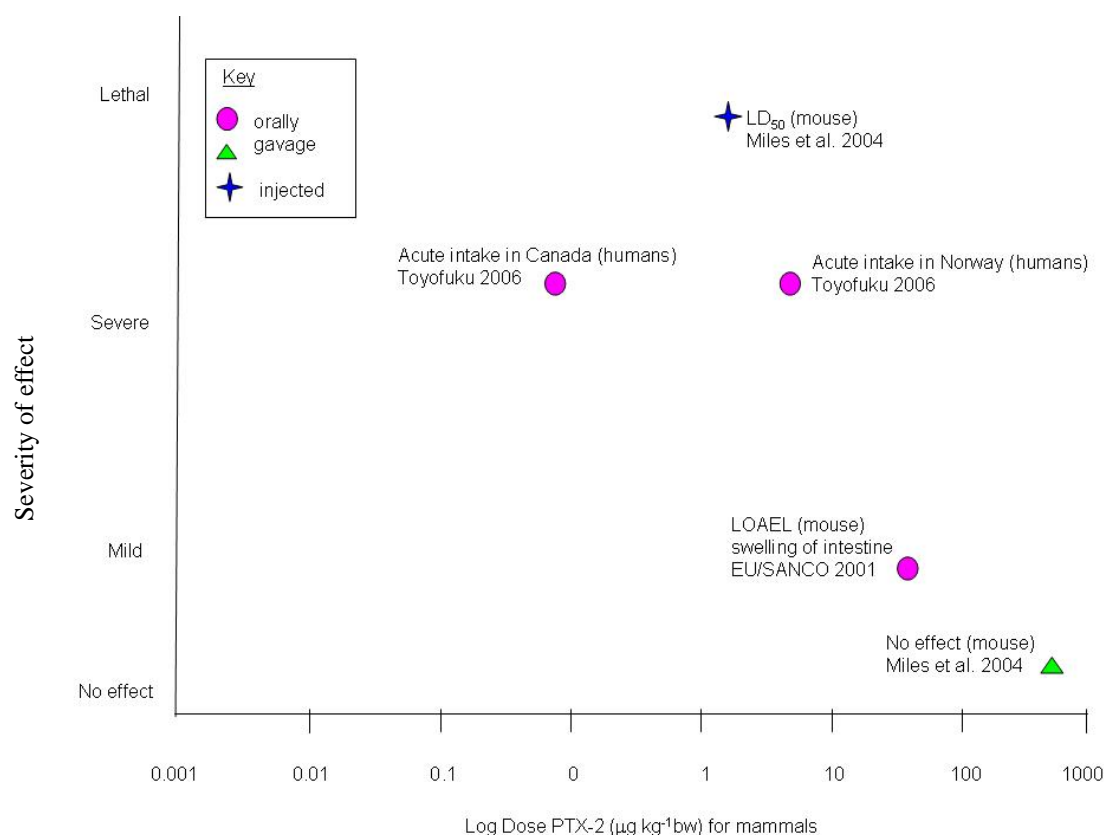


Figure 5.4. Dose of Pectenotoxin-2 (PTX-2) compared to severity of effects on mice.

5.4 Discussion

Since all data on humans are from accidental consumption, there are a limited number of occurrences and no intraperitoneal information. It is also important to note that the doses used for human data may not be as precise as laboratory conducted studies, as they are estimated doses derived from back calculations using the food source, after the event of poisoning.

When the doses of OA applied to mice (Matias & Creppy 1996b) are compared to the concentration accidentally consumed by humans (Yasumoto et al. 1978, Lee et al. 1987), mice showed higher tolerance to OA. This highlights the importance of incorporating safety factors into risk assessment for interspecies comparison, and this point will be discussed further in Chapter 7. The variability of OA dose-response data on human cell lines showed the need to consider the differences in responses between the cell lines. This may relate to differential susceptibility of organs that the cells were derived from.

The domoic acid also showed similar relationships between rodents and humans as mice were more tolerant to DA than were humans when the toxin was orally consumed (Iverson et al. 1990, Todd 1993). With the whole animal studies, sea lions had the lowest tolerance to DA (Scholin et al. 2000). Again, as the sea lion data comes from an accidental consumption incidence in the wild, the consumption rate for DA was estimated from the DA found in anchovies which were consumed by the sea lions, hence it is possible that the consumption rate was under estimated. Nevertheless this was an unexpected outcome since sea lions, being marine organisms, are expected to better adapt to marine toxins, compared to the land mammals. Even more surprising was the study conducted on anchovies (Lefebvre et al. 2001), which showed low tolerance to DA. On the contrary, rainbow trout showed extremely high tolerance to DA compared to any other studied organisms (Hardy et al. 1995). The difference in response, between these two species of fish demonstrates the significance of interspecies variation. The intraperitoneal studies showed less variation between organisms as rodents and monkeys had similar lethal levels (Tryphonas et al. 1990, Fujita et al. 1996). This may be explained by the bioavailability of the toxins when dosed orally compared to i.p. injection. When injected, the toxins go straight to the peritoneal cavity whilst with oral dosing, absorption of toxin is through stomach and intestine, where they may be metabolised.

The dose-response information on GD is limited due to the lack of research on this newly detected toxin. The reason for the scarcity of research may also be related to the lack of acute effects of this toxin when orally dosed to mice, although GD was found to be highly toxic when dosed via gavage (Munday et al. 2004). In Munday's

study (2004) GD was incorporated into cheese for oral dosing, which may have affected the uptake of the toxin, hence showing no potency when orally dosed, however showing high potency when dosed via gavage. Gymnodimine is a relatively lipophilic toxin as evidenced by its detection in the SPATT samplers (Chapter 3). It would therefore be expected that a lipid rich food such as cheese may have reduced uptake due to relatively low partition from the cheese lipids. Such findings imply more studies on GD uptake and its toxicity are required.

Although the presence of PTXs were discovered due to their high acute toxicity when injected into laboratory mice (Toyofuku 2006), the dose-response information for PTX-2 is not as abundant as that for OA and DA. The estimated level of acute toxicity for humans differed 10 fold between Canada and Norway (Toyofuku 2006). This may be due to the extraction and analysis differences in the two locations, or that the levels which provoke diarrhoea, for PTX-2 have a large range. Furthermore, the no effect level by gavage determined by Miles et al.(2004) was higher than the LOAEL when orally dosed (EU/SANCO 2001). Normally gavage dosing is more toxic than oral dosing since in administering by gavage, the toxin does not have the chance to breakdown in the process of reaching the stomach. The reason for this may have occurred due to the differences in the experimental setup, however further investigation is required to understand the effects the dosing methods have on the dose-response analysis.

5.5 Conclusion

From the compiled literature, there is a general trend in the potency of toxins depending on the mechanism of dosing. Toxins are most toxic when they are directly injected into the vein (intravenous) compared to other methods of dosing as there will be direct uptake to the circulation and limited metabolism of the toxins. Dosing by gavage showed higher toxicity than oral dosing in food. Again, this may imply that toxins may have lower bioavailability from certain foods or degrade during the process of being consumed by the organism. For example Munday et al. (2004) has shown a significant increase in the toxicity of GD when the toxin was applied via gavage compared to when applied orally.

The toxins GD and PTX-2s were highly toxic when injected, but showed little toxicity when orally dosed. This makes it difficult to compare the different toxins. If the oral dosing studies were to be compared for all the toxins, OA would be the most toxic, followed by PTX-2, then DA.

The assessment of literature has also shown that the sensitivity of the subject to the toxins vary depending on the subject and the toxins. The studies listed in Table 5.1 were chosen as the most relevant for risk assessment analysis as they showed the LOAEL through oral consumption for humans, when available, or mouse. The comparison between dose-response data of the organisms has shown that humans are normally more susceptible to algal toxins than the rodents, although other primates may share similar toxicity to the rodents. It was also difficult to conclude which group of animals were more sensitive to the toxins. For example, the anchovies were more susceptible to DA compared to mammals, except for sea lions, but the rainbow trout was not affected by high dose of DA.

Table 5.1. List of most relevant orally dose-response studies for the analysed toxins

Toxin	Dose ($\mu\text{g kg}^{-1}$)	Effects	Organism	Reference
OA	40	diarrhoea	Human	Hamano 1986
DA	1000	vomiting	Human	Perl 1990
GD	7500	NOAEL	Mouse	Munday et al. 2004
PTX-2	250	LOAEL	Mouse	EU/SANCO 2001
PTX-2SA	5000	NOAEL	Mouse	Miles et al. 2004

Chapter 6

Dose-Response Component : Cytotoxicity and Gene Regulation

6.1 Introduction

Traditionally, toxicologists have used animal bioassays to identify potentially hazardous substances, including carcinogens, immunotoxins and neurotoxins, but these assays require high doses, which are not always comparable to what is found in the environment (Afshari et al. 1999). For such reasons, testing of toxins on animals has been challenged and alternative methods for dose-response studies are needed (Afshari et al. 1999). Cytotoxicity testing by dosing cell lines has become a popular alternative method to animal testing. Cytotoxicity tests not only provide information for lethal levels on cells, but can also be used to study toxicity related to gene regulation (Akerman et al. 2004).

Cytotoxicity refers to the effects of a substance which is toxic to cells. The cytotoxicity can also be determined by dosing the cells and observing the effects of the toxin at particular concentration. It is normally measured using an assay, such as the MTS assay, which is a colorimetric assay (Mosmann 1983). Initially, MTS (2-(4,5-Dimethylthiazol-2-yl)-2,5-dephenyltetrasolium bromide) is yellow and it is reduced to formazan, which is purple in colour. This reduction takes place in the mitochondrial membrane, and is part of the oxidative reductase pathway (Berridge & Tan 1993). The reduction will only take place if the cells are functioning, hence the MTS assay indirectly measures cell metabolism, from which the inhibitory concentration (IC) can be determined. This is an important step leading to the mechanistic toxicity analysis, which investigates the effects of toxins on gene regulation, as the appropriate IC needs to be determined for such analysis.

Various methods are available for detecting and quantifying gene expression levels, including northern blots and DNA sequencing (Duggan et al. 1999). However, they are time consuming. DNA microarray is a technique which allows for profiling gene expression patterns and screening tens of thousands of genes in a single experiment (Duggan et al. 1999, Burezynski et al. 2000). This method can help define important target molecules for toxicity and complex regulatory networks within a cell, tissue and/or organ that is responding to the toxin (NIEHS 2003). Microarray is a relatively new technique, as the first array was made in 1991 (Fodor et al. 1991), and the first

microarray experiment was performed with cDNA microarray by Schena et al. in 1995 (Schena 1995). It has, however, been utilised successfully in a variety of studies from detecting cancer genes (DeRisi et al. 1996, Tzang 2003) and discovery of disease-related genes (Heller et al. 1997), to gene comparison between different strains of yeast (Lashkari et al. 1997), and toxicological studies (Nuwaysir et al. 1999, Bartosiewicz et al. 2000, Burgess 2003, Shen et al. 2003, Vrana et al. 2003). The gene expression signatures are used to determine for various types of tissue-specific toxicants (Nuwaysir et al. 1999).

Microarrays are constructed by spotting PCR-amplified cDNA onto a solid matrix, such as glass slides (Figure 6.1) (Heller et al. 1997, Hedge et al. 2000). They are then coupled to two or more fluorescently labelled probes, prepared from mRNA from the cellular phenotypes of interest. By performing parallel hybridization of two different reverse transcribed cDNAs from individual mRNA samples, with arrayed cDNAs, the samples can be compared and analysed by comparing means of the ratio of two different coloured fluorescence intensities, which are measured by a confocal laser scanning (Schena et al. 1998, Tzang 2003).

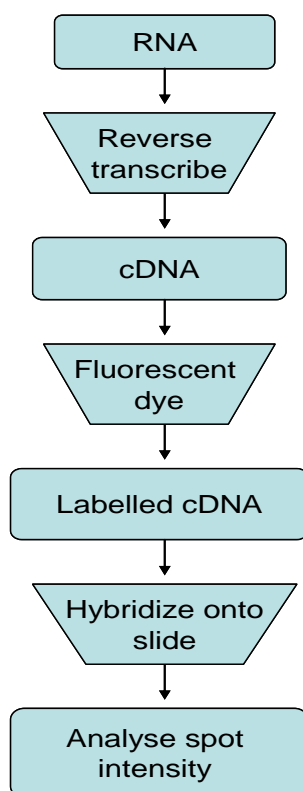


Figure 6.1. Flow chart showing steps involved in microarray analysis.

Since microarray analysis is a qualitative measure, it is important to quantify the results obtained by standard methodologies such as northern or western blotting and quantitative real time polymerase chain reaction (QRT-PCR) (Freeman 1999, Bartosiewicz et al. 2000, Snider et al. 2001). In QRT-PCR, the fluorescence signal is generated as the sequence is getting amplified and this is measured in real time (Snider et al. 2001). Examples of dyes used are SYBR Green, which binds to double stranded DNA or a specific probe which binds to the target sequence (Snider et al. 2001, Tzang 2003).

One software program used for screening genes of interest post microarray analysis is “GeneSpring” (Agilent Technologies). This software can be linked to numerous cellular pathways available on the web, such as Keggs (Kyoto Encyclopedia of Genes and Genomes: <http://www.genome.jp/kegg/pathway.html>) and BioCarta pathways (<http://www.biocarta.com/index.asp>). By linking various pathways to GeneSprings either the whole pathway or individual genes on the pathway can be studied. Some examples of pathways that will be investigated in this study are: oxidative phosphorylation, apoptosis, mitogen activating protein kinase MAPK and Wnt signalling pathways.

Oxidative phosphorylation takes place within the mitochondrial membrane of the cell and is an important step for generating ATP for the cells. Electrons are transferred by the electron carriers, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH), within the inner mitochondrial membrane (Alberts et al. 1994).

Mitogen activating protein kinase (MAPK) pathway plays a major role in cellular processes and are evolutionary conserved (Orton et al. 2005). It is involved in cellular proliferation, differentiation, development and transcription (Pearson et al. 2001). MAPK pathway consists of three kinases, activated by sequentially phosphorylating one another: MAPKKK, MAPKK, MAPK (Orton et al. 2005).

Apoptosis is a process of programmed cell death (Murphy et al. 2000). In contrast to necrosis, which is a form of cell death that results from acute toxicity or injury, apoptosis is a programmed death which is required to maintain the homeostasis within an organism (Murphy et al. 2000).

The Wnt signalling pathway is a major regulator of developmental processes and can lead to cancer formation (Lustig et al. 2002, Nusse 2005). This pathway branches into three sections: β -catenin pathway or canonical pathway in the nucleus; the planar cell polarity pathway and the Wnt/ Ca^{2+} pathway (Huelsenken & Behrens 2002).

In the previous chapter (Chapter 5), the published literature was sourced to compile data on dose-response analysis. There was limited data on cytotoxicity information for algal toxins, especially for gymnodimine (GD). For this reason, this chapter will investigate the dose-response study on the cellular level, and mechanistic toxicity study, investigating the effects of gene regulation by GD. This involves conducting toxicity test on the cell lines to obtain the IC_{30} level, and then to perform cDNA microarray analysis using RNA extracted from such cells. Determining the effects of toxins on gene regulation may serve to provide insight into the long term chronic effects of the toxins, which were lacking from the sourced published literature. The aim of this study is to measure the regulation of genes caused by algal toxins, especially GD, which may be related to chronic effects of these toxins.

6.2 Methods

6.2.1 Cytotoxicity

Cell culture

A human neuroblastoma cell line (BE-2) was kindly donated from Queensland Institute for Medical Research (QIMR). The human liver cancer cell line (HepG₂) was kindly donated from Mr. Peter Bain, Griffith University. Both cell lines were grown in 75ml flasks using Opti-mem1 media with FBC and Multicel. Cells were grown in the incubator with the temperature maintained at 37°C and CO₂ level at 5%.

MTS assay

Cell metabolism can be measured by using the MTS assay which measures the production of fumarate by the cells. Fumarate reductase occurs in the mitochondrial matrix of the cells (Marshall et al. 1995). For this experiment Promega MTS assay was used.

Cell cultures of BE-2 cells were seeded into six 96 well plates at a concentration of 5×10^4 cells per ml with 100 μ L media per well (5×10^3 cells per well). Protocol for seeding cells can be found in Appendix B. Cells were allowed to adhere to the wells overnight in the incubator. Media within the wells were pipetted out and replaced with 100 μ L of media containing three different concentrations of the toxins, okadaic acid (OA), domoic acid (DA) and GD (Table 6.1). There were triplicates for each concentration, and each plate also contained triplicates of blank and the control wells (blank = media only; control = cells and media). After incubation for 24 and 48 hours, 20 μ L of Cell Titer 96^R Aqueous One Solution Reagent was added to each well and read on the plate reader between 1-4 hours of incubation, at wavelength of 450 nm.

Table 6.1. Concentrations of the toxins tested BE-2 cells.

Blank	Control	Okadaic acid (nM)	Gymnodimine (nM)	Domoic acid (μ M)
media	media + cells	1	10	50
media	media + cells	10	50	100
media	media + cells	100	100	1000

Data analysis for MTS assay

To obtain the level of the toxins at which 30% of the cells stopped metabolizing (IC_{30}) on BE-2 cells, the 450 nm wavelength reading for the blank was subtracted from data. Average reading was obtained for the control and the toxin containing wells. Each of the readings for the sample well was divided by the readings from the control well to obtain the survival rate (%).

6.2.2 Mechanistic toxicity

The initial study was conducted for three toxins; OA, DA and GD, by using the human neuroblastoma cell line (BE-2: QIMR). This was a pilot study and the information on the initial study is reported in Appendix A. The second part of the study, which will be reported later in this chapter, was performed using GD alone on the human liver cancer cell line (HepG₂).

Cell dosing

The HepG₂ cells were grown in 96 well plates (Appendix B). When cells reached 80% confluence, they were dosed with GD at the IC₃₀ (determined on BE-2 cells). Toxicity level of IC₃₀ was selected to determine the long term - low dose exposure of toxins on the gene regulation, without cell deaths signals being apparent. These cells were then incubated for a further 24 hours prior to RNA extraction.

RNA extraction and quantification

Total RNA from HepG₂ cells was isolated using QIAGEN RNeasy mini kit plus by following their in house protocol (Qiagen Inc., Valencia, CA) (Appendix C). For the homogenisation step, 2 mm sterile needle was used. For the final elution step, RNA was eluted in 80 µL RNase free water. RNA 6000 Nano assay was used to test for the purity, quality and the quantity of the RNA samples using LabChip Kit (Agilent Technology: <http://microarray.imb.uq.edu.au/Protocols/RNA6000Nano.pdf>). Agilent 2100 bioanalyzer was used for measurement of RNA.

Microarray preparation

RNA was reversed transcribed into cDNA, purified, transcribed back into amplified RNA (aRNA), purified, labeled with fluorescent dye and hybridized onto the microarray slides following the protocol provided by Ambion and Invitrogen (Appendix D).

Data imaging and analysis

Microarrays were scanned by a ScanArray 400TM. For every microarray, initial images were scanned at low resolution (50 µm pixel⁻¹). When the appropriate photo multiple tube (PMT) was selected, images were scanned at high resolution (10 µm pixel⁻¹). Mean and standard deviation of the three replicates were calculated for both the control and the treated samples. Any spots with less than double the background intensity were eliminated. For up-regulated genes, those that were expressed 2 fold higher than the control were selected, and for down-regulated genes, those that were 3 fold higher than the control were selected.

Gene identification

The genes of interest were identified using the specific Genbank number matched to individual genes listed on the website:

(Source: <http://genome-www5.stanford.edu/cgi-bin/source/sourcesSearch>).

Information was accumulated for all genes showing at least two fold up or down-regulation in all three replicate microarray slides. These genes were also grouped into functions using the Function Annotation Clustering within David 2006 Functional Annotation Bioinformatics (<http://david.abcc.ncifcrf.gov/home.jsp>).

Several available pathways from GeneSpring were also matched to the genes of interest. The pathway oxidative phosphorylation, which includes the fumarate reductase involved in the MTS assay, was investigated. Other pathways investigated were MAPK signaling pathway, apoptosis and Wnt signaling pathway which were related to the functions of the genes of interest.

Designing primers

For each of the genes of interest, their gene sequences were “blasted” using GeneBank, NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) to check for any other similar sequences of genes. Forward and reverse primers were designed using Primer Designer (School of Integrated Biology, UQ)

In doing so, the following rules were followed:

1. Primers should be 18 – 30 nucleotides long
2. GC content 40- 60%
3. T_m (melting temperature) should be similar in both primer pairs.
4. Use annealing temp 5C below T_m
5. No primer dimers – avoided by using primer without complementary sequence, especially in the 3' ends.
6. Avoid three or more G or C at the 3' end

Reverse Transcriptase

To test for the quality and quantity of RNA, the Nano drop test was conducted (NanoDrop[®] ND-1000 Spectrophotometer <http://www.nanodrop.com/>). The original RNA was reverse transcribed into cDNA following the protocol provided by Invitrogen (Appendix E).

Quantitative real time PCR

Quantitative real time PCR (QRT-PCR) was run on ABI 7900HT, using a 96-well plate. There were three biological replicates for controls (C5, C6, C7), and for gymnodimine treated samples (G5, G6, G7) (Appendix E). Human Actin gene (donated by Peter Bain; School of Biomolecular and Biomedical, Griffith University) was used as a house keeping gene for this experiment. Each of the six samples possessed duplicates from the same 6 well-plate where the cells were grown.

Data from real time PCR was analysed using software “linkeg PCR”, a program which works with linear data and calculations (Appendix E). The difference in the intensity of expression between the housekeeping gene, human actin, and the genes of interest were compared.

Validation of PCR products

Quantitative real time -PCR samples were validated by running the PCR product on a 2% Agarose gel at 100V for 45 min. To test whether the desired genes were amplified during the PCR reaction, the bands for each gene were cut out from the agarose gel and purified using GelSpin DNA Purification Kit (MO BIO ULTRA CLEAN) and sent for sequencing analysis (AGRF) (Appendix E).

6.3 Results

6.3.1 Cytotoxicity

From the MTS assay, the toxin concentrations at which cells stop metabolizing was obtained (Table 6.2). This shows that OA (10 nM) is the most toxic to BE-2 cells, having IC₃₀ level of 10 nM which is 10 fold lower than that of GD (100 nM). Domoic acid was least toxic, with an IC₃₀ level of 100,000 nM.

Table 6.2. Doses of toxins resulting in IC₃₀ levels on BE-2 cells for RNA work

	Toxin Type		
	Okadaic acid	Gymnodimine	Domoic acid
IC ₃₀ dose (nM)	10	100	100,000

6.3.2 Mechanistic toxicity

6.3.2.1 Microarray

Three sets of RNA from control and GD dosed cells, with highest purity and quality were selected for microarray analysis (Figure 6.2).

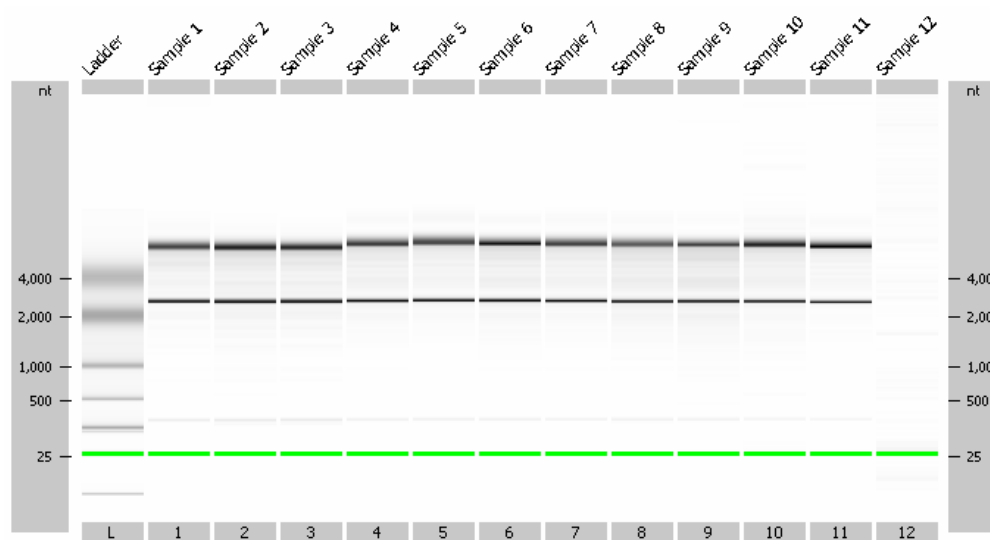


Figure 6.2. RNA 6000 Nano assay result; showing the 14s and 28s bands for the high purity RNA used for microarray analysis (samples 5,6,7).

The hybridized slides of the control and the treated spots can be seen in Figure 6.3. Genes showing either two fold up-regulation or three fold down-regulations are listed in Appendix F. The genes of interest were then selected depending on the significance of their regulation (Table 6.3a-b). In general, genes that were up-regulated were related to functions of transport, physiological process, catalytic, binding and membrane, whilst those down-regulated were related to phosphorylation, signal transduction and cell communication.

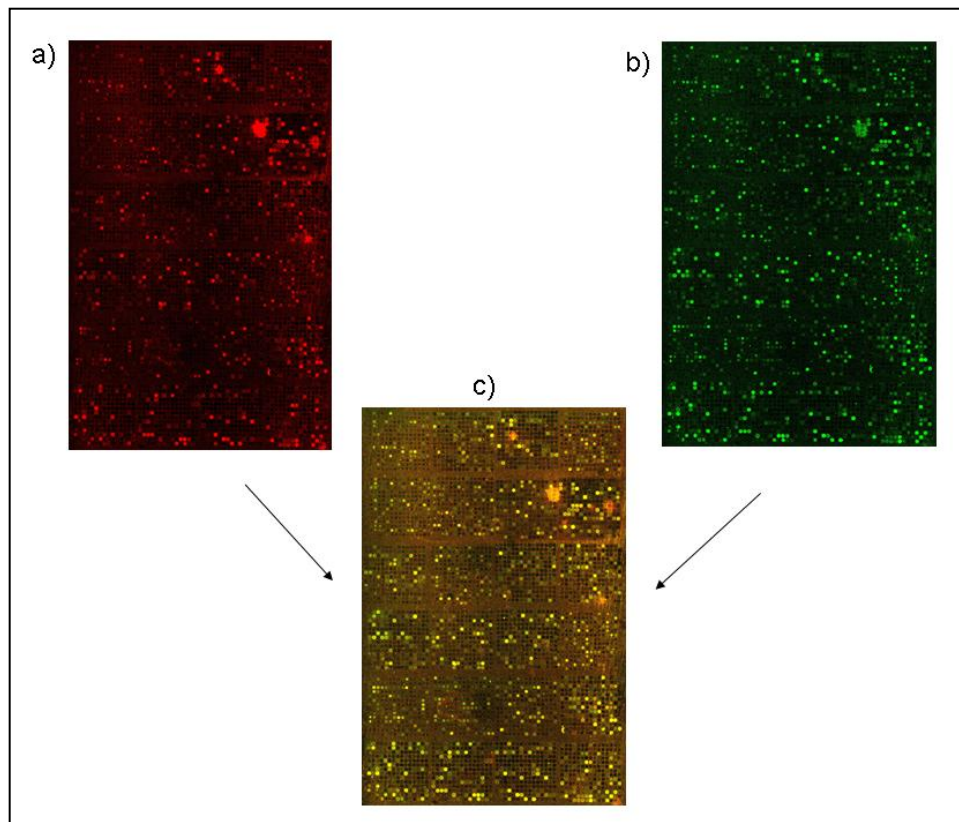


Figure 6.3. Scan of microarray chip showing genes from a) control cells, b) gymnodimine-treated cells, and c) combined image.

Table 6.3a. List of genes up-regulated in all three slides by two fold or higher. The function group derived from “Function Annotation Clustering” program in David Bioinformatics (<http://david.abcc.ncifcrf.gov/home.jsp>).

Symbol	Name	Function Group
KL	Klotho	Binding, membrane, catalytic, physiological
TM9SF1	Transmembrane 9 superfamily member 1	Transport, physiological
SSFA2	Sperm specific antigen 2	Membrane,
BATF	Basic leucine zipper transcription factor atf-like	Physiological, binding
NLK	Nemo-like kinase	Physiological, binding, /MAPK, WNT pathways
SCN8A	Sodium channel voltage gated	Transport, physiological, membrane, binding
MKP-5	DUSP10 dual specificity phosphatase	Catalytic, physiological/ MAPK pathways
PP1665	Glycerophosphodiester domain 5	Catalytic, physiological
APG4B	Atg4 autophagy related 4 homolog b	Transport, binding, physiological
SEC23IP	Sec23 interacting protein	Transport, binding, physiological
AKR1B1	Aldo-keto reductase family 1 member 1	Catalytic, physiological/ Pentose, fructose, glycerine, pyruvate pathways

Table 6.3b. List of genes down-regulated in all three slides by three fold or higher. The function group derived from “Function Annotation Clustering” program in David Bioinformatics (<http://david.abcc.ncifcrf.gov/home.jsp>).

Symbol	Name	Function Group
MAPK8IP2	Mitogen activated protein kinase 8 interacting protein 2	Signal transduction, cell communication/ MAPK pathways
PTK6	Ptk6 protein tyrosine kinase	Signal transduction, cell communication, phosphorylation
CSF2RA	Colony stimulating factor 2 receptor low affinity	Signal transduction, Cytokine receptor
FDXI	Ferredoxin 1	
PGR	Progesterone receptor	Phosphorylation, signal transduction, cell communication
RGS7	Regulator of G-protein signaling 7	Signal transduction, cell communication
FGD6	Five rho GTPase domain	
PMCH	Pro-melanin concentrating hormone	Signal transduction, cell communication
Cd248	Cd248 antigen, endosialin	

6.3.2.2 Gene Pathways

Oxidative Phosphorylation Pathway

Genes of interest were mapped onto different pathways using GeneSpring. The oxidative phosphorylation pathway is involved in the MTS assay in the inner mitochondrial membrane and matrix. The main gene for conversion of succinate to fumarate (succinate dehydrogenase fumarate reductase SDH/ 1.3.5.1) was down-regulated (Figure 6.4; Table 6.4). The ATP synthesis was down-regulated and so was Cytochrome c oxidase. The NADH dehydrogenase: Ndufv1, Ndubf4, 5 and 10 and Ndufa3,8 were up-regulated but Ndubf1, 7, Ndufc 1 were down-regulated.

Apoptosis

In general the genes related to the initial stage of the death ligand are slightly up-regulated (Figures 6.5a-b). Initial stages of survival factors on the other hand are down-regulated, however the final stages are up-regulated. Specifically, genes within the cell membrane, Fas genes and TNF genes, were up-regulated whilst cytoplasm genes were mostly down-regulated (Figure 6.5b; Table 6.4). Both protein kinase apoptosis inhibitor Akt/PKB and anti-apoptosis genes, Bcl-2 were up-regulated.

MAPK Signalling

Mitogen-activated protein kinase (MAPK) signalling is related to apoptosis and Wnt signalling. In general, those genes responsible for binding GTP to the activator gene was up-regulated, however the key genes such as RAS gene was down-regulated, with most genes not expressed by the toxin (Figure 6.6a; Table 6.4). Within the MAPK pathway, the p38 MAPK pathway was further investigated (Figure 6.6b).

Wnt Signalling

There are canonical pathway, planar cell polarity pathway and Wnt/Ca²⁺ pathway involved in this signalling pathway (Figures 6.7a-b; Table 6.4). Initial stages of all three sections of the pathway were up-regulated, however there were no clear up or down-regulation of the whole pathway. The canonical pathway regulates cell fate determination and primary axis formation through gene transcription. MAPK signalling pathway has a direct effect on this pathway.

Table 6.4. Regulation of four different signalling pathways, oxidative phosphorylation, apoptosis, MAPK and Wnt pathways

OXIDATIVE PHOSPHORYLATION		
Up-regulated	Gene name	Function
Ndufv1	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa (NM_007103)	Transfer of electrons from NADH to the respiratory chain. Immediate electron acceptor for the enzyme is believed to be ubiquinone.
Ndufa3	NADH dehydrogenase (ubiquinone) flavoprotein 1 alpha subcomplex 3, 9kDa NM_004542	As above
Ndufa8	NADH dehydrogenase (ubiquinone) flavoprotein 1 alpha subcomplex, 8, 19kDa NM_014222	As above
Ndufb4	NADH dehydrogenase (ubiquinone) flavoprotein 1 beta subcomplex, 4, 15kDa NM_004547	As above
Ndufb5	NADH dehydrogenase (ubiquinone) flavoprotein 1 beta subcomplex, 5, 16kDa NM_002492	As above
Ndufb10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa NM_004548	As above
Gamma/ ATP5C	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1 NM_005174	Produces ATP from ADP in the presence of a proton gradient across the membrane. The gamma chain is believed to be important in regulating ATPase activity and the flow of protons through the cf(0) complex.
Delta/ ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit NM_001687	Produces ATP from ADP in the presence of a proton gradient across the membrane.
d/ ATP5H	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d NM_006356	This is one of the chains of the nonenzymatic component of the mitochondrial ATPase complex.

OXIDATIVE PHOSPHORYLATION

Down-regulated	Gene name	Function
SDHA/ 1.3.5.1	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp) NM_004168	Produce fumarate from succinate
Ndufb1	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 1, 7kDa NM_004545	Transfer of electrons from NADH to the respiratory chain. the immediate electron acceptor for the enzyme is believed to be ubiquinone.
Ndufb7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa NM_004146	As above
Ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa NM_005004	As above
Ndufc1	NADH dehydrogenase (ubiquinone) 1 beta subcomplex unknown, 1, 6kDa NM_002494	As above
ATP12A/3.6.3.10	ATPase, H ⁺ /K ⁺ transporting, alpha polypeptide NM_001676	Catalyses the hydrolysis of ATP coupled with the exchange of h(+) and k(+) ions across the plasma membrane. Responsible for potassium absorption in various tissues.
QCR6	Ubiquinol-cytochrome c reductase hinge protein	This protein may mediate formation of the complex between cytochromes c and c1.
Cox10	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase	Converts protoheme ix and farnesyl diphosphate to heme, NM_001303
F ATP6V1F	ATPase, H ⁺ transporting, lysosomal 14kDa, V1 subunit F NM_004231	Vascular-ATPase is responsible for acidifying a variety of intracellular compartments
H ATP6V0E	lysosomal 9kDa, V0 subunit NM_003945	As above
Lipid/ ATP6V0C	lysosomal 16kDa, V0 subunit c NM_001694	Proton-conducting pore forming subunit. As above

APOPTOSIS		
Up-regulated	Gene name	Function
TNF α FAS	Tumor necrosis factor NM_000594	It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. Receptor, resulting death- inducing signalling complex performs caspase-8 proteolytic activation, which initiates the mediating of apoptosis.
	Fas (TNF receptor superfamily, member 6) NM_152875	
FAD D	Fas (TNFRSF6)-associated via death domain, NM_003824	Apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated fas (cd95) or tnfr-1 receptors. Resulting aggregate called the death-inducing signalling complex (disc) performs caspase-8 proteolytic activation/ mediating of apoptosis
FLIP/ CFLAR	CASP8 and FADD-like apoptosis regulator, NM_003879	Apoptosis regulator protein which may function as a crucial link between cell survival and cell death pathways in mammalian cells. Acts as an inhibitor of tnfrsf6 mediated apoptosis. A proteolytic fragment (p43) is likely retained in the death-inducing signalling complex (disc) thereby blocking further recruitment and processing of caspase-8 at the complex.
TRAF2	TNF receptor-associated factor 2, NM_021138	Adaptor protein and signal transducer that links members of the tumour necrosis factor receptor family to different signalling pathways by association with the receptor cytoplasmic domain and kinases.
Bcl-2/XL	B-cell CLL/lymphoma 2, NM_000633	Suppresses apoptosis in a variety of cell systems including factor-dependent lymphohematopoietic and neural cells. Regulates cell death by controlling the mitochondrial membrane permeability. Appears to function in a feedback loop system with caspases. Inhibits caspase activity either by preventing the release of cytochrome c from the mitochondria and/or by binding to the apoptosis-activating factor (apaf-1).
AKT3/PKB	V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma), NM_005465	Leads to the activation of akt3, which may play a role in regulating cell survival. Capable of phosphorylating several known proteins. Truncated isoform 2/pkb gamma 1 without the second serine phosphorylation site could still be stimulated but to a lesser extent.

APOPTOSIS

Down-regulated	Gene name	Function
DFF 45	DNA fragmentation factor, 45kDa, alpha polypeptide, NM_213566	Inhibitor of the caspase-activated DNase
DFF 40/	DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase), NM_001004286	Nuclease that induces dna fragmentation and chromatin condensation during apoptosis. Degrades naked dna and induces apoptotic morphology.
ENDO-G	Endonuclease G, NM_004435	Cleaves DNA at double-stranded (dg)n.(dc)n and at single-stranded (dc)n tracts. In addition to deoxyribonuclease activities, also has ribonuclease (RNase) and RNase h activities.
NGF	Nerve growth factor, beta polypeptide, NM_002506	Nerve growth factor is important for the development and maintenance of the sympathetic and sensory nervous systems. It stimulates division and differentiation of sympathetic and embryonic sensory neurons.
P13K/PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5, NM_014308	
IL-3R/CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity, NM_000395	High affinity receptor for interleukin-3, interleukin-5 and granulocyte-macrophage colony-stimulating factor.
BAX	BCL2-associated X protein, NM_138763	This protein forms a heterodimer with BCL2, and functions as an apoptotic activator. This protein is reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss in membrane potential and the release of cytochrome c. The expression of this gene is regulated by the tumor suppressor P53 and has been shown to be involved in P53-mediated apoptosis.

MAPK		
Up-regulated	Gene name	Function
NLK	Nemo-like kinase, NM_016231	Transcriptional activator; DNA-binding protein. Plays an important role in the control of proliferation and differentiation of hematopoietic progenitor cells.
BDNF	Brain-derived neurotrophic factor, NM_170735	Promotes the survival of neuronal populations that are all located either in the central nervous system or directly connected to it. Expression of this gene is reduced in both Alzheimer's and Huntington disease patients. This gene may play a role in the regulation of stress response and in the biology of mood disorders
EGFR	Epidermal growth factor receptor, NM_005228	Receptor for EGF, but also for other members of the EGF family. Involved in the control of cell growth and differentiation.
GBR2	Growth factor receptor-bound protein 2, NM_002086	Involved in the signal transduction pathway
MKK4/ MAP2K4	Mitogen-activated protein kinase kinase 4, NM_003010	Dual specificity kinase that activates the jun kinases mapk8 (jnk1) and mapk9 (jnk2) as well as mapk14 (p38) but not mapk1 (erk2) or mapk3 (erk1).
ERK/MAPK1	Mitogen-activated protein kinase 1, NM_138957	Involved in both the initiation and regulation of meiosis, mitosis, and postmitotic functions in differentiated cells by phosphorylating a number of transcription factors such as ELK1.
TNF	Tumor necrosis factor, NM_000594	Mainly secreted by macrophages and can induce cell death of certain tumor cell lines.
BDNF	Brain-derived neurotrophic factor, NM_170735	Promotes the survival of neuronal populations that are all located either in the central nervous system or directly connected to it.
P38/MAPK14	Mitogen-activated protein kinase 14, NM_001315	Responds to activation by environmental stress, pro- inflammatory cytokines and lipopolysaccharide by phosphorylating a number of transcription factors, such as elk1 and atf2 and several downstream kinases, such as mapkapk2 and mapkapk5. Plays a critical role in the production of some cytokines.
Sapla / ELK4	ETS-domain protein, SRF accessory protein 1, NM_001973	Forms a ternary complex with the serum response factor srf. Requires DNA-bound srf for ternary complex formation and makes extensive DNA contacts to the 5' side of srf, but does not bind DNA autonomously.
ATF-2	Activating transcription factor 2, NM_001880	protein binds the camp response element (cre) (consensus: 5'-gtgacgt[ac][ag]-3'), a sequence present in many viral and cellular promoters.

MAPK

Down-regulated		Function
JNK/ MAPK8IP2	Mitogen-activated protein kinase 8 interacting protein 2, NM_012324	Responds to activation by environmental stress and pro- inflammatory cytokines by phosphorylating a number of transcription factors, primarily components of ap-1 such as c-jun and atf2 and thus regulates ap-1 transcriptional activity. In t- cells, jnk1 and jnk2 are required for polarized differentiation of t-helper cells into th1 cells
RAS/RRAS	Related RAS viral (r-ras) oncogene homolog 2, NM_012250	GTP binding
JIP1/2NGF	Nerve growth factor, beta polypeptide, NM_002506	Important for the development and maintenance of the sympathetic and sensory nervous systems. Stimulates division and differentiation of sympathetic and embryonic sensory neurons.
G12/ GNA12	Guanine nucleotide binding protein (Gprotein) alpha 12, NM_007353	Guanine nucleotide-binding proteins (g proteins) are involved as modulators or transducers in various transmembrane signalling systems.
MEK1/ MAP2K1	Mitogen-activated protein kinase kinase 1, NM_002755	Catalyses the concomitant phosphorylation of a threonine and a tyrosine residue in a thr- glu-tyr sequence located in map kinases. activates erk1 and erk2 map kinases.
Tau/ MAPT	Microtubule-associated protein tau, NM_016834	Promotes microtubule assembly and stability, and might be involved in the establishment and maintenance of neuronal polarity.
NFAT-2/ NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2, NM_173091	Plays a role in the inducible expression of cytokine genes in t-cells
c-JUN MEF2C	Jun oncogene, NM_002228 MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C), NM_002397	Transcription factor, recognizes and binds to the enhancer heptamer motif 5'-tga[cg]tca-3'. Transcription activator which binds specifically to the mef2 element present in the regulatory regions of many muscle- specific genes. Controls cardiac morphogenesis and myogenesis, and is also involved in vascular development. may also be involved in neurogenesis and in the development of cortical architecture
PP2CB	Protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform, NM_177968	Enzyme with a broad specificity. dephosphorylates cdk2 and cdk6 in vitro

WNT		
Up-regulated	Gene name	
Proc	Porcupine homolog, NM_022825	Modulates the processing of WNT proteins. Probable n-palmitoyltransferase that palmitoylates wnt family members.
Dvl1	Dishevelled, dsh homolog 1, NM_004421	May play a role in the signal transduction pathway mediated by multiple WNT genes.
Cer-1	Cerberus 1, cysteine knot superfamily, NM_005454	Cytokine that may play a role in anterior neural induction and somite formation during embryogenesis in part through a bmp-inhibitory mechanism
APC2	Adenomatosis polyposis coli 2, NM_005883	Tumor suppressor. promotes rapid degradation of ctnnb1 and participates in wnt signalling.
FRP/ SFRP1	Secreted frizzled-related protein 1, NM_003012	Modulators of WNT signalling through direct interaction with WNTs. they have a role in regulating cell growth and differentiation in specific cell types. sfrp1 decreases intracellular beta-catenin levels
PS-1/ PSEN1	Presenilin 1 (Alzheimer disease 3), NM_007319	may play a role in intracellular signalling and gene expression or in linking chromatin to the nuclear membrane
NLK	nemo-like kinase, NM_016231	Transcriptional activator; DNA-binding protein. Plays an important role in the control of proliferation and differentiation of hematopoietic progenitor cells.
CK2/CSNK2A1	Casein kinase 2, alpha 1 polypeptide, NM_177559	a serine/threonine protein kinase that phosphorylates acidic proteins such as casein
GSK-3β	Glycogen synthase kinase 3 beta, NM_002093	Involved in energy metabolism, neuronal cell development, and body pattern formation
Daam1	Dishevelled associated activator of morphogenesis 1, NM_014992	Binds to disheveled (dvl) and rho, and mediates WNT- induced dvl-rho complex formation. May play a role as a scaffolding protein to recruit rho-gdp and rho-gef, thereby, enhancing rho-gtp formation.
ICAT/CTNNBIP1	Catenin, beta interacting protein 1, NM_020248	The encoded protein is a negative regulator of the WNT signalling pathway
PKC/ PRKCA	Protein kinase C, alpha, NM_002737	This is a calcium-activated, phospholipid-dependent, serine- and threonine-specific enzyme.
NFAT	Nuclear factor of activated T-cells 5, tonicity-responsive, NM_138714	May play a role in cell motility by phosphorylating cspg4
Uterine/MMP7	Matrix metalloproteinase 7 (matrilysin, uterine), NM_002423	Plays a role in the inducible expression of genes. Regulates hypertonicity-induced cellular accumulation of osmolytes. Degrades casein, gelatins and fibronectin. Activates procollagenase.

WNT**Down-regulated**

Wnt	Wingless-type MMTV integration site family, NM_016087	These proteins have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis This gene encodes a cytoplasmic protein which contains a regulation of G-protein signaling (RGS) domain and a dishevelled and axin (DIX) domain. The encoded protein down-regulates B-catenin. Can induce apoptosis. Essential for cell proliferation. May be required for the activation of transcriptional programs associated with oncogene and proto-oncogene mediated growth induction, tumour suppressor mediated growth arrest and replicative senescence, apoptosis, and DNA repair Acetyltransferase enzyme. Acetylates histones, giving a specific tag for transcriptional activation Functions together with arm to transduce the wingless (wg) signal in embryos and in developing adult tissues. Acts as a transcriptional activator, but in the absence of arm, it binds to gro and acts as a transcriptional repressor of wg-responsive genes. Binds to WNT proteins and inhibits their activities. May be involved in mesoderm segmentation. WNT proteins are extracellular signalling molecules involved in the control of embryonic development. This gene encodes a secreted protein, which binds WNT proteins and inhibits their activities. This protein contains a WNT inhibitory factor (WIF) domain and 5 epidermal growth factor (EGF)-like domains. It may be involved in mesoderm segmentation. This protein is found to be present in fish, amphibia and mammals.
Axin1	Axin 1, NM_003502	
Protein52/ RUVBL1	RuvB-like 1, NM_003707	
CBP/CREBBP,	CREB binding protein (Rubinstein-Taybi syndrome), NM_004380	
Wif-1	Lymphoid enhancer-binding factor 1, NM_016269 WNT inhibitory factor 1, NM_007191	
c-JUN JNK/ MAPK8IP2	Jun oncogene, NM_002228 Mitogen-activated protein kinase 8 interacting protein 2, NM_012324	Transcription factor, recognizes and binds to the enhancer heptamer motif 5'-tga[cg]tca-3'. Responds to activation by environmental stress and pro- inflammatory cytokines by phosphorylating a number of transcription factors, primarily components of ap-1 such as c-jun and atf2 and thus regulates ap-1 transcriptional activity. In t- cells, jnk1 and jnk2 are required for polarized differentiation of t-helper cells into th1 cells
fra-1/FOSL1	FOS-like antigen 1, NM_005438	These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1

* The genes showing up or down-regulation were extrapolated from the pathways

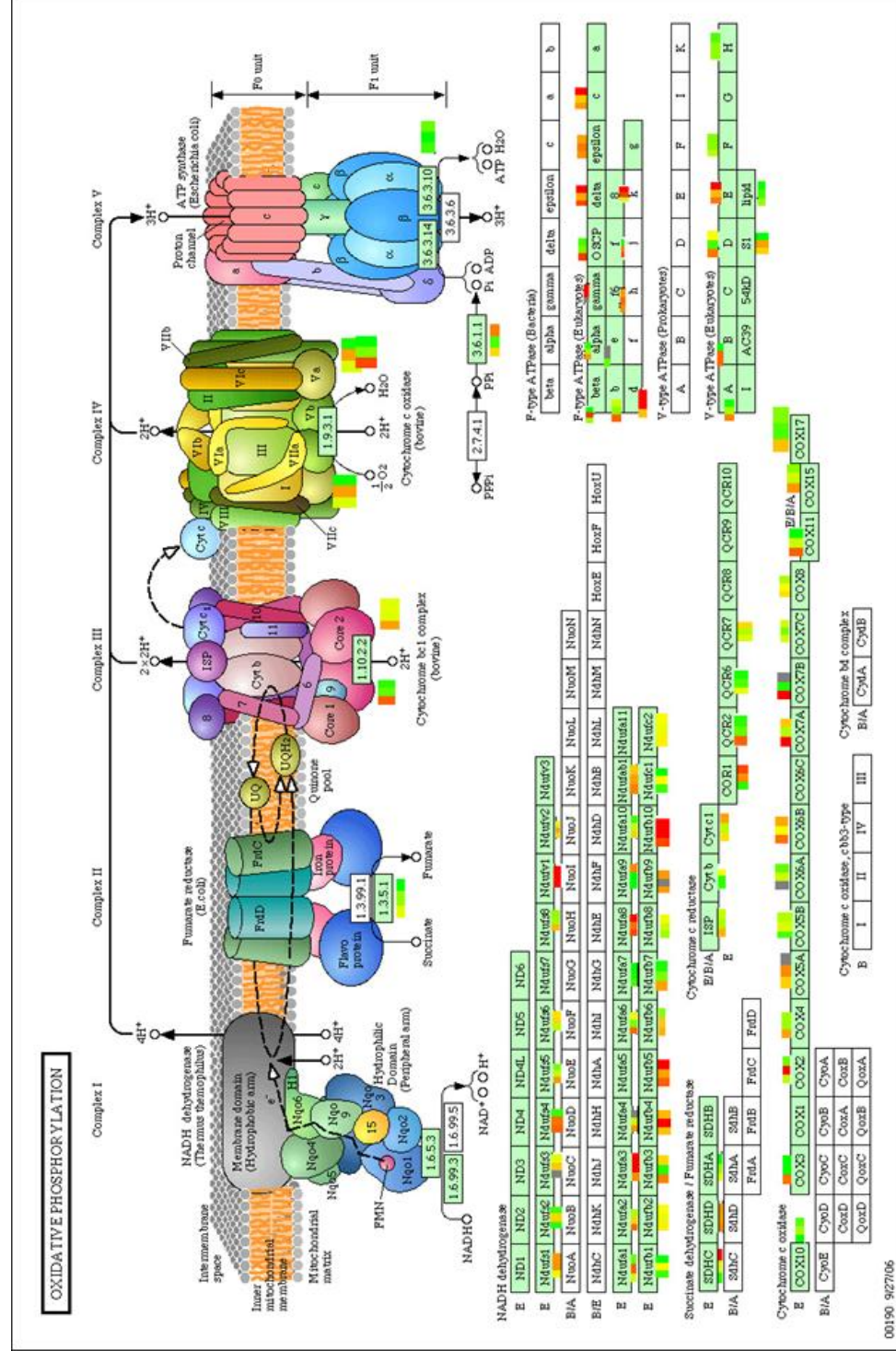


Figure 6.4. Oxidative phosphorylation pathway (altered version of KEGG pathway: <http://www.genome.jp/kegg/pathway.html>). The boxes adjacent to the genes represent the gene regulation effects by gymnodimine. The three divisions within each box represent the three replicated microarray slides). The colour schemes are representing the gene regulation: green (down regulated) through yellow (neutral) to red (up regulated)

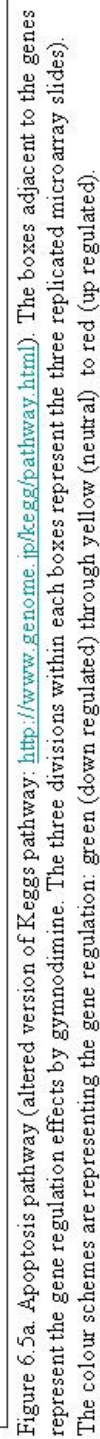


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Figure 6.5b. Apoptosis pathway showing the physical location of the genes within the cell. (reproduced from Proteinlounge: <http://www.merckbiosciences.co.uk>).
U = upregulated, D = down-regulated.

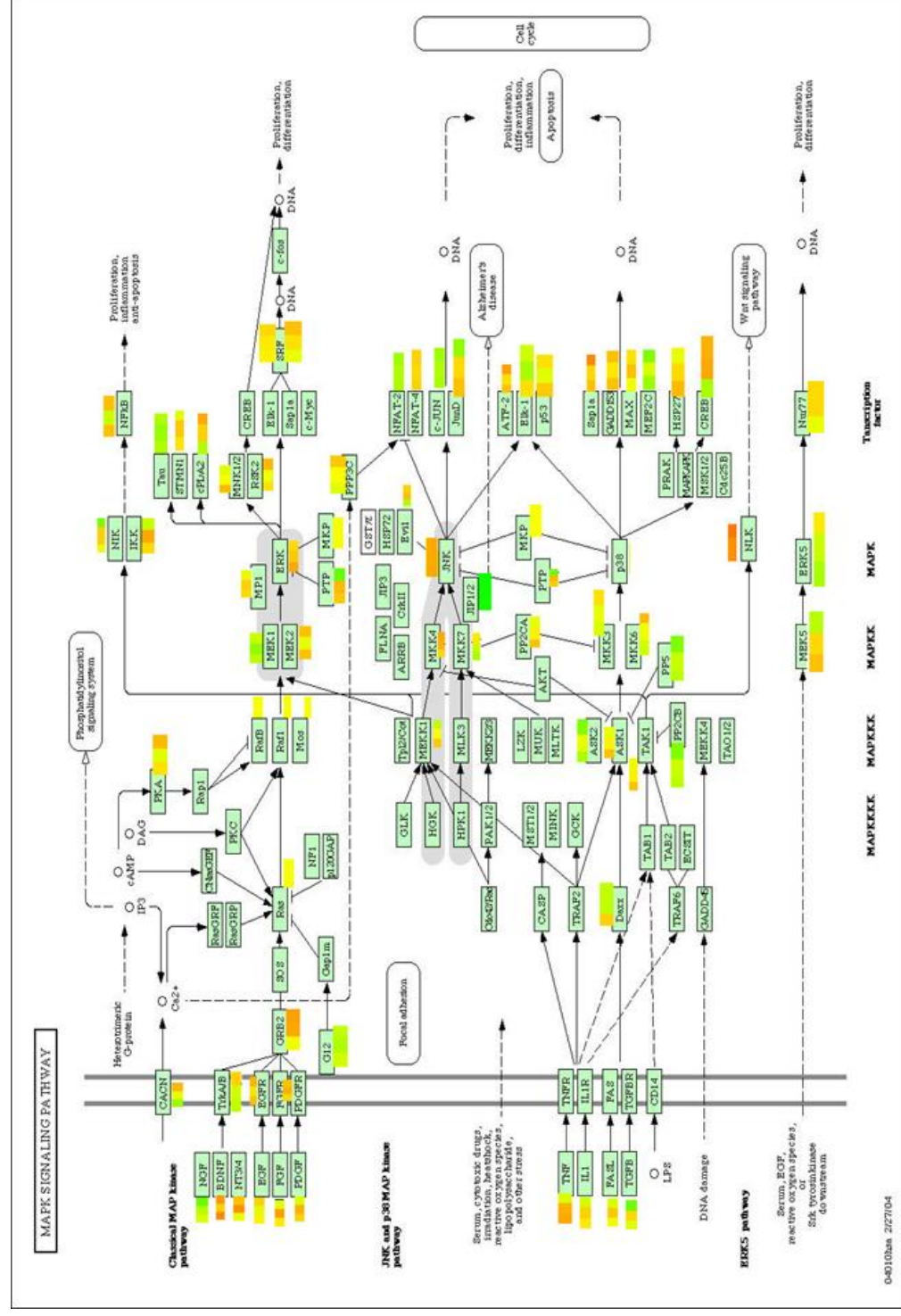


Figure 6.6a. MAPK pathway (altered version of KEGGS pathway: <http://www.genome.jp/kegg/pathway.html>). The boxes adjacent to the genes represent the gene regulation effects by gymnomimide. The three divisions within each box represent the three replicated microarray slides. The colour schemes are representing the gene regulation: green (down regulated) through yellow (neutral) to red (up regulated)

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Figure 6.6b p38 MAPK pathway showing the physical location, within the cell, of the genes involved in the pathway (reproduced from BioCarta: <http://www.biocarta.com>).
D = down-regulated by gymnodimine; U = up-regulated by gymnodimine.

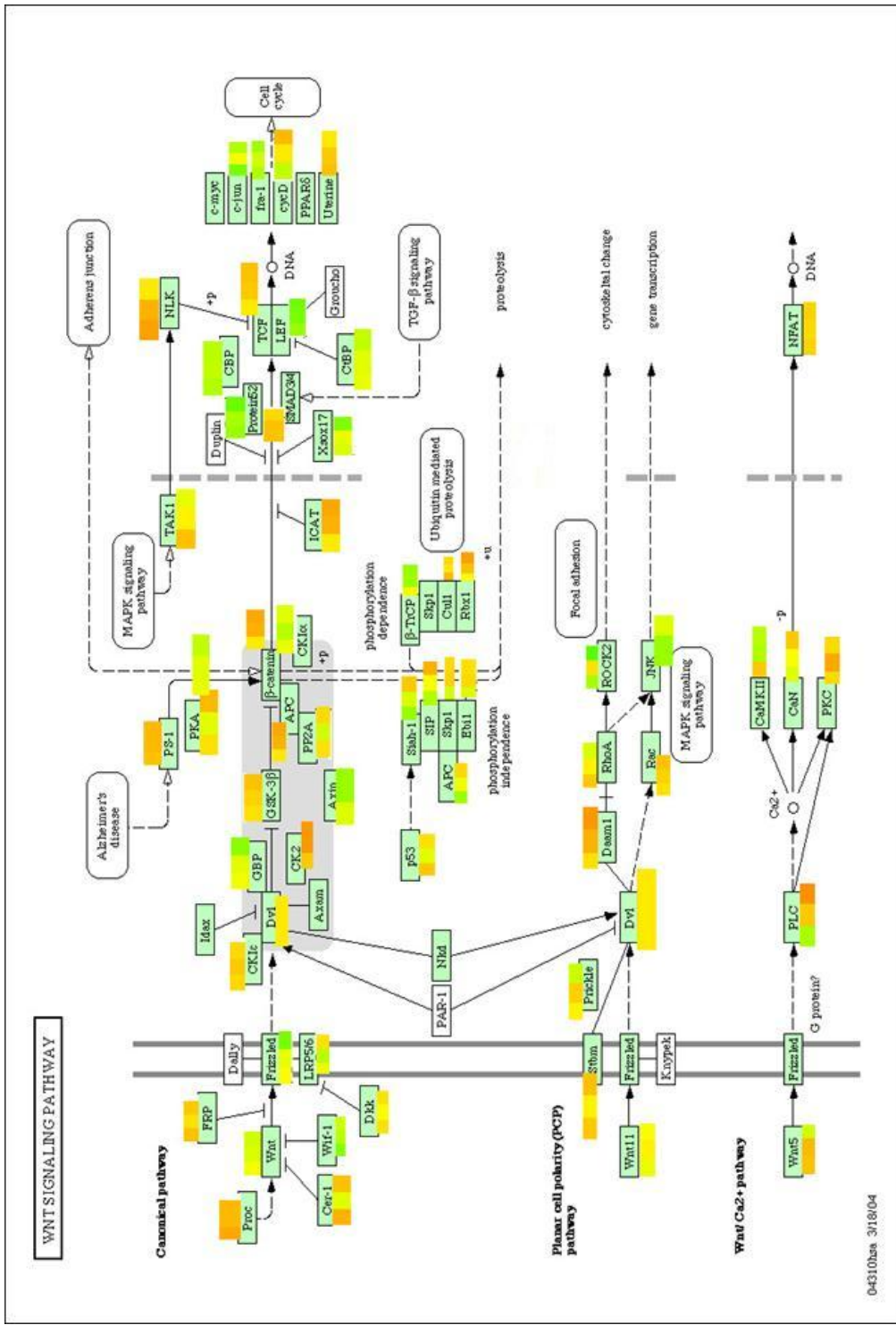


Figure 6.7a. Wnt pathway (altered version of Kegg pathway <http://www.genome.jp/kegg/pathway.html>). The boxes adjacent to the genes represent the gene regulation effects by gymnodimine. The three divisions within each box represent the three replicated microarray slides). The colour schemes are representing the gene regulation: green (down regulated) through yellow (neutral) to red (up regulated)

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Figure 6.7b Wnt pathway showing the physical location, within the cell, of the genes involved in the pathway (reproduced from BioCarta: <http://www.biocarta.com>).

D = down-regulated by gymnodimine; U = up-regulated by gymnodimine.

6.3.2.3 Real-Time PCR

To validate the microarray analysis, real time PCR was performed on several genes of interest. For each PCR analysis, their CT (cycle threshold) was corrected using the house keeping gene (actin). The amplification of genes is measured by PCR efficiency at the time of CT. Genes listed in Figure 6.8 showed agreement with the microarray data, however none of the genes were significantly up or down-regulated.

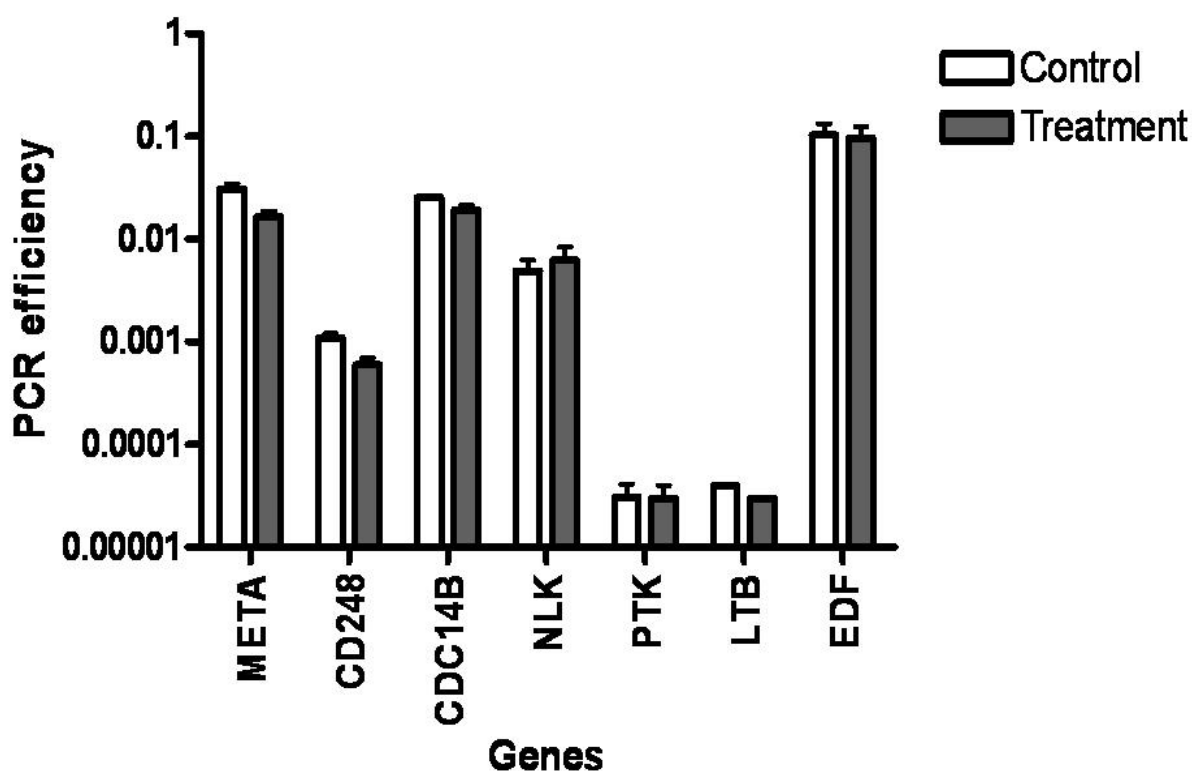


Figure 6.8 Results of Real time PCR. Genes of interest are shown on the x-axis and the PCR efficiency is shown on the Y-axis.

6.4 Discussion

Numerous assays using cell lines have been developed as alternative methods to animal testing. The work conducted on cells lines have shown to be useful in cytotoxicity and mechanistic studies (Amzil et al. 1992, Naline et al. 1994), however there are limits in that the concentration of toxins administered on cells cannot be compared to the doses used on whole organisms. Methods such as microarray analysis and cellular toxicity are useful tools in investigating potential chronic effects of toxins.

6.4.1 Cytotoxicity

Although there were a number of published articles on cell toxicity of algal toxins, the toxicity levels varied between species and between cell types. It was therefore, critical to perform the MTS assay on human liver cells (HepG₂) to derive the IC₃₀ levels for all toxins prior to the microarray analysis (Figure 6.9).

In the present study, the IC₃₀ level for OA was 10 nM, however, a higher concentration of OA on human bronchus cells showed no sign of toxicity (Naline et al. 1994), whilst the LC₃₀ level for KB cells was as low as 3.7 nM (Amzil et al. 1992) (Figure 6.9). This difference implies that cell lines may have differing tolerance levels to toxins, highlighting the importance of working with varying cell lines whenever possible.

The IC₃₀ level for DA in the present study was 100,000 nM, which was 10 fold higher than the toxicity seen in human and mice brain slices (Ross et al. 2000, Erin et al. 2003) al). Again, this may be due to the differences between brain slices and cell lines, showing the difficulty in comparing the doses-response relation between the cells and tissues, let alone a whole organism.

In a study conducted using MTS, GD was toxic at 10,000 nM on neuroblastoma cells (Dragunow et al. 2005). This was 10 fold higher than the IC₃₀ level of 100 nM for human neuroblastoma and liver cells obtained in this study. It is difficult to explain this difference except for the fact that the former study was conducted to determine the lethal level of toxicity whilst this study aimed to obtain the IC₃₀ level. Dragunow et al. (2005) has also shown that pre-exposition of cells to OA sensitises the cells to GD, hence in the wild where mix of toxins exist, GD may be more potent than when toxin occurs on its own. This is critical in this study as GD was present at the same time as OA, and the combination of toxins could potentially increase the potency of GD.

The LC₃₀ of PTX-2SA on HepG₂ cells has been documented to be 922ng ml⁻¹ (1052 nM) (Burgess 2003). This is significantly higher than OA or GD shown in this study, however lower than the IC₃₀ level of DA. This reveals the order of toxicity of the algal toxins. The

difference in the potency between the toxins as observed between PTX-2s , OA, DA and GD is most probably due to the differences in the mechanism by which toxins affect the cells.

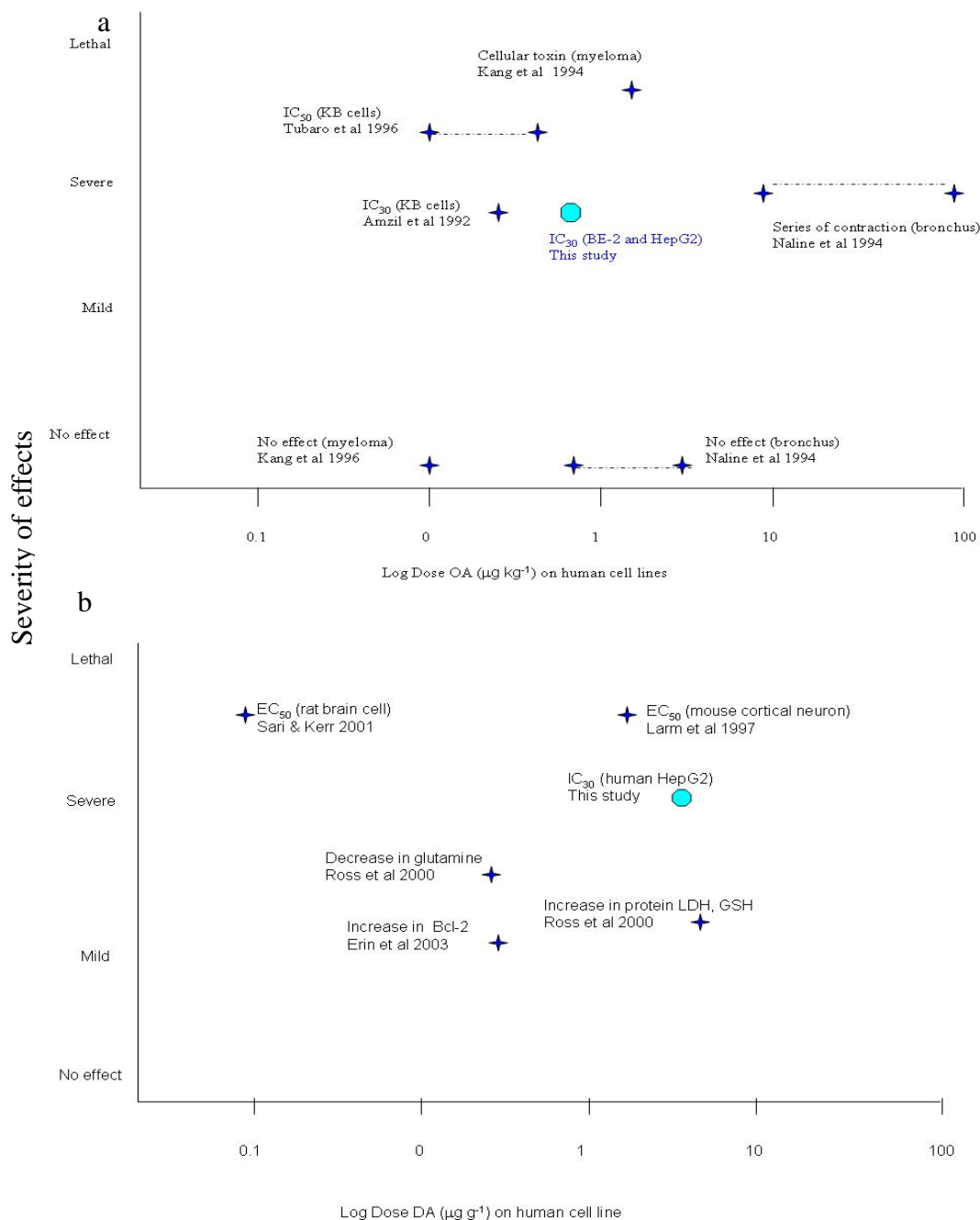


Figure 6.9. IC₃₀ level for HepG₂ cells conducted in this study compared to the dose-response data accumulated from the literature: a) okadaic acid; b) domoic acid. The circle represents the results from this study.

6.4.2 Mechanistic toxicity from Be-2 cell study

The bulk of this pilot study is presented in Appendix A of this thesis. Results from this pilot study show the potential target genes for OA, DA and GD. As there was limited dose-response study on GD, further investigation on this toxin was conducted using HepG2 cells with three biological replicates.

6.4.2.1 Okadaic acid treated cells

One of the genes up-regulated by OA treatment codes for the dual specific phosphatase 4 (DUSP4). The phosphatases are known to inactivate their target kinases by dephosphorylating the phosphoserine. This finding can be supported by the fact that the OA group of toxins is potent and selective inhibitors of three of the four protein serine/threonine phosphatase; type 1, 2A and 2B (PP1, PP2A, PP2B) (Bialojan & Takai 1988, Arias et al. 1993). Their relative toxicity is related to their affinity for protein phosphates (Holmes & Teo 2002). Another up-regulated gene of interest codes for STX4A syntaxin 4A, as the ratio of expression between the control and the treated cells are the highest for this gene. This gene is potentially involved in docking of synaptic vesicles of presynaptic active zones.

Previous studies has shown OA to induce DNA adducts and it is therefore, suggested to be a carcinogen (Creppy et al. 2002). Even without changing or mutating DNA base sequence epigenetic alterations in DNA methylation can occur (Creppy et al. 2002). Such changes in DNA methylation patterns can strongly affect the regulation of expression of many gene (Cerdeira & Weitzman 1997). Low concentration of OA increases DNA methylation (Matias & Creppy 1996b), hence OA may interfere with gene regulation expression and cellular proliferation and participate in promotion of epigenetically-induced tumours by gap junction communication (GJIC) inhibition (Creppy et al. 2002). This inhibition of GJIC is believed to be important in promotion stage of carcinogenesis (Budunova et al. 1996).

Another gene which was down-regulated by OA was GABRB3 gamma-aminobutyric acid A receptor, beta 3. This is a multi-subunit chloride channel that mediates the fastest inhibitory synaptic transmission in the central nervous system.

6.4.2.2 Domoic acid treated cells

One of the up-regulated genes codes for KCNAB1, potassium voltage-gated channel, shaker-related subfamily, beta member 1. Some of the known potassium channel functions are the regulation of neurotransmitter release. As DA is a known excitatory toxin which affects the glutamate receptor, this up-regulation of the gene may explain the mechanism of this effect.

Two down-regulated genes, related to Alzheimer disease were also present. One codes for the APP amyloid beta precursor protein. It is implied that mutation in this gene can affect Alzheimer disease and cerebroarterial amyloidosis. The second gene codes for UBB (ubiquitin B). This covalently bonds to protein to be degraded. An aberrant form of this protein has been noticed in Alzheimer's and Down syndrome patients. Domoic acid is especially known to affect the glutamate receptors in the CA3 region of the hippocampus, a region responsible for functional memory (Cendes et al. 1995, Sari & Kerr 2001). This region of the brain is also affected in patients with Alzheimer disease.

Additionally there were two genes which had similar functions, yet one, PSMB4 proteasome subunit beta type 4, was up-regulated by DA while other gene, PSMD1 proteasome, was down-regulated (Table A.3). Both of these genes code for proteasome, a multicatalytic proteinase complex, known to cleave peptides in an ATP/ubiquitin dependent process in non-lysosomal pathway. This protein may not be affected by DA since the up and down-regulation for these genes will cancel each other out. It may therefore not be a significant protein for the mechanism of this toxin.

6.4.3 Mechanistic toxicity for gymnodimine

Gymnodimine is a relatively newly discovered toxin. Although GD is toxic when injected,

it is not toxic to mice when dosed orally with food, but has been shown to be toxic when dosed orally via gavage. It is a cyclic imine, functionally related to spirolides, pinnatoxins and prorocentrolides, all of which are known as “fast-acting toxins” when they are injected into mice (Seki et al 1995, Stewart et al 1997, Stirling & Kharrat 2002). This project provides a novel insight into the possible chronic effects of GD by investigating the gene regulations affected by the toxin. Several pathways were chosen as GD showed significant regulation of some of the genes within the pathways.

Oxidative phosphorylation

There are five main complexes within the oxidative phosphorylation pathway (Figure 6.4). The MTS assay used to assess the IC₃₀ level of algal toxins in this study measures the complex II, succinate dehydrogenase (SDH) (Berridge & Tan 1993, Marshall et al. 1995). Succinate dehydrogenase complex catalyses the oxidation of succinate to fumarate in the Krebs cycle and the MTS assay measures the amount of fumarate after dosing of cells. It is therefore not surprising to find the gene responsible for SDH to be down-regulated in this study. The down-regulation of the SDH gene is significant to the cell survival as this step is critical in ATP production..

Gymnodimine also down-regulated the ATPase gene (complex V) which is responsible for ATP production (Figure 6.4). This is a critical step in cell metabolism and production of energy for survival. If gymnodimine reduces the ATP production, the energy supply for cells will be reduced, leading to death. This is a critical gene and further study to validate the down-regulation of this gene will be essential.

Apoptosis

The initial stage of the apoptosis pathway includes the Fas and Fas receptor genes, which are members of the tumour necrosis factor receptor (TNFR) (Wajant 2002). This subfamily of TNF genes are known to trigger apoptosis of tumour cells (Chen & Goeddel 2002). Gymnodimine slightly up-regulated these genes, showing signs of mediating apoptosis (Figure 6.5b).

Another interesting gene within this pathway is the protein kinase B gamma (AKT/ PI3K) gene which belongs to the Akt family. This family of genes are known to affect cell survival by antagonising apoptosis by phosphorylation (Kim et al. 2001). The activity of Akt have been reported to be increased in different tumours, suggesting its role in inhibition of cell apoptosis and promoting tumours (Cristiano et al. 2006). Therefore GD may be up-regulating this gene (Figure 6.5a-b). This may be an implication for chronic effects of GD in shellfish consumers.

In another study, PTX-2 has been shown to affect the Bim/BAM genes within the apoptosis pathway, and lethal at 100ng ml⁻¹ (117 nM) (Chae et al. 2005). This level is similar to that of GD in this study.

MAPK Signalling Pathway

The mitogen activating protein kinase (MAPK) was also affected by PTX-2SA as the gene CDC42 was up-regulated (Burgess 2003). In the initial stage of the pathway, GTP binding protein (RAS) is activated by adapter molecule (GRB2) and guanine nucleotide exchanger (mSOS) (Seger & Krebs 1995). The RAS subfamily within the MAPK signalling pathway has been reported as essential for the long –term memory formation as shown in Figure 6.6b (Orban et al. 1999). Gymnodimine was seen to up-regulate GRB2, however the RAS gene was down-regulated. The RAS gene may be of interest for future study as long term exposure to GD may have a chronic effect on the long term memory of the consumers.

The Raf genes, responsible for activating the MEK genes were not effected by the toxins, and the MEK genes, responsible for passing the signal into the nucleus of the cell (Seger & Krebs 1995), showed both up and down-regulation, hence difficult to predict if GD has any chronic effect on the MAPK pathway actions in the nucleus.

WNT Signalling Pathway

In the Wnt signalling pathway, the protein β -catenin plays a major role in this pathway as

it moves from the cytoplasm into the nucleus when it is activated. Within the canonical Wnt pathway, when the gene dishevelled (Dv1) is activated, it inhibits the second complex of proteins Axin, GSKs and APC (Liu et al. 2005, Nusse 2005).

In this project, Dv-1 was not expressed by GD, and GSK-3 β and APC were up-regulated, while Axin2 was down-regulated. This shows the complex interactions of genes within the pathway. It is also known that when Wnt is absent, β -catenin protein is destroyed by phosphorylation by Axin, GSKs and APC (Nusse 2005). In this study, gymnodimine down-regulated Wnt, Axin 1 and up-regulated APC2 gene β -catenin inhibitor, hence it is likely that β -catenin would not be abundant. The Wnt pathway signalling normally blocks degradation of β -catenin, however GD may have a reversing effect to this pathway and inhibit β -catenin from entering the nucleus. Since this Wnt signalling pathway is considered a major oncogenic mechanism, if one was to only consider this pathway, GD may act in favour of the consumers of this toxin and exert an anti-carcinogenic effect. However, with the complexity of signalling pathways and gene regulation, it is difficult to predict how individual genes will interact.

6.4.4 Validation

In this study, the quantitative real time PCR (QRT-PCR) was utilised to validate the microarray data. The gene NLK was up-regulated in both microarray and QRT-PCR whilst other genes tested were down-regulated in both methods used, however none of the QRT-PCR showed significant results.

Careful experimental design was undertaken for the study of effects of gymnodimine on gene regulation of cell lines. Three biological replicates were used for the cell lines, within which there were three experimental replicates. A high quality RNA were used for the microarray and QRT-PCR analysis. Several experiments were conducted to obtain the purest set of RNA and the highest quality of PCR runs, however the microarray analysis could not be validated using QRT-PCR. Although microarrays provide an unprecedented capacity for screening vast amount of genes, validation by QRT-PCR can result in disagreement (Morey et al. 2006) and there are several cited problems with microarray

comparison to QRT-PCR. The fluorescent dye which labels and distinguishes the control and the treated genes may have slightly different efficiency (Yang et al. 2002). The non-specificity and cross hybridizations of labelled probes are also problematic in microarray experiments (Chuaqui et al. 2002). It is possible that genes with some homology may have also hybridised with the spots on the array, which may be different to the annotated genes, for which the primers for QRT-PCR were designed. This implies that future work is required in validation of the microarray work by analysing multiple genes within the same family of genes which were significantly up or down-regulated by gymnodimine.

This study provided insight into, and a screening process on gene regulation by OA, DA and GD. As further knowledge on chronic toxicity becomes available for these algal toxins, it will be possible to investigate further into individual genes and pathways.

Chapter 7

Risk characterization for humans and dugongs

7.1 Introduction

Risk characterisation is the final step of health risk assessment (Renwick et al. 2003). From the dose-response information, tolerable daily intake (TDI) can be derived from the lowest observed adverse effect level (LOAEL) or the no observed adverse effect level (NOAEL), and dividing those values by the uncertainty factor (Formula 7.1) (Duy et al. 2000, Gundert-Remy et al. 2002). The guideline value (GV) and Reference dose (RfD) can be calculated from the TDI. These numbers can then be compared to the exposure values, such as daily intake (DI), obtained from the exposure assessment step. If the DI is lower than the RfD, then the amount of exposure is considered “safe” while if the DI is higher than the RfD, then the exposure is considered to be “unsafe” (Renwick et al. 2003). The degree of unit however depends on the magnitude of the quotient of TDI and RfD. Additionally, variations between individuals in a population (interspecies) and between different species (intraspecies) need to be accounted for by incorporating uncertainty factors (UF) (Gundert-Remy et al. 2002).

There are at least two ways of undertaking risk characterisation. One approach is to calculate the TDI through LOAEL and UF. This method is normally used to characterise the human health risk (Duy et al. 2000). The second approach is to obtain the risk quotient through taking the exposure and dividing this by the toxicity. This approach is sometimes called the deterministic approach (US-EPA 2006), and is normally used to assess the environmental risk.

In this study, risks related to acute effects caused by algal toxins: okadaic acid (OA), domoic acid (DA), gymnodimine (GD) and pectenotoxin-2 (PTX-2), from consumption of seafood for humans and dugongs were characterized by comparing the exposure levels determined in the field (Chapters 3 and 4) to the acute levels of dose-response data interpolated from the literature (Chapter 5). For comparison, both approaches of risk characterisation are utilised for both humans and dugongs.

For chronic risk characterisation, information compiled from the literature, and data from microarray analysis conducted during this project, were assessed. With the information on effects of toxins on cellular and molecular targets, there are no direct methods for comparing the concentrations used and the exposure levels, however, the toxicodynamic study on cellular levels can provide an important endpoint of the toxins (Gundert-Remy et al. 2002). Most tests conducted at the cellular and molecular levels are aimed at obtaining information which may be related to a long term exposure leading to chronic effects. The aim of this chapter is to perform risk characterisation based on acute effects and chronic effects and the exposures determined from this study.

7.2 Methods

For both humans and dugongs, the acute effects were used to conduct the risk assessment. Information on chronic effects was investigated to provide mechanistic information for qualitative use in characterisation of chronic effects. This was however not incorporated in the calculation of the risk.

7.2.1 Calculation for daily intake & guideline values for humans

The daily exposures (DE) of humans to the toxin were calculated in Chapter 3a. This produced mean and maximum DE (Formula 7.1). These data were used to calculate the estimated daily intake (DI), or toxin level in tissue, by dividing DE by the body weight (BW) (Formula 7.2). Mean body weight for humans was estimated to be 70 kg.

$$\text{Formula 7.1: DE } (\mu\text{g day}^{-1}) = \text{Toxin in food } (\mu\text{g g}^{-1}) \times \text{daily consumption (g day}^{-1})$$

$$\text{Formula 7.2: DI } (\mu\text{g day}^{-1} \text{ kg}^{-1}) = \frac{\text{DE } (\mu\text{g day}^{-1})}{\text{mean BW (kg)}}$$

Tolerable daily intake (TDI; $\mu\text{g kg}^{-1} \text{ day}^{-1}$) was calculated using the lowest observable adverse effect level (LOAEL; $\mu\text{g kg}^{-1} \text{ day}^{-1}$) from the literature divided by the uncertainty factor (UF; Formula 7.3) (Duy et al. 2000). The uncertainty factor comprises of

interspecies variation (factor of 10), intra-species variation (factor of 10) and an additional factor for extrapolating from less than chronic results from non-long-term data (factor of 10), which comes to the total of 1000 (Duy et al. 2000).

Guideline values (GV; $\mu\text{g kg}^{-1}$) were then calculated using the TDI, multiplied by the body weight (BW; kg) and then divided by daily consumption of seagrass (C; kg day^{-1}) (Duy 2000) (Formula 7.4). This was compared to the estimated DE for humans. Data are presented as mean value \pm standard deviation.

$$\text{Formula 7.3: } TDI = \frac{LOAEL}{UF}$$

$$\text{Formula 7.4: } GV = \frac{(TDI \times BW) + P}{C}$$

Tolerable daily intakes were calculated for acute effects, using information extrapolated from the literature, whilst for chronic effects, both information from the literature and the microarray analysis were used (Chapter 2).

For deriving reference dose (RfD), an additional 10 fold factor is applied if using a LOAEL instead of an NOAEL. This is to account for the uncertainty involved in conversion of LOAEL to NOAEL (EPA 1986). A modifying factor (MF) can also be used depending on the scientific uncertainties of the study used (EPA 1986). Numbers between one and ten are used for MF depending on the study.

To characterise the risks using the deterministic approach, the reference dose and risk quotient were obtained by incorporating list of variables (Table 7.1). Using this method, the uncertainty factor is not incorporated as this is a precautionary measure for acute toxicity (Hung et al. 2006). The toxicity reference value (TRV) takes into account the differences in the body weight of the test and the receptor animals. Test animals for algal toxins in most cases are laboratory rodents and primates. The receptor animals for this study were humans. These values were calculated using varying contamination concentration

7.2.2 Calculation for daily intake & guideline values for dugongs

Concentration of OA per seagrass was calculated for each seagrass extract by dividing the OA concentration by the volume of extract and then multiplying by the wet weight of seagrass (Chapter 4; Formula 4.1 and 4.2). In brief, estimate of potential dugong exposure to OA was based on daily seagrass intake of 40 kg day^{-1} (Lanyon 1991, Bjorndal 1997) and the calculated abundance of toxin production of *P. lima* on each seagrass species. Tolerable daily intake and guideline values were calculated, as above (Formula 7.3 and 7.4). The proportion of source (P) will be 1 for this analysis as it is assumed that all the source of OA is from seagrass alone. The reference dose and risk quotient were calculated using the equations listed in Table 7.1. Some of the values were substituted for dugongs.

Table 7.1. Definition and equations for calculation of risk associated information. The mean, the maximum and 5, 50 and 95 percentile values were chosen.

Humans	Definition	Equations and values
CF*	Contamination concentration	Toxin mean, maximum
IR	Ingestion rate	0.25 kg day^{-1}
FI	Fraction ingested	1
EF	Exposure frequency	52 day year^{-1}
ED	Exposure duration	80 years
BWt	Body weight of test species (mice)	0.2kg
BWr	Body weight of receptor species	70 kg
AT	Average time	4,171 days
LOAEL*	Lowest observed adverse effect level	Toxin level
TRV	Toxicity reference value	$\text{LOAEL (BWt/BWr)}^{0.25}$
MAC	Maximum allowable concentration	$(\text{TRV} \times \text{BW} \times \text{AT})/(\text{IR} \times \text{FI} \times \text{EF} \times \text{ED})$
RfD	Reference dose	$(\text{MAC} \times \text{IR} \times \text{FI} \times \text{EF} \times \text{ED})/(\text{BW} \times \text{AT})$
RQ	Risk quotient	$(\text{CF/food})/\text{MAC}$

Table 7.1 (cont.)

Dugongs	Definition	Equations and values
IR	Ingestion rate	40 kg day ⁻¹
EF	Exposure frequency	365 day year ⁻¹
ED	Exposure duration	70 years
BWr	Body weight of receptor species	700 kg
AT	Average time	25,550

* CF and LOAEL are toxin dependent; CF is also species dependent

7.3 Results

7.3.1 Humans

The field data on OA, DA, GD and PTX-2s were compared to the dose-response data accumulated from the literature (Figure 7.1 – 7.4). The estimated dose of OA ($\mu\text{g kg}^{-1}$ bw) in field-collected shellfish samples was at least 10 fold lower than the level known to cause human diarrhoea. As for DA, the highest estimated dose from the field-collected shellfish was closed to 100 fold lower than the TDI for DA (Marien 1996, Toyofuku 2006). The range for GD found in the field was small and the highest estimated dose was at least 1000 fold lower than the oral dose corresponding to the NOAEL in mice.

As dose-response data for PTX-2s are limited, the information on PTX-2 and PTX-2SA are plotted on the same graph (Figure 7.4). The estimated dose for PTX-2 in field-collected shellfish is ten fold lower than the predicted acute intake level issued in Canada (Toyofuku 2006). As PTX-2SA showed no toxicity when administered both via gavage and by i.p (Miles et al. 2004a), the estimated dose for PTX-2SA from the field shows no health risk when compared to the dose-response data. When PTX-2SA levels in field-collected shellfish were compared to dose-response data for PTX-2, it overlapped with the acute intake level for PTX-2 in Canada, but was lower than the acute uptake level for PTX-2 in Norway (Figure 7.4) (Toyofuku 2006).

Lowest observed adverse effect levels (LOAEL) were extrapolated from the accumulated

literature, and TDI and GV were calculated for each of the toxins (Table 7.2). The maximum daily exposure and maximum toxin concentration present in shellfish tissue were obtained for both Queensland (QLD) and South Australia (SA) (Table 7.2). LOAELs used for risk assessment in the later section, are in bold letters. The risk quotient calculated using the deterministic approach is summarised in Table 7.3.

When the TDI and the maximum daily exposure of toxins around North Stradbroke Island (QLD) are compared, all the daily exposures are lower than the TDI except for PTX-2 (Table 7.2). The TDI for PTX-2 varies between 0.05 to 5, and the maximum daily exposure level obtained from this study is one, which is still lower than the upper limit of TDI. Similarly, when the maximum toxin concentrations detected in shellfish are compared to GV (Table 7.2), the two toxins in which maximum toxin level in shellfish exceeds the GV are PTX-2 and PTX-2SA.

The maximum daily exposure levels for both OA and PTX-2 exceeded TDI, and the maximum PTX-2 levels in shellfish exceeded the GV in South Australian samples during *Dinophysis* bloom (Table 7.2).

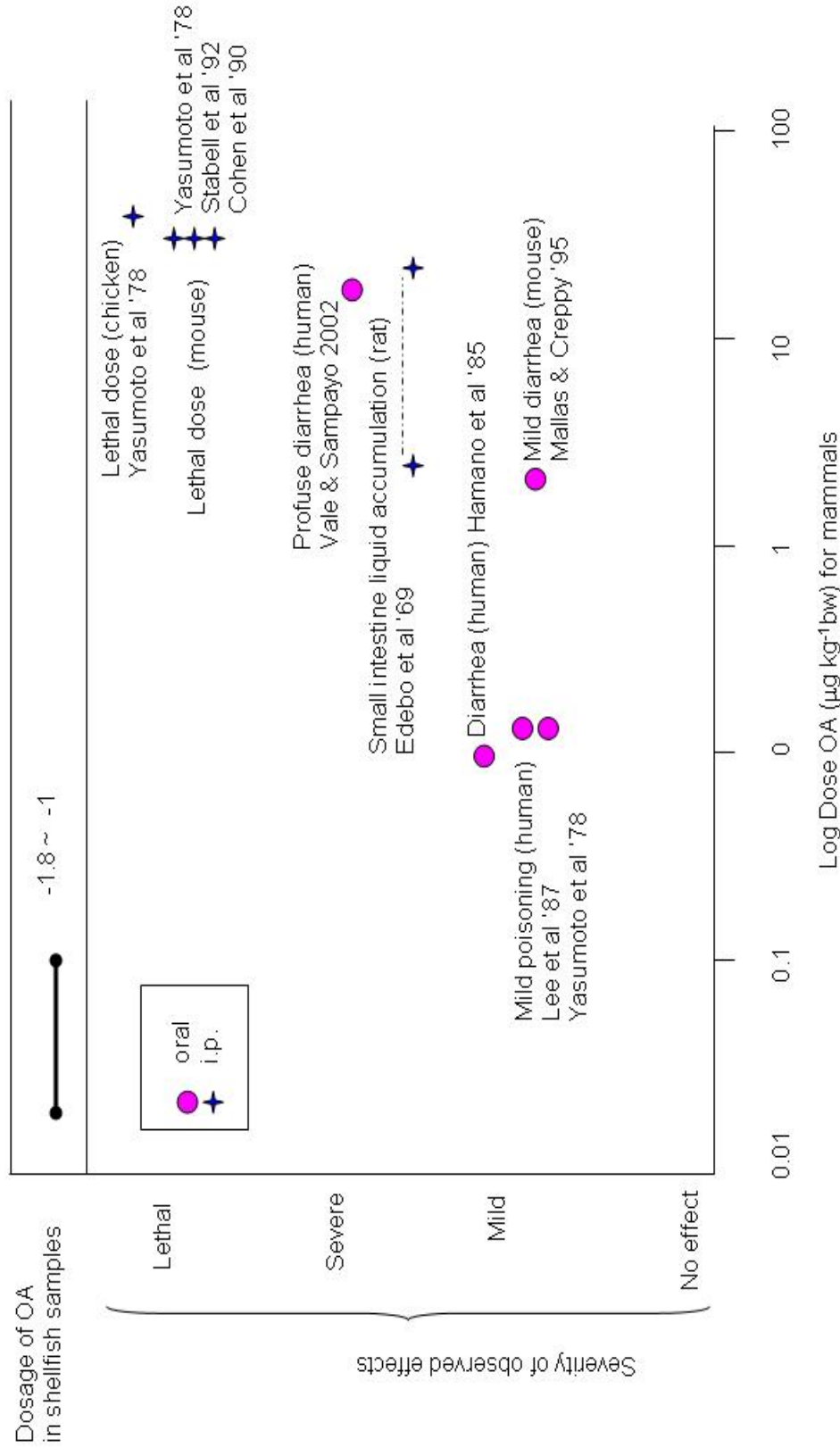


Figure 7.1. Dose-response data for OA collated from the literature. Dose per body weight of the mammals (µg kg⁻¹bw on log scale) are plotted qualitatively according to the severity of the effects. OA in shellfish in the field, assuming consumption of 250 g per human body weight of 70 kg (µg kg⁻¹bw).

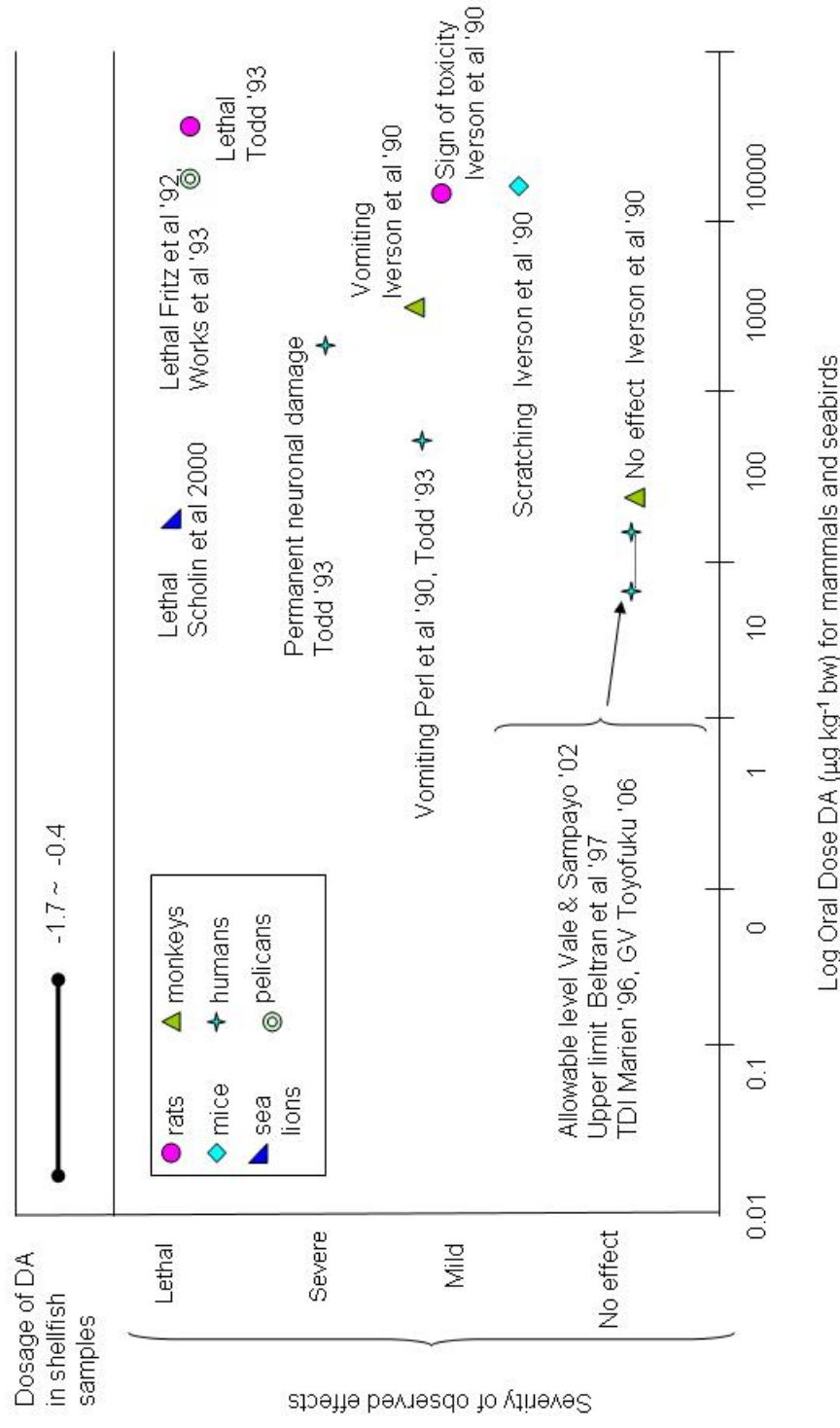


Figure 7.2. Oral dose-response data for DA collated from the literature. Dose per body weight of the mammals ($\mu\text{g kg}^{-1} \text{bw}$) on a log scale) plotted qualitatively according to the severity of the affects. DA collected from the shellfish in the field, assuming consumption of 250 g per human body weight of 70 kg ($\mu\text{g kg}^{-1} \text{bw}$). TDI = tolerable daily intake, GV = guideline value.

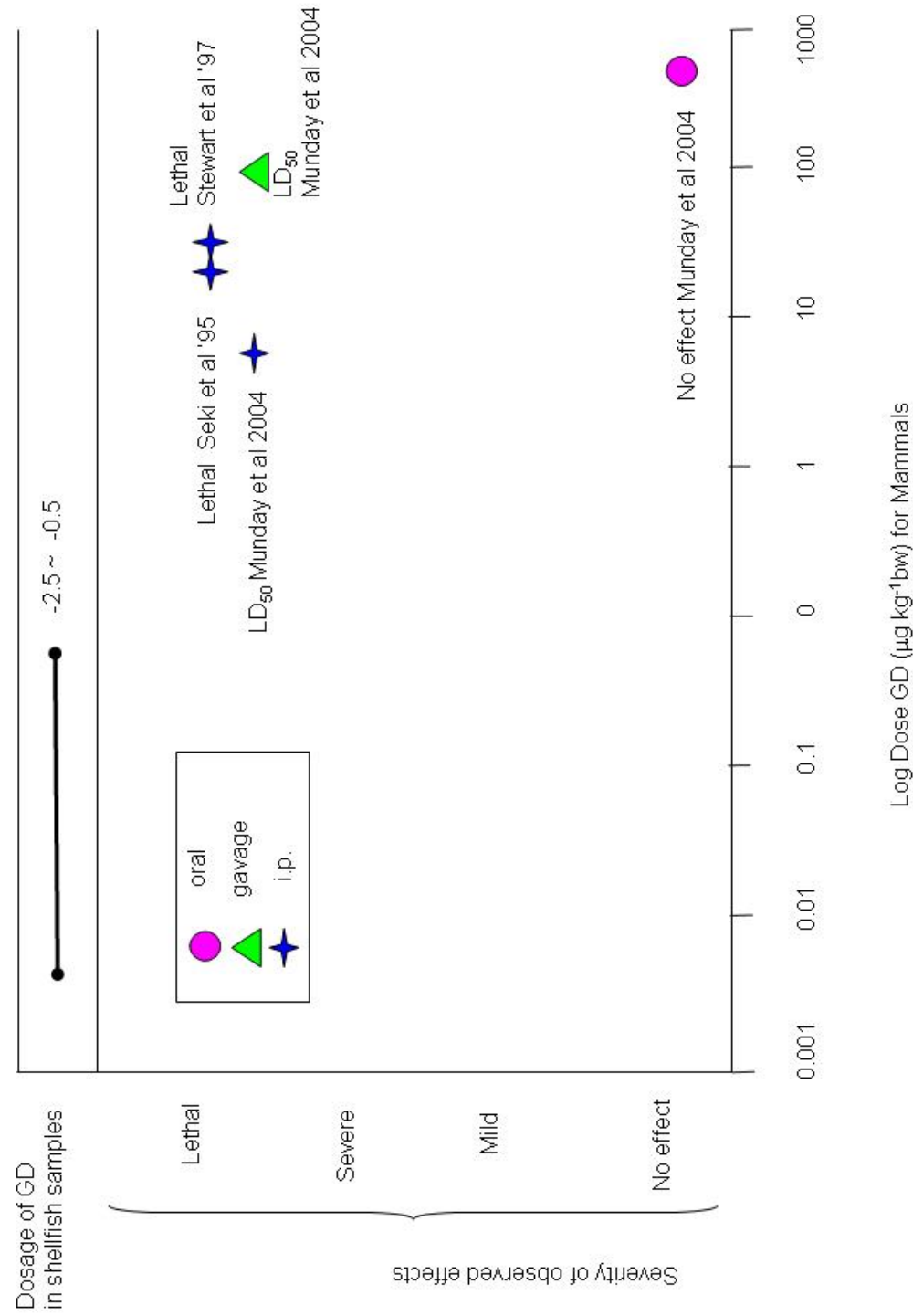


Figure 7.3. Dose-response data for GD collated from the literature. Dose per body weight of mice ($\mu\text{g kg}^{-1}\text{bw}$ on a log scale) plotted qualitatively according to the severity of the affects. GD in shellfish in the field, assuming consumption of 250 g per human body weight of 70 kg ($\mu\text{g kg}^{-1}\text{bw}$).

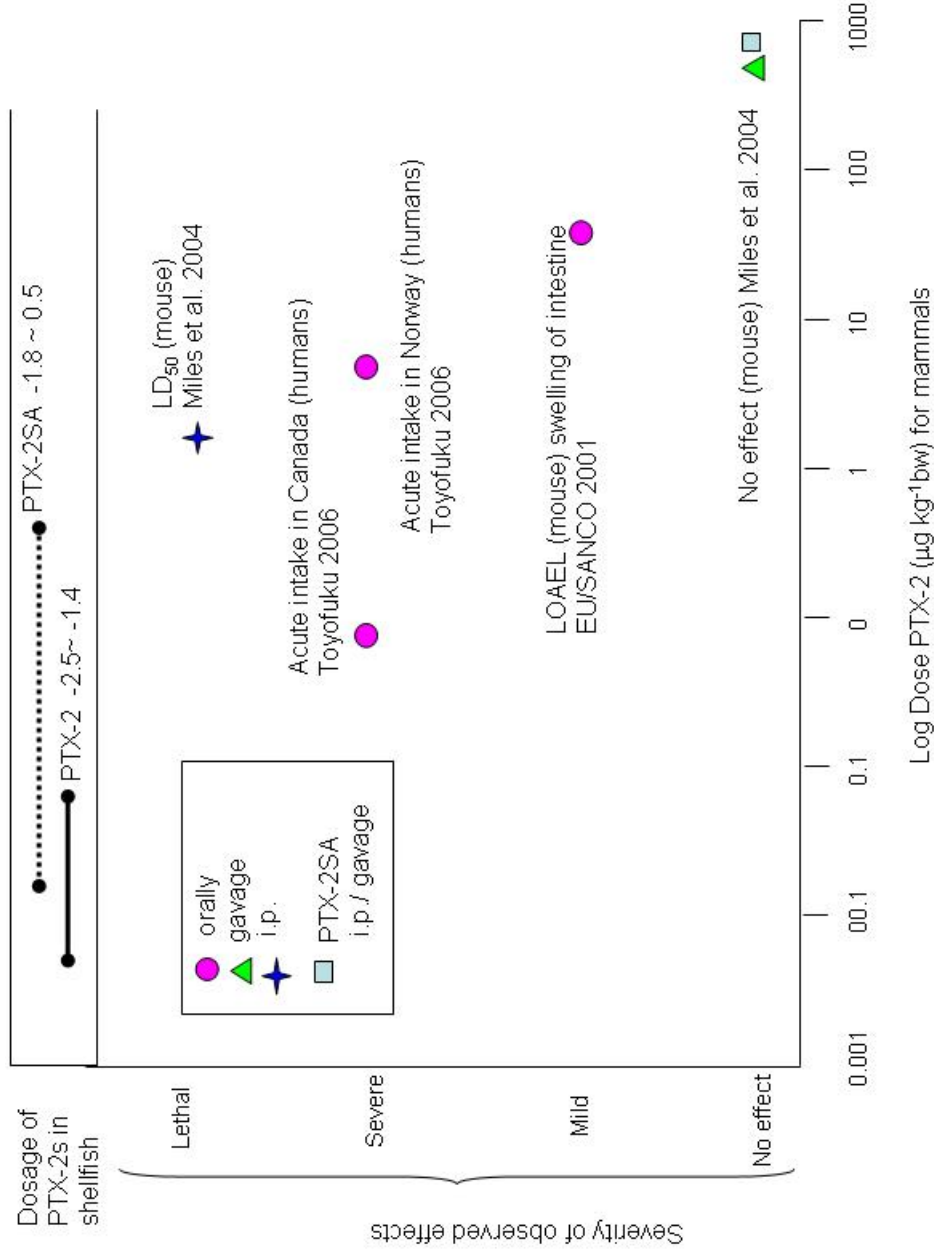


Figure 7.4. Dose-response data for PTX-2 collated from the literature. Dose per body weight of the mammals ($\mu\text{g kg}^{-1}\text{bw}$ on log scale) are plotted qualitatively according to the severity of the effects. PTX-2 and PTX-2SA in shellfish in the field, assuming consumption of 250 g per human body weight of 70 kg ($\mu\text{g kg}^{-1}\text{bw}$).

Table 7.2. Calculated tolerable daily intake (TDI) and guideline value (GV) for OA, DA, GD and PTX-2s using doses used on whole organisms. Doses were given orally unless otherwise stated. TDI and GV shown in bold were used for risk analysis. Uncertainty factor (UF) was based on the variations. OCD = ornithine decarboxylase. The last row of each toxin shows the maximum daily intake and maximum OA concentration found in shellfish.

Dose $\mu\text{g kg}^{-1}$	Effects	UF	TDI $\mu\text{g kg}^{-1}\text{d}^{-1}$	GV $\mu\text{g kg}^{-1}$	References
OA					
1	sign of toxicity *	100	0.01	2.8	Toyofuku 2006
40	diarrhoea*	100	0.4	112	Hamano 1986
50	diarrhoea***	5000	0.05	14	Matias & Creppy 1996
500	induce ODC ***	1000	0.1	28	Suganuma et al. 1992
14.3	skin tumour ***	1000	2.9 ng	0.8	Fujiki 1991
0.84	PP2A inhibition	5000	0.17 ng	47 ng	Haystead et al. 1989
QLD: Daily exposure = 0.2 ; maximum OA concentration in food = 45 $\mu\text{g kg}^{-1}$					
SA: Daily exposure = 1 ; maximum OA concentration in food = 280 $\mu\text{g kg}^{-1}$					
DA					
500	NOAEL **	1000	0.5	140	Iverson et al 1990
5000	vomiting **	1000	5	1400	Iverson et al 1990
1000	vomiting *	100	10	2800	Perl 1990
2250	scratching i.p.***	5000	2.3	630	Iverson et al 1990
50	vomiting i.v.**	1000	0.05	14	Truelove & Iverson 1994
4000	lethal i.p.**	10000	0.4	112	Tryphonas et al 1990
QLD: Daily exposure = 0.9 ; maximum DA concentration in food = 256 $\mu\text{g kg}^{-1}$					
GD ***					
7500	NOAEL	1000	8	2100	Munday et al. 2004
755	LD ₅₀ gavage	10000	0.08	21	Munday et al. 2004
96	LD ₅₀ i.p.	10000	0.01	3	Munday et al. 2004
455	LD ₅₀ i.p.	5000	0.09	25	Seki et al. 1995
QLD: Daily exposure = 0.8 ; maximum GD concentration in food = 220 $\mu\text{g kg}^{-1}$					
PTX-2 ***					
220	LD ₅₀ i.p.	10000	0.02	6	Miles et al. 2004
5000	NOAEL	1000	5	1400	Miles et al. 2004
	gavage				
250	Intestine swell	5000	0.05	14	EU/SANCO 2001
0.1	Cell death	500	0	0.002	Chase et al. 2005
QLD: Daily exposure = 1 ; maximum PTX-2 concentration in food = 270 $\mu\text{g kg}^{-1}$					
SA: Daily exposure = 34.2 ; maximum OA concentration in food = 9600 $\mu\text{g kg}^{-1}$					
PTX-2SA ***					
5000	NOAEL	1000	5	1400	Miles et al. 2004
	gavage/i.p.				
QLD: Daily exposure = 7 ; maximum PTX-2SA concentration in food = 1940 $\mu\text{g kg}^{-1}$					
SA: Daily exposure = 0.2 ; maximum OA concentration in food = 45 $\mu\text{g kg}^{-1}$					

Uncertainty factor (UF) was based on the following variations:

interspecies = 10, intraspecies = 10, less than life time study = 10, severity =5-10

Studies performed on different organisms: *=human, ** = monkey, *** = mice

Table 7.3. The calculated reference dose (RfD) and risk quotient (RQ) for toxins found in the field. For OA, there are some data for both humans and dugongs.

	RfD ($\mu\text{g kg}^{-1}$)	RQ				
		Mean	Max	5%	50%	95%
OA (human)	5.2	0.004	0.031	0.003	0.008	0.021
OA (dugong)	3.4	0.002	0.014	0.001	0.002	0.004
DA	130	0.0002	0.007	0.0001	0.009	0.004
GD	975	0.00005	0.001	<0.00001	0.00003	0.0002
PTX-2	250	0.0002	0.03	0.0001	0.001	0.03
PTX-2SA	650	0.0001	0.01	<0.00001	0.0005	0.01

7.3.2 Dugongs

In Chapter 3, when estimating the maximum potential OA exposure to dugongs, the maximum concentration of OA extracted from *H. spinulosa* was used as it had the highest concentration of OA. For estimating the mean OA exposure, the mean concentration of OA extracted from *H. ovalis* was used since *H. ovalis* has been known to be preferred species of seagrass for both dugongs in this area.

The maximum and mean daily exposures (DE) of dugongs to OA were calculated for dugongs (Formula 7.1). The mean daily wet weight consumption of seagrass by dugongs is 35 - 40 $\text{kg}^{-1} \text{day}^{-1}$ (Lanyon 1991; Gaus 2002). The estimated maximum DE of dugongs to OA, using maximum OA concentration determined for *H. spinulosa*, is 18.4 $\mu\text{g OA day}^{-1}$. The estimated mean DE of dugongs to OA, determined for *H. ovalis* is 2.4 $\mu\text{g OA day}^{-1}$. For an adult dugong weighing 350 - 400 kg (Lanyon 1991; Gaus 2002), the maximum estimated daily intake (DI) of OA was 0.046 $\mu\text{g kg}^{-1}_{(\text{bw})}$ and the mean was 0.006 $\mu\text{g kg}^{-1}_{(\text{bw})}$ (Chapter 4).

To obtain the TDI and GV, the same LOAEL was used as for human risk characterisation (Table 7.2). All information would be the same except for the TDI and GV calculation for human diarrhoea data (Hamano et al. 1985), used for LOAEL since there will be an additional factor of 10 for interspecies variation to the uncertainty factor ($\text{UF} = 1000$; $\text{TDI} = 0.04 \mu\text{g kg}^{-1} \text{d}^{-1}$; $\text{GV} = 112 \mu\text{g kg}^{-1}$). The maximum DI for dugongs (0.046 $\mu\text{g kg}^{-1}$) is slightly

higher than the calculated TDI (Table 7.4). It is difficult to compare the dermal study (Fujiki 1991) and the oral consumption.

Table 7.4. Tolerable daily intake (TDI) and guideline values (GV) from the calculated from the dose-response data compared to the numbers derived by FAO/WHO/IOC*. The maximum OA daily intake (DI) by dugongs, estimated from field-collected seagrass is shown.

Toxins	TDI ($\mu\text{g kg}^{-1} \text{ d}^{-1}$)	GV ($\mu\text{g kg}^{-1}$)	RfD* ($\mu\text{g kg}^{-1}$)	GV * ($\mu\text{g kg}^{-1}$)	Max DI ($\mu\text{g kg}^{-1} \text{ d}^{-1}$)	Max OA ($\mu\text{g kg}^{-1}$)
OA	0.04	0.5	0.33	80	0.046	0.5

*(Toyofuku 2006)

7.4 Discussion

For the exposure data, analysis conducted using consumption rate of 250 g shellfish day⁻¹ from FAO data (Toyofuku 2006) was used. Although the local survey showed that residents of North Stradbroke Island consume, on average 12 g shellfish day⁻¹, the decision to use 250 g was made based on the fact that the larger number, 250 g, would provide a more conservative value for risk assessment. If no acute or chronic effects are shown using the larger number, then the lower consumption rate will not be relevant for risk assessment.

7.4.1 Acute effects of okadaic acid

The RfD derived by Toyofuku (2006) and TDI for OA derived from this study are similar (Table 7.5), however the TDI from this study gave a slightly higher GV than that derived by Toyofuku's study. This may be due to the difference in the body weight used to derive the values (60 kg vs 70 kg). When, however, the same LOAEL was used ($1 \mu\text{g kg}^{-1}$), the numbers derived by Toyofuku (2006) are higher due to the difference in the UF used. In the present study, an additional factor of 10 for non long-term study was incorporated, giving more conservative results.

Data obtained from QLD shellfish showed lower levels of OA in shellfish than the GV, and the daily exposure was also below TDI. However, in shellfish from SA, both exceeded the TDI and GV. The samples collected from SA were taken during a known *Dinophysis* bloom. This determines the risks involved in shellfish consumption during an algal bloom.

For dugongs, the calculated maximum daily intake of OA is within the TDI, and the maximum OA concentration on seagrass is also within the GV. This implies that it is unlikely for the dugongs to be suffering from acute effects.

7.4.2 Acute effects of domoic acid

The LOAEL for DA was $1 \text{ mg kg}^{-1} \text{ bw}$, based on DA accidentally consumed by humans in Canada (Perl et al. 1990). In the present study, a factor of 100 was incorporated for intra-species variation and for the less than life-time study being used for LOAEL. Toyofuku (2006), however, only calculated the RfD since DA has not proven to bioaccumulate, hence he concluded that this RfD can be considered a “provisional chronic TDI”. Toyofuku (2006) points out that the evidence for chronic effects of DA, such as memory loss are due to a one off acute dose of DA, and no study, to this day, has shown any accumulation, however, since molecular studies are still limited, there may be other chronic effects that we are not aware of, hence the present study had the extra factor of ten to account for any unknown long term effects.

7.4.3 Acute effects of gymnodimine

The TDI and GV were calculated in this study, extrapolating limited information from the literature, and comparing the results to the field data. Unlike OA and DA, GD is not as potent and did not show toxicity when dosed orally (Munday et al. 2004).

7.4.4 Acute effects of pectenotoxin-2s

Similar to GD, PTX-2s is not as potent as OA and DA when orally dosed (Miles et al. 2004a). For the purpose of this study, TDI and GV were calculated as references for the field measured exposures. The PTX-2 concentration within shellfish collected from SA during an algal bloom exceeded GV, and the maximum daily exposure also exceeded the TDI. Other studies have investigated the toxicity and risks involved in consuming PTX-2SA and have shown that there are no risks of acute toxicity (Miles et al. 2004). This demonstrates the importance of maintaining algal toxin monitoring by either conducting monitoring for toxins or/ and monitoring for toxic algae specie. The daily exposure also

exceeded the TDI, however NOAEL was used as the value to obtain TDI and GV for PTX-2SA, hence the LOAEL for PTX-2SA may be higher than the value obtained.

Table 7.5. Tolerable daily intake (TDI) and guideline values (GV) for humans, calculated from the dose-response data compared to the numbers derived by FAO/WHO/IOC*.

Toxins	TDI ($\mu\text{g kg}^{-1} \text{ d}^{-1}$)	GV ($\mu\text{g kg}^{-1}$)	RfD ($\mu\text{g kg}^{-1}$) Toyofuku '06	GV ($\mu\text{g kg}^{-1}$) Toyofuku '06	Max DI** ($\mu\text{g kg}^{-1} \text{ d}^{-1}$)
OA	0.01~0.4	2.8~112	0.33	80	0.2 & 1
DA	5~10	2,800	100	24,000	0.9
GD	8	2100	N/A	N/A	0.8
PTX-2	0.05	14	N/A	N/A	0.9 & 34***
PTX-2SA	> 5	1400	N/A	N/A	7

*(Toyofuku 2006)

**Max DI estimated from field-collected shellfish; for OA and PTX-2, 2nd number of Max DI are from South Australia, whilst the rest of the data are from Queensland.

*** Max DI from the field data exceeds the TDI

7.4.5 Other source of exposure

The SPATT bags deployed within the bay had shown dissolved OA in the water column (Chapter 3). The lipophilic toxins were extracted from the passive sampler, however this was a non-quantitative study, and for this reason, when calculating the tolerable daily intake (TDI) for this study, one is given for P (P = proportion of source). The OA consumed by the dugongs are assumed to be result of the OA from seagrass epiphytes alone. In reality however, it has been demonstrated that this lipophilic toxin is present in the water and there is an uncalculated possibility that consumption of water may add to the uptake of OA.

7.4.6 Chronic Effects

The cellular and molecular studies provide insight into toxicodynamic effects of the toxins (Gundert-Remy et al. 2002), and what the long term exposure to toxins could result in. In this study, chronic effects relating to different pathways, such as apoptosis, MAPK and Wnt pathways, which can relate to uncontrolled cell proliferation and tumour promotion were assessed.

One of the up-regulated genes for OA treatment (IC_{30}) codes for the DUSP4 dual specific phosphatase 4. The phosphatases are known to inactivate their target kinases by dephosphorylating the phosphoserine (Appendix A). This finding can be supported by the fact that the OA group of toxins is a potent and selective inhibitor of three of the four protein serine/threonine phosphatase; type 1, 2A and 2B (PP1, PP2A, PP2B) (Bialojan & Takai 1988, Arias et al. 1993). Their relative toxicity is related to their affinity for protein phosphates (Holmes & Teo 2002). Another gene which was down-regulated by OA was GABRB3 gamma-aminobutyric acid A receptor, beta 3. This is a multi-subunit chloride channel that mediates the fastest inhibitory synaptic transmission in the central nervous system. As previous studies have found OA to be a tumour promoter, it is not surprising to find OA having effects on the genes regulating PP2A and channels since the PP2A inhibition can lead to tumour promotion (Messner et al. 2006).

Domoic acid down-regulated two genes related to Alzheimer's disease. One gene codes for the APP amyloid beta precursor protein. It is implied that mutation in this gene can affect Alzheimer disease and cerebroarterial amyloidosis. The second gene codes for UBB ubiquitin B. This covalently bonds to proteins to be degraded. An aberrant form of this protein has been noticed in Alzheimer's and Down syndrome patients. DA is especially known to affect the glutamate receptors in the CA3 region of the hippocampus, a region responsible for functional memory (Cendes et al. 1995, Sari & Kerr 2001). This region of the brain is also affected in patients with Alzheimer disease. Although DA has not determined to affect Alzheimer disease, this chronic disease may require long term exposure of DA. It is therefore critical to investigate the long term effects of DA on genes related to this disease, other than acute effects.

As for GD, most genes were down-regulated, except for one significant up-regulated gene, NLK which is known to control transcription of some genes. This gene may be controlling or suppressing other genes. It is natural for genes to be down-regulated when toxins are applied as the cells are being intoxicated. As discussed in Chapter 6, GD may potentially have chronic effects as this toxin affects the various pathways related to apoptosis and growth factors.

Similar to GD, PTX-2SA has not been proven to be highly toxic when orally dosed,

however the microarray study conducted previously by Burgess (2003) has shown that this toxin can also affect various genes within different pathways at the IC_{30} cytotoxicity level. It is therefore important to investigate further the chronic effects of this toxin. Furthermore phytoplankton are only known to produce PTX-2 form and, the seco acid form is the metabolic form of PTX-2, by the shellfish. It is, therefore essential to monitor the presence of PTX-2SA as the original form of PTX-2 can be more potent than the seco acid form, and the seco acid form can be used as an indicator of the presence of PTX-2.

The microarray analysis has provided extensive insight into the possible effects algal toxins on the regulation of the genes, and alternately on the production of proteins. As Gundert-Remy et al. (2002) states, a toxicodynamic study can provide a “good surrogate” to assess the toxicological effects. The present study is novel as it focussed on the gene regulation of gymnodimine, which could provide the mechanism of toxicity for this toxin. There is limited available information on GD, as there are only few oral, gavage and i.p. dose-response study on whole organisms. There is also one another study on cytotoxicity besides the current study (Dragunow et al. 2005), however the mechanism by which the GD poses its toxicity is still unknown.

The complexity of genes regulated by the single toxin can be further analysed by investigating the effects caused by mix of toxins. The previous study by Dragunow et al. (2005) has shown, that the pre-exposure of BE-2 cells to OA can shift the levels of toxicity of GD. As determined in this study, presence of multiple toxins in the environment is evident, hence the levels of toxicity for individual toxins may alter. Such toxicodynamic study show that the acute risk calculations, conducted in this study, may not be applicable to the chronic long term effects, and there may be a potential for chronic effects with continued exposure to sub-acute levels. This study has demonstrated the potential of chronic risk of GD, and further evaluation is required to quantify chronic risk assessment.

Chapter 8

Overall Conclusion

Research undertaken in this project has contributed significantly to the understanding of algal toxin dynamics in Moreton Bay. An evaluation of the relationship between collated literature derived dose-response data and the exposure levels for the toxins of interest. This has provided knowledge of acute health risks involved in consumption of shellfish for humans and seagrass for dugongs

The published literature on the background information and characteristics of the algal toxins; okadaic acid (OA), domoic acid (DA) gymnodimine (GD), pectenotoxin-2 (PTX-2) and pectenotoxin-2 seco acid (PTX-2SA) is reviewed. Recorded information on outbreaks of toxic incidents and the algae responsible for each of the toxins and their geographical location were investigated. Toxicity levels were derived to be used in the following work.

The exposure assessment study was divided into two sections; humans and dugongs. The findings of human exposure to algal toxins through consumption of shellfish around North Stradbroke Island are reported in Chapter 3. There were OA and PTX-2 producing *Dinophysis* detected on the ocean beach side of the island, both from the phytoplankton samples and from shellfish tissue during the summer months (Figure 8.1). However, the levels of toxins detected were low and the daily exposures of toxins to humans were below the guideline values established by the joint agreement between IOC, WHO and FAO (Toyofuku 2006). In winter OA and PTX-2s were not detected in the bay or the beach, and neither were *Dinophysis* species (Figure 8.2). Although no OA was detected from the shellfish samples in winter, it was detected from seagrass samples throughout the year, implying *P. lima* may be less prone to the temperature difference than *D. acuminata*.

Domoic acid was present both in summer and winter, at about the same maximum concentration in the ocean, but slightly higher in the bay for winter months (Figures 8.1 & 8.2). The maximum abundance of *Pseudo-nitzschia* were higher in the summer than winter. Although DA produced by *Pseudo-nitzschia* and GD produced by *K. selliformis* were present within the bay and the beach, levels of these toxins in shellfish were lower than the levels known to cause any acute effects. Both DA and GD were present in winter and were slightly higher in the bay than the beach, however, their concentration was again, lower than the guideline values.

The exposure analyses were also conducted using levels of OA detected in South Australian

shellfish. This evaluation showed that the daily exposure exceeded the guideline levels when blooms of *Dinophysis acuta* were seen in South Australian sites. This confirms the importance of monitoring of algal toxins and phytoplankton to ensure safety of shellfish consumption.

An additional component of Chapter 3 included the study conducted to assess the availability of dissolved algal toxins in the water column. By deploying a passive sampling device (SPATT), the study demonstrated the availability of dissolved okadaic acid, gymnodimine and pectenotoxin-2 in the water column around the island. This is a critical finding providing further evidence supporting a previous study by Mackenzie et al. (Mackenzie et al. 2004) indicating that dissolved toxins were available in the water column, thus providing an early warning of available toxins for uptake. More information is required before it can be determined whether these dissolved toxins are bioconcentrated in marine organisms by equilibrium partitioning between water and organism lipids. These toxins detected from SPATT bags were lipophilic, hence may bioconcentrate by the equilibrium partitioning process.

The exposure assessment for dugongs is reported in Chapter 4. Okadaic acid was detected from epiphytes of seagrass blades within Moreton Bay both in winter and in summer (Figures 8.1& 8.2). Using the levels of extracted OA, the daily exposure to dugongs feeding on seagrass within the area was evaluated. Dugong tissue samples were also obtained for the okadaic acid investigation, however no OA was detected from these samples with a detection limit of $10\mu\text{g kg}^{-1}$ tissue. It was concluded that the lack of OA detected does not imply absence of toxin in dugongs, moreover, estimated levels of OA in the tissue were most likely too low to be detected by the HPLC-MS/MS method used.

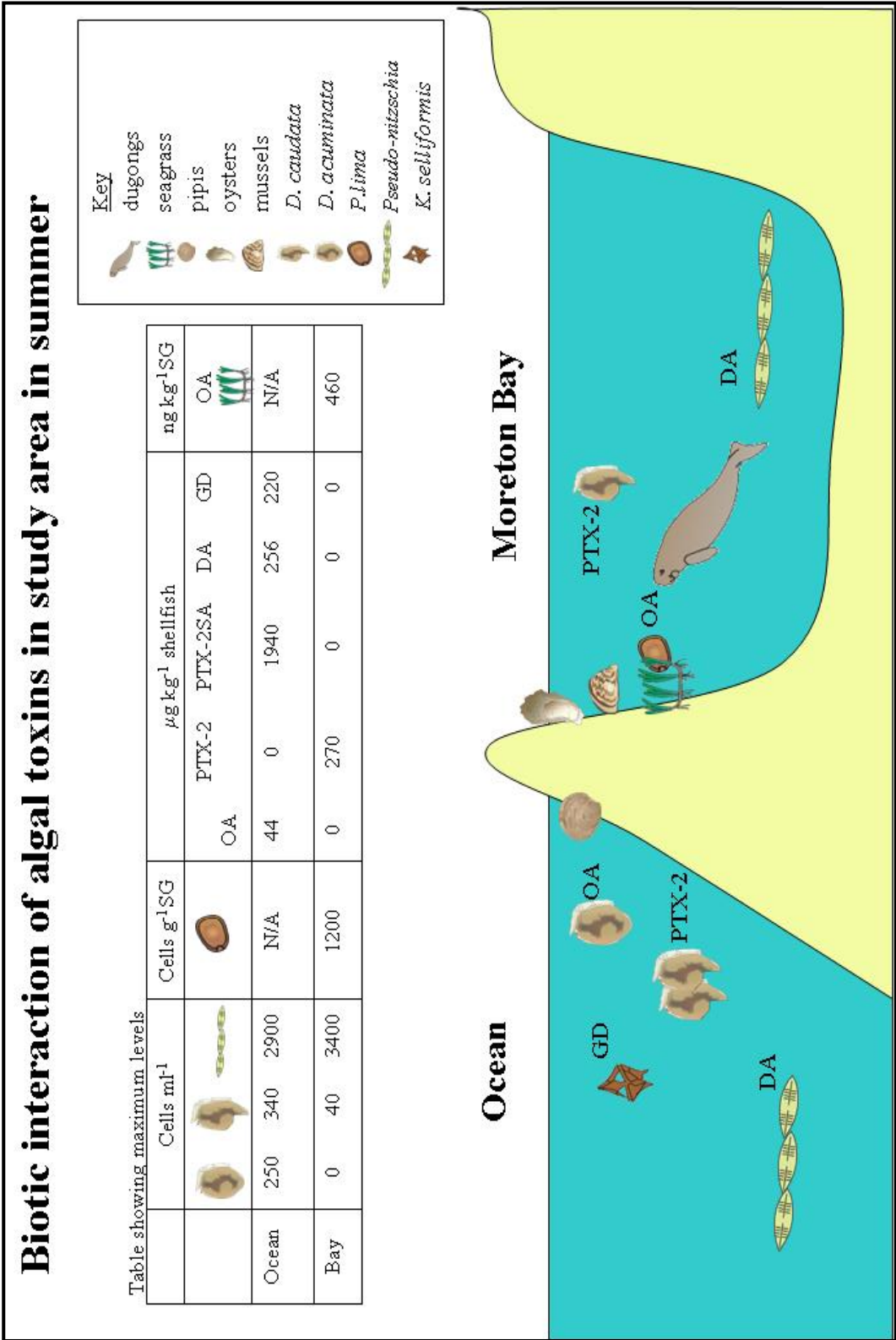


Figure 8.1. Conceptual diagram demonstrating the presence of toxic algae and their toxins in summer months.

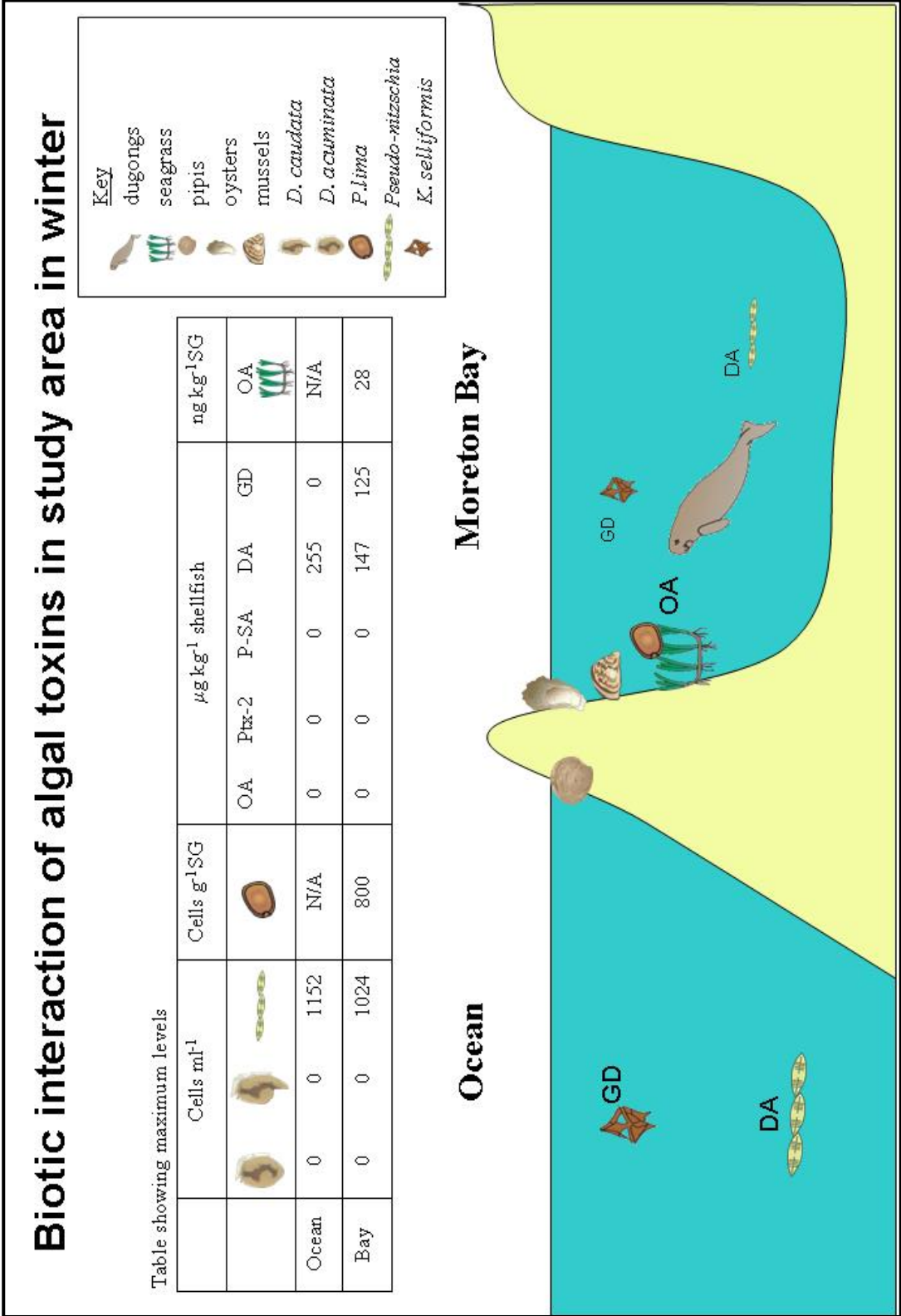


Figure 8.2. Conceptual diagram demonstrating the presence of toxic algae and their toxin in winter months.

In Chapter 5, dose-response analyses were conducted by utilisation of data from the literature to compare the dose-effect relationship. This allowed for comparison between the different mechanism of dosing and the difference in effects depending on the organisms being tested on. For example, humans are more susceptible to algal toxins than the laboratory tested rodents which highlights the importance of incorporating uncertainty factors for interspecies variation. It also accentuated the difficulty of comparing experiments performed on cell lines to the whole organism.

In Chapter 6, dose-response analysis was conducted on a cellular level, investigating the cytotoxicity of the three toxins; OA, DA and GD. The IC₃₀ levels for each of the three toxins were obtained using the MTS assay, and additionally, RNA from the cell lines were extracted for microarray analysis. This resulted in compilation of lists of genes affected by the toxins. The microarray study was repeated for GD with three biological replicates. Results showed the potency of this toxin as most genes were down regulated, slowing transcription of proteins, and affecting several pathways regulating cell deaths and relating to tumour development. Pathways relating to apoptosis and ATP synthesis were also found to be affected by GD at IC₃₀ levels. Although GD has not been shown to be toxic via feeding of mice with the toxin incorporated into a food matrix, these results demonstrate that it can have adverse effects depending on the route of exposure and consequent bioavailability. It is interesting that previous studies have demonstrated that some toxins may be more potent when in presence of another toxin. For example, OA can affect the potency of GD on cell lines (Dragunow et al. 2005). This is an important observation as toxins in the environment normally coexist. Future study on the effects of combination of toxins may be an important step in health risk assessment.

In Chapter 7, the exposure assessment and the literature-based dose-response evaluation of acute toxicity (Chapter 5) were compared to assess the risks involved for humans and dugongs from consumption of shellfish and seagrass around North Stradbroke Island. Since the levels of toxins detected from sampling sites were below the guideline values (derived in this study), there were no acute risks of toxicity for these organisms determined in this study. However, risks of chronic effects from consumption of seafood around the island still remains, especially from consumption of OA, which is a known tumour promoter (Fujiki et al. 1989). Additionally the mix

of algal toxins, such as OA and GD, and other organic pollutants in the water such as lingbyatoxins and dioxins (Osborne et al. 2001, Gaus 2004, Dragunow et al. 2005) can potential enhance the toxicity of tumour promoters. As the indigenous community on the island also consume higher levels of shellfish compared to the consumers on the mainland, hence they too may be at a higher risk of chronic effects. Similarly dugongs feed on seagrass on a daily basis, therefore they are exposed to OA for a prolong period of time.

The project was conducted between 2003~2005, during which no algal blooms were detected, and there were no acute health risks determined during this period. However, as South Australian data has shown, in cases of algal blooms, the chances of shellfish poisonings are high. With the changes in climate and global warming, potential for occurrence of algal bloom may be increasing (Van Dolah 2000b), hence it is important to investigate the levels of toxins and abundance of toxic algae in such events.

The study has provided the basis for investigation of the human health and ecological risk of algal toxins in the waters around North Stradbroke Island, with data on concentration of toxins in phytoplankton tows, shellfish and the water column. It has provided further evidence of toxin availability in the water column, in addition to the toxic algal cells. It has demonstrated that acute health risks to humans and dugongs around the island are unlikely. However the occurrence of chronic effects are possible as algal toxins are present in the area, and this is the limitation of the study as it requires further long term studies. With changes in the environment (Scheffer et al. 2001), there is also a potential for an increase in the production of algal toxins with toxic algal blooms in the area (Hallegraeff 1993b, Pritchard et al. 2003).

This study has demonstrated that there is little chance of acute health risks to humans and dugongs from algal toxins in the study area, provided that environmental conditions do not change significantly. On the contrary, this research has shown the presence of algal toxins that have adverse chronic effects, in the abiotic and biotic environment of Moreton Bay and North Stradbroke Island ocean waters. It is therefore possible that in conjunction with other chronic toxins present in the environment, these algal toxins may cause undefined chronic health effects in humans and dugongs.

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APPENDIX A: Complimentary DNA microarray analysis on three algal toxins using human neuroblastoma (BE-2) cell line

Methods

Dosing of cells in flasks

Human neuroblastoma cells (BE-2; QIMR) were subcultured to obtain six 70-mL flasks for each of the three toxins. Three of these flasks were used as controls where no toxins were added and separate flasks were used for each of the three toxins (OA, GD, and DA). Cells were left to grow in the flasks and on the fourth day toxins at IC₃₀ levels were added to the appropriate flasks. Flasks were further incubated for 24 hours.

RNA extraction

Total RNA from BE-2 samples was isolated using QIAGEN RNeasy mini kit by following their in house protocol (Qiagen Inc., Valencia, CA). For the homogenisation step, a 2 mm sterile needle was used. For the final elution step, RNA was eluted twice in 50 µL RNase free water.

Assessing RNA purity

To test for the purity of the RNA samples, 5 µL from each of the samples were dissolved in 955 µL RNase free Milli Q water (1:200). Total RNA concentrations were determined by the absorbance at 260 nm on the spectrophotometer (Cary 300). The purity of the RNA samples was determined using the ratio of optical densities measured at 260 and 280 nm.

Quality of the RNA samples was based on the integrity of the 28S and 18S bands, run on denaturing gels. In brief: Denaturing buffer containing formamide, formaldehyde and 10x MOPS were added to the RNA samples (5 µg:25 µL) and heated for 20 minutes at 60°C, and immediately placed on ice. Loading dye 5 µL, including ethidium bromide, was added to the sample and run on 1x MOPS gel at 100V for 1 hour.

RNA drying & re-suspension

Sodium acetate (3M pH 5-5.3) was added into the RNA water sample at a 0.1 volume (18:180 μ L sample) and vortexed. Two volumes of 99% ethanol (360 μ L) were added and vortexed. Samples were then placed in a -80°C freezer overnight. Tubes were centrifuged at 13k rpm for one hour at 4°C. Supernatant was pipetted and pellet was washed with 500 μ L of 70% ethanol. Samples were centrifuged at 13k rpm for 30 minutes at 4°C. Supernatant was pipetted and the sample was air dried for 15 minutes and stored at -80°C.

After transferring of RNA samples in dry ice for 16 hours and stored at -20°C for 12 hours, RNA pellets were re-suspended in RNA secure water.

RNA quantification

To re-quantify the samples, 5 μ L was diluted in 995 μ L RNase free water (1:200) and the absorption was recorded at 260 nm using a spectrophotometer. This measurement was used to calculate the amount of RNA in the sample. Samples were also run on 1x TBE gel to test for the quality of the RNA.

This step was repeated after the samples were combined to obtain the total amount of RNA in each of the OA, GD, DA and control samples.

Nano 6000 was also used for the second set of samples (RNA extracted from HepG2) to test for the purity of the RNA.

Reverse Transcription & dye labeling for 5K chips

Samples were placed into new tubes and diluted to obtain aliquots of 4.67 μ g RNA in 15 μ L RNA secure solution. Oligo dT (8 μ L) was added to each of the samples. Samples were heated to 70°C for 10 minutes and then placed on ice for a further 10 minutes. Master buffer were added to each tubes (Table A.1).

Table A.1: Master buffer for reverse transcription

	Volume per sample (μL)	Total volume (μL) for 5K chip
5 x RT buffer	8	32.8
Low T dntp	4	16.4
0.1M DTT	4	16.4
SS II enzyme	2	8.2
RNase Inhibitor	1	4.1
Total	19	77.9

The fluorescent dyes: Cy3 dUTP dye and Cy5 DUTP dye (2 μL), were incorporated into the appropriate tubes (Table A.2) and heated at 65°C for 2 hours.

Table A.2: Cy3 and Cy5 dUTP labeling

Cy3 labelled samples	Cy5 labelled samples
DA	Control
Control	DA
OA	Control
GYM	Control

To denature the remaining RNA that did not transcribe to cDNA, 0.5M NaOH/ 100 mM EDTA (20 μL) was added and this was heated to 65°C for 10 minutes. To adjust the pH, 0.5M HCl /1M Tris (20 μL) was added.

Purification

RNA samples from control tubes and treatment tubes were combined. A 1x TE buffer (100 μL) was added to each of the four tubes. This was then transferred onto the column (Microcon-YM-30) and centrifuged for 3 minutes at 14,000 g. The flow through was discarded and another 1xTE (400 μL) was added and centrifuged for 5 minutes. After the flow through was discarded, 1x TE (200 μL) was again added and

centrifuged for 3 minutes. This step was repeated approximately 5 times, until the colour of the dye was no longer visible. For the final centrifugation step, the column was spun dry. The column was inverted and centrifuged at 10,000 g for 3 minutes into a new collection tube. The sample was brought up to 16 μL with 1x TE and placed into a new tube.

Hybridization and washing

Hybridization buffer (5 x concentrations, 4 μL) was added to each sample. This was immediately followed by addition of 10% SDS (0.2 μL). Samples were heated at 100°C for 2 minutes to uncoil the cDNA strands and then placed on ice for 2 minutes. A total of 16 μL of the samples were placed on the B-chip slide. Cover slip was carefully placed on top and the slide was placed in the chamber, and incubated overnight at 65°C for hybridization.

The slides were washed twice in buffer 1 (2xSSC and 0.1% SDS) and twice in buffer 2 (2x SSC) at 37°C for ten minutes. After a quick rinse in the water, slides were dried using the N₂ blower.

Data imaging and analysis

Microarrays were scanned by ScanArray 400TM. For every microarray, initial images were scanned at low resolution (50 $\mu\text{m pixel}^{-1}$). When the appropriate PMT (photo multiple tube) was selected, images were scanned at high resolution (10 $\mu\text{m pixel}^{-1}$) and saved as TIF format.

GenePix 3.0 was used to analyze the images. Both Cy3 (wavelength 532) and Cy5 (wavelength 635) images were loaded into the software and assigned with two colours: green and red respectively. To input the size and location of each spot, gridding was performed, where each spot was automatically aligned with the grids. This was fine tuned manually to ensure that the spots and the grids aligned themselves as the algorithm takes the median value of inside pixels of the circle.

The digital format was transferred into data and graphed in Microsoft Excel. The ratio of Cy3 and Cy5 dyes were calculated and the ratio falling between 2 and 0.5 were filtered out. For domoic acid, a dye swap test was performed, and information from

the two microarrays was compared.

The genes of interest were identified using the website (Source: [Http://genome-www5.stanford.edu/cgi-bin/source/sourcesSearch](http://genome-www5.stanford.edu/cgi-bin/source/sourcesSearch)).

Quantitative real time PCR

Reverse transcriptase (RT-PCR)

Total RNA, extracted from dosed cells with OA, DA and GD were used as a template for cDNA (Table A.3). Protocol provided by the supplier of RT, Invitrogen, was followed. The mixture was incubated at 70°C for 10 minutes using a DNA Thermal Cycler (Perkin Elmer Cetus) and then chilled on ice to disrupt the secondary structure of RNA and to anneal the Oligo dT to the poly(A) tail. Master Mix was prepared and 8 µL of this was added to each of the tubes containing the initial mix of template RNA. This was then pre-incubated for 5 minutes at 42°C. 1 µL Reverse transcriptase (SuperScript II Reverse Transcriptase; Invitrogen) was added to each of the tubes, giving a total volume of 20 µL per tube. The enzyme was activated by heating at 42°C for 50 minutes. The enzyme activity was terminated by heating to 70°C for 15 minutes, and then chilled on ice for minimum of 1 minute.

Table A.3: RNA quantification using spectrophotometer (A260nm) to be used as template for cDNA transcription

Type of toxin	Initial A260/A280	ratio	Initial (µg)	RNA	Final (µg)	RNA
Control	1.92		1082		524	
Okadaic acid	1.78		231		78	
Gymnodimine	1.61		323		76	
Domoic acid	1.88		462		208	

Designing primers

Five genes of interest were selected from the results obtained from the microarray experiment. The sequence was “blasted” using GeneBank, NCBI

(<http://www.ncbi.nlm.nih.gov/BLAST/>) to check for any other similar sequences of genes. Forward and reverse primers were designed using both Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Sigma-Genosys (<http://orders.sigma-genosys.eu.com/probedesign/>). The primers were then ordered from Sigma-Aldrich.

Quantitative real-time polymerase chain reaction (QRT-PCR)

cDNA synthesized (reverse transcriptase section) was used in this analysis. Analysis was performed in triplicates on Rotor-Gene 3000 (Corbette Research), with Platinum SYBR Green q PCR SuperMix-UDG (Invitrogen). GAP and B-Actine (kindly provided by Mr Peter Pain, Griffith University), used as house-keeping primers. Master mix was prepared and 12 μ L was pipetted into appropriate tubes. Tubes 1-24 were prepared for specific primers while tubes 25- 48 were for house-keeping genes. The tubes were set up in triplicates to cater for genes of interest, positive control for the dosing experiment, a control for RT-PCR and a negative control (water) for the current experiment. Reactions were performed for x cycles (95°C/ 15 sec denaturing step; 60°C/1min annealing and extension step).

Results

Microarray

From the MTS assay, the toxin concentrations at which cells stop metabolizing was obtained (Chapter 5).

Each of the three replicates for OA, DA and GD were combined per toxin. Nine control replicates were also combined into one.

The quality and quantity of RNA decreased between the original and the final amount of RNA. This vast difference in the amount of RNA may be due to several reasons, including loss of RNA during the process of RNA drying and re-suspension, loss of RNA whilst transferring from one tube to another and pipetting error.

For cells dosed with OA and GD, there were just enough RNA to hybridize one 5K chip for microarray analysis, while for DA-dosed cells, there was enough to do a dye

swap test using two 5K chips.

For OA-dosed samples, there were five up-regulated genes and nine down-regulated genes (Table A.4). For cells treated with GD, there were 18 down-regulated genes but no up-regulated genes. For DA treated samples there were five up-regulated genes and nine down-regulated genes when Cy₃ = control vs Cy₅ = DA, and five up-regulated genes when Cy₅ = control and Cy₃ = DA. When the dye swap test was performed on the DA samples, the gene regulation did not match as none of the oppositely labelled genes showed the same results. Nevertheless, there were some genes which coincide with the preexisting knowledge on this toxin.

Real-Time PCR

Out of the five genes chosen from the microarray experiment, regulations of three genes were confirmed by QRT-PCR. GABRB3 is down regulated by OA, KCNAB1 is up-regulated by DA, while LASS5 is down regulated by GD (Table A.4). Microarray results showed down regulation of HYAL3 by OA, while repeated QRT-PCR showed up regulation of this gene. Similarly, microarray showed down regulation of IGF1R by GD, while repeated QRT-PCR showed up-regulation of this gene by GD.

Table A.4 Summary of the individual genes either up or down regulated by the three toxins; okadaic acid (OA), domoic acid (DA) and gymnodimine (GD).

Toxin/ regulation	Reporter Biosequence	#	Name of Gene (Source)	Function
OA up	NM_001394	DU	SP4 dual specificity phosphatase 4	Phosphatase inactivate their target kinases by dephosphorylating both the phosphoserine and threonine and phosphotyrosine residues
	NM_002687	PNN	Pinin demosome	General translation initiation by promoting the binding of tRNA to ribosome.
	BE394944	PLAT	plasminogen activator	High enzymatic activity causes hyperfibrinolysis, which manifests as excess bleeding.
	NM_004604	STX4A	Syntaxin 4A (placental)	Potentially involved in docking of synaptic vesicles of presynaptic active zones.
OA down	*NM_003549	HYAL	3 hyaluronoglucosaminidase	Protein similar in structure to hyaluronidases, degrade hyaluronan, one of the major glycosaminoglycans of the extracellular matrix. In the region of chromosome 3p21.3, this is associated with tumour suppression.
	AW172605	cDNA	FLJ37123 fis clone BRACE2022450	This may be a result from a cloning artefact or may be due to misinterpretation of the sequence data.
	AL514784	SNX15	sorting nexin 15	Member of this family contains phox domain, which is phosphoinositide binding domain and involved with intracellular trafficking.
	AI874346	VDR	vitamin D receptor	Nuclear hormone receptor vdr mediates the action of vitamin d3 by controlling the expression of hormone sensitive genes.
* NM_000814		GABRB3	gamma-aminobutyric acid A receptor, beta 3	Multi-subunit chloride channel that mediates the fastest inhibitory synaptic transmission in the central nervous system (CNS). Mediates neuronal inhibition by binding to the gaba/benzodiazepine receptor and opening an integral chloride channel
	NM_004541	ZNF183	zinc finger protein	Transfer of electron from nadh to the respiratory chain
	AA886389	Transcribed	sequence with strong similarity to protein HSPC189 protein	Krueppel is a gap class segmentation protein involved in the segmentation of the malpighian tubules
	JO5682	ATP6VIC1	ATPase, H transporting lysosomal 42kDa VI subunit C isoform1	Encodes a component of vacuolar ATPase a multisubunit enzyme that mediate acidification of eukaryotic intracellular organelles. Subunit c necessary for the assembly of catalytic sector of the enzyme and likely to have specific function in its catalytic activity. Responsible for acidifying intracellular compartment.

Toxin/ regulation	Reporter Biosequence	#	Name of Gene (Source)	Function
GYM down	AA557388		SRP 46 splicing, arginite/serine -rich	Contain number of phosphoproteins that function as splicing factors.
	* NM_147190		LASS5 LAG1 longevity assurance homolog 5	
	* NM_000875		IGF1R Insulin-like growth factor 1 receptor	Critical role in transformation events. Highly overexpressed in most malignant tissues where it functions as an anti-apoptotic agent by enhancing cell survival.
	NM_006819		STIP1 stress induced- phosphoprotein 1	
	NM_004255		COX5A cytochrome C oxidase subunit Va	Terminal enzyme of the mitochondrial respiratory chain. Couples the transfer of e- from cytochrome c to molecular oxygen and contributes to a proton electrochemical gradient across inner mitochondrial membrane.
	BE746861		PDAP1 associated protein 1	Enhances pdgfa-stimulated cell growth in fibroblasts, but inhibits the mitogenic
	XM_013042		MSN moesin	Probably involved in connections of major skeletal structures to the plasma membrane
	NM_001445		GPC4 glypican 4	Cell surface proteoglycan that bears heparin sulfate. Maybe involved in development of kidney tubules and of the CNS.
	NM_005900		SMAD1 mother against DPP homolog 1	Transcriptional modular activated by bone morphogenetic proteins.
	AF125188		ADAT1 adenosine deamine tRNA-specific 1	Using site specific adenosine modification proteins encoded by these genes participate in the pre-mRNA editing of nuclear transcripts.
	AW372543		ATF6 activating transcription factor 6	Endoplasm reticulum stress-regulated transmembrane transcription factor that activates the transcription of ER molecules
	NM_005584		MAB21L1 mab-21	May be involved in eye & cerebellum development. Has been proposed that expansions of repeat region of 5'UTR may play a role in psychiatric disorders.
	NM_006316		MYCNOS v-myc myelocytomatosis viral	N-myc oncogene implicated in the pathogenesis of a number of human tumours including child neuroblastoma and adult cell lung cancer.

Appendix

Toxin/ regulation	Reporter Bioscience	#	Name of Gene (Source)	Function
DA up	* NM_172159		KCNAB1 gated channel, shaker-related subfamily, beta member 1	Potassium channel functions: regulating neurotransmitter release, heart rate, insulin secretion, neuronal transcript, smooth muscle contraction.
	AA293676		FLOT1 flotillin 1	Caveolae are small domains on the inner cell membrane involved in vesicular trafficking and signal transduction.
	NM_017712		PGPEP1 peptidase1	Removes 5-oxoproline from various penultimate amino acid residues except 1-proline
	NM_002796		PSMB4 proteasome subunit beta type 4	Proteasome is a multicatalytic proteinase complex with highly ordered ring-shaped 20s core structure. It cleaves peptides in an ATP/ubiquitin-dependant process in a non- lysosomal pathway.
	NM_005625		SDCBP syndecan binding protein	An adaptor protein in adherent junctions, may function to couple syndecans to cytoskeletal proteins or signalling components. Highly expressed in heart and placenta in adults and in foetal kidney, liver, lung and brain.
	AW 474551		CAMLG calcium modulating ligand	Involved in mobilization of calcium as a result of the tcr/cd3 complex interaction binds to cyclophilin b.
	BF966476		STOM stomatin	Regulate cation conductance
	NM002184		IL6ST interleukin 6 signal transducer	Signal transducer shared by many cytokines, including interleukin 6, ciliary neurotrophic factor, leukaemia inhibitory factor and oncostatin M.
	NM_00615		NCAM1 Neural cell adhesion molecule	Cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation.

Toxin/ regulation	Reporter Biosequence	#	Name of Gene (Source)	Function
DA down	* NM_201413		APP amyloid beta precursor protein (protease nexin-II, Alzheimer disease)	Cell surface receptor and transmembrane precursor protein that is cleaved by secretases to form a number of peptides. Some peptides form the protein basis of the amyloid plaques found in the brains of patients with Alzheimer's disease. Mutation in this gene has been implicated in autosomal dominant Alzheimer's disease and cerebroarterial amyloidosis.
	NM_198970		AES	Amino terminal enhancer
	NM_002574		PRDX1 peroxiredoxin 1	Member of the peroxiredoxin family of antioxidant enzymes which reduces hydrogen peroxide and alkyl hydroperoxides. May have proliferative effect and play a role in cancer development or progression.
	NM_002807		PSMD1 proteasome (similar to NM_002796 which is up regulated)	Prosome, macropain/26s subunit, non ATPse 1. Codes for the largest non-ATPase subunit of the 19s regulator lid. Cleave peptides in an ATP/ubiquitin dependent process in non-lysosomal pathway.
	AL536237		TUBB5 tubulin beta 5	Major constituent of microtubules, binds two moles of GTP, one at exchangeable site on the beta chain and the other at nonexchangeable site on the alpha chain.
	NM_006761		YWHAE tyrosine monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide	Belongs to 14-3-3 family of protein which mediates signal transduction by binding to phosphoserine-containing proteins. Suggested role in diverse biochemical activities related to signal transduction such as cell division and regulation of insulin sensitivity. Also has been implicated in the pathogenesis of small cell lung cancer.
	NM_018955		UBB ubiquitin B	Required for ATP-dependent nonlysosomal intracellular protein degradation of abnormal protein and normal protein with rapid tumour. Covalently bound to protein to be degraded. Aberrant form of this protein has been noticed in Alzheimer's and Down syndrome patients.

Table A.5. Quantitative real time polymerase chain reaction (QRT-PCR).

Genes of interest	Toxin used	Up-regulated	Down-regulated	Microarray results	QRT-PCR # trials
HYAL 3	OA	2- 4 x		Down	3/3
GABRB3	OA		30 x	Down	1/1
KCNAB1	DA	0.5 - 2 x		Up	2/2
LASS5	GD		30 x	Down	2/3
IGF1R	GD	2 x		Down	2/2

For GABRB3, KCNAB1 and LASS5 QRT-PCR confirmed the microarray analysis, while for HYAL3 and IF1R genes, these QRT-PCR results did not confirm the microarray data.

Conclusions

This preliminary study has shown the potential to use the cDNA microarray technique in determining gene regulation of the three algal toxins reviewed. Results have shown that the genes being up or down regulated by OA and DA are related to the already known mechanisms of the toxins. Since knowledge on the mechanisms of GD is limited, this study has the potential to propose the pathway of this toxin.

APPENDIX B: Seeding cells

Seeding cells into 96 well plates

	24 H test						48 H test					
	1	2	3	4	5	6	7	8	9	10	11	12
A	media	media	media	media	media	media	media	media	media	media	media	media
B	media	ctrl	ctrl	toxin a	toxin b	media	media	ctrl	ctrl	toxin a	toxin b	media
C	media	ctrl	ctrl	toxin a	toxin b	media	media	ctrl	ctrl	toxin a	toxin b	media
D	media	ctrl	ctrl	toxin a	toxin b	media	media	ctrl	ctrl	toxin a	toxin b	media
E	media	ctrl	ctrl	toxin a	toxin b	media	media	ctrl	ctrl	toxin a	toxin b	media
F	media	ctrl	ctrl	toxin a	toxin b	media	media	ctrl	ctrl	toxin a	toxin b	media
G	media	media	media	media	media	media	media	media	media	media	media	media
H												

200ul into each well

5x replicates for each dose

3 different doses + 1 ctrl = 4

2 different days

Calculation: $200\text{ul} \times 5 \times 4 \times 2 = 8\text{ ml}$ of cells needed

After PBS, Trypsin, dislodge – “Tube A”

Place into 15ml tube

Take 10ul and dilute into 30ul media

Count cells

Cell count	
1	
2	
3	
4	

TOTAL “X” $\times 10^4$ cells

$((5 \times 10^4) / X \times 10^4 \text{ cells}) \times 8\text{ml} = \text{Zml}$
above

$8\text{ml} - \text{Zml} = \text{Yml}$

NB: 8ml comes from

Z = amt of cell/media

*Y = extra media to
make up the volume*

Take Zml from cell mix (tube A) and

Place it into tube B

Place Yml media into tube B

Vortex and seed wells

Seeding cells into 6 well plates

	1	2	3
1	control	control	control
2	GD	GD	GD

3ml into each well

Calculation: $3\text{ml} \times 6 = 18\text{ ml}$ of cells needed

After PBS, Trypsin, dislodge – “Tube A”
Place into 15ml tube

Cell count	
1	
2	
3	
4	

TOTAL “X”/4 $\times 10^4$ cells

$$((5 \times 10^4) / X \times 10^4 \text{ cells}) \times 18\text{ml} = Z\text{ml}$$

Z = amt of cell/media

$$18\text{ml} - Z\text{ml} = Y\text{ml}$$

*Y = extra media to
make up the volume*

Take Zml from cell mix (tube A) and

Place it into tube B

Place Yml media into tube B

Vortex and seed

APPENDIX C: RNA Extraction using Qiagen RNeasy Mini Protocol

1. Dislodge cells
 - Discard the old media
 - Add PBS (10ml) – wash/ mix gently and discard
 - Scrap cells into eppendorf – pipet the end bit
 - Determine # cells – 10ul slide
2. Harvest cells
 - Centrifuge 5 min @ 1500rpm (20-25°C)
 - Remove all supernatant
3. Disrupt cells
 - Loosen the cell pellet by flicking the tube
 - Add 350µl Buffer RLT (50ul B-ME into 5ml RLT)
 - Vortex
4. Homogenise the sample
 - 20x with needle homogeniser – making sure to get all the cells
5. Add 1 volume (350 µl) 70% ethanol to lysate and mix well by pipeting
6. Apply up to 700 µl of the sample each to 2 column, placed in 2ml collection tube
 - Close the tube carefully
 - Centrifuge 15sec @ 8000g (10,000rpm) (20-25°C)
 - Re-apply the flow through and centrifuge again
 - Discard the flow-through – keep collection tube for #6
7. Add 700 µl Buffer RW1 to each column
 - Close tube gently
 - Centrifuge 15 sec @ 8000g
 - Discard flow-through and collection tube
8. Transfer column into new 2ml collection tube
 - Pipet 500 µl Buffer RPE onto each column
 - Centrifuge 15 sec @ 8000g (to wash)
 - Discard flow-through, keep collection tube for #9
9. Add another 500 µl Buffer RPE to each column
 - Centrifuge 2 min @ 8000g (to dry)
 - Discard flow-through and collection tube
 - Place a new collection tube (not provided)
 - Centrifuge in a microcentrifuge @ full speed 1 min

10. To elute

- Transfer column into a new 1.5ml collection tube
- Pipet 50µl or 30µl RNase free water into the membrane
- Centrifuge 1min @ 8000g

11. Add another 50µl RNase free water and centrifuge 1mn

12. Combine the two samples into one.

OR in ethanol

13. Store at 70°C if stored in water

APPENDIX D: MessageAmp II aRNA Amplification (ambion)

Reverse Transcription to synthesize 1st strand cDNA

RNA	x μ l for 750 μ g RNA
T7 Oligo (dT) primer	1 μ l
Water	up tp 12 μ l

↓
Incubate 10min at 70°C

↓
Ice

Master Mix - 8 μ l to each tube

	Vol (μ l)	For 7 samples
10x 1 st strand buffer	2	14
dNTP Mix	4	28
RNase inhibitor	1	7
Array Script	1	7
<i>Total vol</i>	8	56

↓
42°C 2 hours

↓
Spin

↓
Ice (Freezer -20°C)

Second Strand cDNA Synthesis

Need: 16°C incubator

Add Master Mix: 80 μ l to each tube

	Vol (μ l)	For 7 samples
Nuclease-free water	63	441
10x 2 nd strand buffer	10	70
dNTP Mix	4	28
RNA polymerase	2	14
RNase H	1	7
<i>Total vol</i>	80	56

↓
16°C 2 hours

↓
Spin

↓
Ice (Freezer -20°C)

cDNA Purification

- Preheat water to 50-55°C
 - Check for precipitate in cDNA binding buffer
1. Add 250µl cDNA binding buffer to each sample
↓ pipette mix and spin
 2. Pass through cDNA filter cartridge (onto centre of filter)
↓ spin 10,000 x g
1 min
↓ Discard flowthrough
 3. Wash with 500µl Wash Buffer
↓ 10,000 x g
1 min
↓ Dry spin, new tube for elution
 4. Elute with 10µl 50-55°C water
↓ Let it sit for 2 min
10,000 x g
1.5 min
↓ ICE
- Repeat twice

***In Vitro* Transcription**

Need: 37°C

For 20µl IVT RXN, 12µl master mix into each tube

	Vol (µl)	X 7 samples Master Mix
ATP/CTP/GTP mix (25mM)	6	42
UTP (50mM)	1.3	9
Biotin-II-UTP (50mM)	0.7	5
T7 10x Rxn Buffer	2	14
T7 Enzyme Mix	2	14
<i>Total Volume</i>	<i>12</i>	<i>84</i>

↓
37 °C

↓
14 hours (O/N)

↓
Stop RXN by adding nuclease-free water to bring volume to 100 µl

↓
Store at -20°C

aRNA Purification

- Preheat water to 50-55°C

1. Add 350µl aRNA Binding Buffer to each

2. 250µl 100% ethanol to each sample
pipette mix **No vortex or spin*



3. Pipet each sample mixture onto center of filter in aRNA Filter Cartridge



10,000 x g 1 min
Discard flowthrough



4. 650µl Wash Buffer to each cartridge



10,000 x g 1 min
Discard flowthrough



Dry spin 10,000 x g 1 min
New tube for elution

5. Elute with 100µl 50-55°C water



Let it sit for 2 min



10,000 x g 1.5 min

Keep elution

Aliquot into 5-20ug
Store -80°C

Labelling with fluorescent dye (Invitrogen)

Alexa Fluor Dyes

1. Calculate yield of aa-aRNA and place 5 μ g RNA into 1.5ml tube
 2. Speed vac until volume is <3 μ l
 3. Add 2 μ l DMSO directly to Alexa Fluor Reactive Dye (re-suspend dye)
Vortex and spin
 4. Add 5 μ l 2x Coupling Buffer to aRNA
 5. Add dye solution to the tube - vortex
 6. incubate @ rm temp in the dark for 30min
 7. purify dye-coupled aa-aRNA using aRNA purification
- * minimize light exposure
 - * use amber-collection tube

Hybridization

Blocking buffer: 2x SSC
 0.1% SDS
 0.1% BSA

- Place slides onto the racks in 55°C blocking buffer
- Incubate 30 min w/ agitation as it cools to room temp
- Rinse slide with water
- Dry spin in centrifuge
- Let it sit for 15min
- Speedi vac until <10ul
- Add: 8 µL 20x ssc
 20µL DEIONISED FORMAMIDE
 2 µL 10% sds
- Mix then spin
- Heat to 95°C 5 min
- Cool to Rm temp 10 min
- Spin
- Add 30µL coverslip or slide
- Carefully slide into hybridizing chamber at 42°C water for O/N
- Wash with Wash buffer 1 (20 minutes on a gentle shaker with foil)

(SDS, SSC, up to 2L water)
- Wash with wash buffer 2 (5-15 minutes on a shaker with foil)

(SSC, up to 2L water)
Dip in water (1 second) spin dry

APPENDIX E: Real Time PCR

The quality and quantity of RNA was measured using the Nano drop test (table E.1). The volume required for 1 μg RNA was calculated using the quantity of RNA from this test. Once the volume of RNA was calculated, the volume of water required for the total volume of 14.5 μL was calculated.

Table E.1 Nano drop test for initial RNA concentration

RNA samples	RNA concentration (ng mL ⁻¹)	260/280	For 1 μg RNA (μL)	Water (to make up to 14.5 μL)
C5	231.6	2.05	4.3	9.2
G5	296.6	2.04	3.4	10.1
C6	221.4	2.06	4.5	9
G6	201.8	2.05	4.9	8.6
C7	236	2.04	4.2	9.3
G7	115.6	2.09	8.65	4.82

Invitrogen Superscript III Reverse Transcriptase (cDNA synthesis)

Master Mix 1 was prepared as shown below (table E.2). For each reaction tube, 1.25 μL of Master Mix was aliquoted, and 1 μg RNA and equivalent water was added. This was heated at 70°C for 5min, then placed on ice for 2 min.

At room temperature 5.5 μL of Master Mix 2 was added to each tube and heated at 46°C for 50 min, then to 70°C for 15 min. The cDNA product was then stored at -80°C.

Table E.2. Master Mix 1

For 7 reactions	
dNTP	7 μL
Random hexamers	0.35 μL
Oligo dT	1.4 μL
Total volume	8.75 μL

Table E.2. Master Mix 2

For 7 reactions	
5 x 1 st strand buffer	28 μL
0.1M DIT	7 μL
Superscript III	7 μL
Total volume	42

Real Time PCR

List of Samples

1. C5
2. C5
3. C6
4. C6
5. C7
6. C7
7. G5
8. G5
9. G6
10. G6
11. G7
12. G7
13. Blank (water)
14. Blank (water)
15. Blank (RNA C5)
16. Blank (RNA G5)

List of Primers

1. Actin
2. PGR
3. KL
4. NRP
5. METAP
6. CD248
7. CDC14B
8. MKRN
9. MAWBK
10. RABL7
11. SNRP
12. APOBEC

Primers Mix

Master 100 μM

Working stock: 0.5 μM

Forward: 5 μl

Reverse: 5 μl

In 990 μl water
(5:1000 = 1:200; 5 μM)

Primer Mix (5 μM) was measured out for each primer. The following was added to each tube.

cDNA Mix

cDNA	1 μl
SybrG	5 μl

Primer Mix

Each	4 μl
------	-----------------

Verifying QRT-PCR products

Agarose gel (2%) electrophoresis was conducted. QRT-PCR products were loaded onto the gel (10 µl: 1µl dye; Run 100V 45 min)

<u>List of samples</u>	<u>Results</u>
1. KL	NG – 2 different bands
2. METAP	faint
3. CD 248	NG 1 faint band and 2 different bands (3 bands)
4. CDC 14B	good
5. MKRN	good
6. MAWB	good
7. RABL7	good
8. SNRPN	good
9. NRP	NG no band
10. APOBEC	good

Each of the bands were cleaned using the following protocol

GelSpin DNA Purification Kit (MO BIO ULTRA CLEAN)

1. Place gel in filter column
2. Add 3 volumes of GelBind (3x “p” grams)
Bands cut out = “p” grams
3. Incubate 2min at 55C, invert and incubate longer until gel melts
4. Centrifuge 10 sec 10,000 g
5. Remove spin filter, vortex and re-load all liquid onto same filter column
6. Centrifuge 10 sec at 10,000 g
7. Discard flow-through, replace tube
8. 300ul GelWash
9. Centrifuge 10 sec, discard flow through, replace collection tube
10. Elute with 40ul 80°C water, spin 30 secs 10,000 g (repeat twice)

Gene	Gel (g)	Volume GelBind (µl)
KL a	0.058	0.174
KL b	0.078	0.23
MET	0.12	0.36
CD248 a	0.061	0.18
CD248 b	0.05	0.15
CD 248 c	0.112	0.336
CDC 14B	0.13	0.39
MKRN	0.11	0.33
MAWB	0.114	0.34
RABL7	0.08	0.24
SNRP	0.12	0.36
APOBEC	0.09	0.24

Analysis for QRT-PCR

1. Export files to excel

Results table was exported to excel. Delta RN, which shows the difference between cycles (1= between one and two; 2= between two and three; 3 = between three and four etc....) was exported for analysis

2. “linkeg PCR”

Linkeg PCR program was used for linear data and calculations

NB: when using this program in excel, make sure to have top row as cycle #s and one extra left column.

- Fit Option: between 4 and 6 cycles
- PCR efficiency
- Correlation was calculated to determine how PCR efficiency

3. In Excel

Copy PCR efficiency and CT results (R2)

Paste the following into new sheet

- Well #
- CT
- PCR Effi (delta RN)
- R2

Eliminating PCR with less than 97% efficiency:

If R2 >0.997, PCR “Bad PCR”

This is for quality control, using efficiency of 97% or more.

Average efficiency of primer/PCR, close to 2 is good.

Comparing to actin house keeping gene

How many cycles from zero did it cross the threshold, compared to actin.

$$= \text{PCR of gene}^{\text{gene ct}} / \text{PCR of actin}^{\text{actin ct}}$$

This gives the amount of RNA compared to actin as a ratio(GENE EXPRESSION)

Comparing control to treatment

- Average sample C5.1 and C5.2 (same plate, different wells)
- Ratio between treatment / control for 5, 6, 7
(5, 6 and 7 are biological reps – different passage #s)
- Average of three bio reps of C and std errors
- Average of three bio reps of G and std errors
- t- test was conducted to compare the averages of C and G to obtain the p-value.

APPENDIX F: Genes affected by gymnodimine

Up regulated genes (> 2 folds up regulated)

Gene Name	GeneBank ID#	Description
NLK	U00949	Myb proto-oncogene protein, nemo like kinase Role in cell fate determination, required for differentiation of bone marrow stromal cells. Acts downstream of MAP3K7 and HIPK2 to negatively regulate the canonical Wnt/beta-catenin signalling pathway and the phosphorylation and destruction of the MYB transcription factor. May suppress a wide range of transcription factors by phosphorylation of the coactivator, CREBBP. Belongs to the Ser/Thr protein kinase family. MAP kinase subfamily, subcellular location:Nuclear; predominantly. Transcriptional activator; DNA-binding protein that specifically recognize the sequence 5'-YAAC[GT]G-3'. Plays an important role in the control of proliferation and differentiation of hematopoietic progenitor cells.
KL	NM_004795	Klotho ; Transmembrane protein; type 1 membrane protein that is related to beta-glucosidases Overexpression: extended average life span 19% and 31% (Kuron et al 2005) Klotho gene deficient mice – show premature human aging (Arking et al 2002) Disease: mutation associated with ageing and none loss; coronary artery.
SLC34A1 NaPi2	NM_003052	Solute carrier family 34 (sodium phosphate) member1 Other names: NAP1-3, NPTIIa, NPT2, NaPi2a, sodium phosphate transport Actively transport phosphate into cells via Na cotransport in the renal brush border membrane. Important regulator for Pi reabsorption in renal proximal tubules (Nashiki et al 2005) Disease: defects in slc34a1 cause <u>hypophosphatemia</u> (deficiency in phosphate in blood) which contributes to pathogenesis of hypophosphatemic <u>urolithiasis</u> (kidney stone)as well hypophosphatemic <u>osteoporosis</u> (bone demineralization).
TM9SF1	NM_006405	Transmembrane 9 superfamily member 1 Multi-pass membrane proteinmulti-pass membrane protein; May function as a channel or small molecule transporter.

SSFA2	NM_006751	Sperm specific antigen 2 Sperm surface antigen involved in some step of early cleavage of the fertilized oocyte (Inokuchi et al 2004). Bock <i>in vitro</i> contraception (Naz 1995) “Antigen” = stimulates an immune response especially to the production of antibodies.
DUSP10 MKP 5	AB026436	Dual specificity phosphatase Elevated by stress stimuli. Inactivate target kinases by dephosphorylating both phosphoserine/threonine and phosphatyrine residues. Negatively regulate membrane of MAPK superfamily. Inactivate p348 and MAPK/JNK in cytoplasm and nucleus.
HM 13	AF075033 NM_030789	Histocompatibility (minor) 13 On endoplasmic reticulum catalyses intramembrane proteolysis of some signal peptides after being cleaved from preprotein. An integral membrane protein with sequence motifs characterization of the presenilin type aspartic proteases Disease: Alzheimer’s.
MAWBP	AB049758 NM_001033083	MAWD binding protein = human MAPK Activator with WD repeats A novel protein containing WD-40 repeats (Matsuda et al 2000). Frequently over-expressed in breast cancer, involved in tumour progression Over-expression of genes activates mitogen-activated protein kinases (MAPK) and promotes anchorage-independent growth of cells. Most metazoan cells are anchorage dependent, requiring a surface on which to divide.
NFKBIB	NM_002503	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta <i>Nf-kappa-B</i> = <i>Nuclear factor kappa-B</i> = nuclear transcription factor found in all cell types, involved in cellular responses to stimuli such as stress, cytokines, free radicals, UV, bacteria Pathways: T-cell receptor signalling pathway, B-cell receptor signalling pathway, adipocytokine signalling pathway, hypoxia and p53 in cardiovascular system.
PQLC2	NM_017765	PQ loop repeat contain 2 Function: unknown; May be associated with glycosylation machinery.
FAM5B KIAA 1747	AL035289	Family with sequence similarity 5 member B Playing a physiology functions in development of neurons system.

CTSL	L25628	<p>Cathepsin L-like 2</p> <p>Important for overall degradation of proteins lysosomes</p> <p>“Cathepsin” = family of protease (breaks apart other proteins)</p> <p>There is a premature stop codon, hence NO functional products are likely to arrive from this gene.</p>
RAB7L	NM_003929	<p>RAB7 member RAS oncogene family-like 1</p> <p>RAB or RAS family: protein in this family is important in molecular switches for a wide variety of signal pathways that control cytoskeletal integrity, proliferation, cell adhesion, apoptosis and cell migration. Activates # of pathways including MAP kinases (mitogen-activated protein)</p> <p>GTPases are involved in the regulation of exocytotic and endocytotic pathways.</p>
BATF	NM_006399	<p>Basic leucine zipper transcription factor ATF-like</p> <p>BZIP family, AP-1/ATF superfamily of transcription factors</p> <p>Negative regulator of APT-1 mediated transcription by binding to Jun proteins.</p> <p>BATF transgenic mice reveal a role for AT-1 in NKT cell development. BATF is a nuclear protein which negatively regulates AP-1 activity.</p>
DNMBP TUBA KIA1010	AB023227	<p>Dynamin binding protein</p> <p>It is a scaffold protein that binds dynamin and actin regulating proteins together</p> <p>It is concentrated at the synapses in the brain</p> <p>Diseases: Alzheimer Disease (neuropathologically characterized by loss of synapses, extracellular deposition of amyloid B-protein and cell death. Gene encoding for DNMBP is significantly associated with late-onset AD (LOAD), when the subject is already lacking in APOE-4 allele.</p>
AKT3 PKB	U79271	<p>v-akt murine thymoma viral oncogene homolog 3 (protein kinase b, gamma)</p> <p>AKT kinases are known to be regulators of cell signalling in response to insulin and growth factors.</p> <p>AKT are known to be stimulated by IGF1 (insulin like growth factor), play a role in cell survival, proliferation, differentiation, apoptosis, tumourigenesis and glucose uptake.</p> <p>Protein kinase B is involved in many crucial cellular processes such as metabolism, apoptosis and cell proliferation; this study showed the physiological role of PKB gamma, a crucial role in postnatal brain development</p> <p>Deregulation of PKB leads to diabetes.</p>

SNRPN PARI	NM_003097 NM_022805 AL157455	Small nuclear ribonucleoprotein polypeptide N Involved in tissue-specific alternative RNA processing events Disease: Prader-Willis Syndrome (chromosome 15 missing; short stature, mental retardation, incomplete sexual development, low muscle tone, involuntary urge to eat and reduced need for calories leading to obesity).
SEC23IP	NM_007190	SEC 23 interacting protein May be involved in maintenance of ER-golgi intermediate compartment and golgi structure. Over-expression perturbs the ER-golgi intermediate compartment. And disturbs protein transport between the 2, hence may have a role in early secretory pathway.
KCNJ8	NM_004982	Potassium inwardly rectifying channel subfamily J member 8 Potassium channels are important for range of physiological responses Has greater tendency to allow potassium to flow into a cell rather than out of a cell Potassium channels are central to regulate cell membrane potential and contractility of smooth muscle; expressed more during pregnancy; and down regulation may facilitate myomethial function during pregnancy.
CEP63	AF113697	Centrosomal protein 63kDa Function: muscle contraction, muscle myosin is a hexameric protein.
APOBEC1	NM_001644	Apolipoprotein b mrna editing enzyme, catalytic polypeptide 1 Member of cytidine deaminase enzyme family. Class of apoprotein, that depend on presence of other small molecules of cofactors to function. Converts C to U in the glutamine codon (CAA) to transcriptional stop codon (UAA), resulting in expression of truncated apob48 protein. Over-expression has been shown to hyper-edit other sites on apob mRNA besides the edited base (Teng et al 1993 within Zhong 06). Suspected that over-expression of Apobec 1 result in aberrant hyper-editing which will lead to hepatocellular carcinoma and dysplasia in mice (Yamada 95 within Zhong 06).

AKR1B1 AR	NM_001628	Aldo-keto reductase family 1 member b1 (aldose reductase) AR Catalyses the NADPH-dependent reduction and a number of aldehyde form of glucose and thereby implicated in the development of diabetes complications by catalyzing the reduction of AR is a component of myocardial ischemic injury and that inhibitors of this enzyme protect rat hearts from ischemic injury.
Lamin a/c	M13452	Lamins are component of nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane, thought to provide a framework for the nuclear envelope and may also interact with chromatin. Diseases: defects in lamina cause emery-dreifuss muscular dystrophy, slow progressive muscle wasting and weakness; also causes dilated cardiomyopathy (deterioration of function of myocardium, heart muscle tissue), cardiac dilation and reduced systolic function; generalized <u>lipodystrophy</u> (wasting away of fatty tissue) associated with diabetes, hepatic steatosis; <u>laminopathy</u> , Werner syndrome (premature aging). In lamina/C deficiency mice, slow growth, weak muscles and death (Fong et al 2006) Pathway: cell communication.
SH3GLB2 SEC23B	NM020145	Sec23 homolog B (S. cerevisiae)/ sh-3 domain grab2-like endophilin 2 Involved in vesicle trafficking Component of coat, that covers ER-derived vesicles involved from the ER to Golgi apparatus.
WDR40A	NM_015392 AF085907	WD repeat domain 40A Unknown function Implicated in variety of functions, signal transduction, cell apoptosis and regulation The underlying common factor of all WD repeat proteins is: coordinating multi protein complex assemblies, where repeating units serve as a rigid scaffold for protein interactions.
AKAP5	NM_004857	A kinase (PRKA) anchor protein 5 Bind to the regulating subunit of protein kinase A (PKA) and confining the holoenzyme to discrete locations within the cell May anchor the PKA protein to cytoskeletal and/or organelle associated proteins.

PPME1	AF146798	<p>Protein phosphatase methylesterase 1</p> <p>Demethylates proteins that have been reversibly carboxymethylated. Demethylates ppp2cb</p> <p>Pathway: 2,4-dichlorobenzoate degradation, butanoate metabolism, alkaloid biosynthesis.</p>
Neuropilin 2 NRP2	AF119893	<p>Receptor protein for; Axon guidance, angiogenesis, nervous system development, transport, cellular metabolism, vascular endothelial growth factor (VEGF = important signal protein involved in angiogenesis, belongs to PDGFR superfamily platelet-derived growth factor which regulate cell growth and division);</p> <p>VEGF: over-expressed in PDAC, critical for tumour angiogenesis.</p> <p>VEGF-A promote angiogenesis and may also suppress cancer directed immune responses.</p> <p>NRP1 and 2 co-express angiogenesis and results in poor recovery in non-small cell lung cancer with</p> <p>May directly promote pancreatic cancer cell growth <i>in vivo</i> (<i>Fukahi et al 2004</i>)</p> <p>PDAC = pancreatic ductal adenocarcinoma; deadly malignancy. NRP1/2 are present in cultured cancer cells. NRP1/2 immunoreactivity was also present in tumour microvasculature and adjacent acinar cells.</p> <p>NRP-2 are up regulated in endothelial cells of human neuroblastomas</p> <p>NRP_2 is expressed in pancreatic endocrine islet cells & in endocrine tumours in gastrointestinal tract</p> <p>SEMA3F has a repulsive effect on motile cells, which is mediated by NRP2 (Nassarre).</p>

Down regulated genes (> 3 folds down- regulated)

Gene Name	GeneBank ID#	Description
MAPK8IP2	NM_012324	Mitogen-activated protein kinase 8 interacting protein 2 Closely related to MAPK8IP1 – a scaffold protein that is involved in c-Jun amino-terminal kinase signalling pathway. Role in signalling transduction in cells, including mitogen-induced cell cycle progression through G1 phase, regulation of embryonic development, cell movement, apoptosis and cell and neuronal differentiation.
PTK6	NM_005975	protein tyrosine kinase 6 Cytoplasmic non-receptor protein kinase , may function as an intracellular signal transducer in epithelial tissues. Growth, metabolism and apoptosis.
CSF2RA	NM_006140	Colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage) Controls the production, differentiation and functions of granulocytes and macrophages (white blood cells). Activation of the granulocyte-macrophage colony-stimulating factor (GM-CSF) family of receptors promotes the survival, proliferation, and differentiation of cells of the myeloid compartment (Brown et al 2004).
METAP2	NM_006838	Methionyl aminopeptidase 2 Enzyme. Removes amino-terminal methionine from nascent proteins. Molecular target of angiogenesis inhibitors (inhibit growth of new blood vessels) and fumagillin (block blood vessel formation) and ovalacin. It up regulates telomerase (enzyme adds specific DNA seq repeats, allowing cells to divide forever) and bcl-2 (govern mitochondrial membrane permeability, known to resist cancer treatment by suppressing initiation of cell death process).
MKRN3	NM_005664 NM_011746	Makorin ring finger protein 3 Overlapping antisense transcript in Prader-Willi Syndrome critical region (Jong et 1999) Part of coordinately regulated imprinted domain affected in PW S (Nicholls 1998) So maybe associated with PWS.

SLIT2	NM_004787 O94813	Slit homolog 2 Thought to act as molecular guidance cue in cellular migration, and function appears to be mediated by interaction with roundabout homolog receptors. During neural development involved in axonal navigation at the ventral midline of the neural tube and projection of axons to different regions. SLIT1 and SLIT2 seem to be essential for midline guidance in the forebrain by acting as repulsive signal preventing inappropriate midline crossing by axons projecting from the olfactory bulb.
FDX1	NM_004109	Ferredoxin 1 Iron-sulfur protein. Mediate electron transfer in metabolic transfer. “adrenodoxin type in mitochondrial monooxygenase systems – ferredoxin transfers electron from NADPH to membrane-bound cytochrome P450.
APRN	U50539	Androgen-induced proliferation inhibitor Androgen is a generic term for steroid hormone; usually stimulates or controls the development and maintenance of masculine characteristics; controls proliferation of their target cells firstly by increasing cell proliferation and later by inhibiting the proliferation of those same cells.
BNIP2	NM_004330	BCL2/adenovirus E1B 19KDa interacting protein 2 Unknown function, but it interacts with E1B19KDa protein, which is responsible for protection of virally-induced cell death as well as E1B 19KDa like sequenced BCL2 which are apoptotic receptor
KRTHB5	NM_002283	Keratin, hair basic 5 Type II hair keratin; it is a basic protein which heterodimerizes with type I keratins, form hair/nails
PGR	NM_000926	Progesterone receptor Progesterone = C-21 steroid hormone in female menstrual cycle, pregnancy; supports gestation Decreased in oestrogen receptor and PGR in early pregnancy Down-reg of PGR – associated with development of ovarian epithelial carcinoma.
SLCO1C1	NM_01743	Solute carrier organic anion transporter family, member 1c 1 Oatp 14 plas a role in maintaining the concentration of T4 in the brain by transporting T4 from circulating blood to the brain.
ZWINT	NM_007057	ZW10 interactor Involved in kinetochore function, though exact role is unknown. Possibly play a role in targeting HZW 10 to the kinetochore at prometaphore during mitosis .

SLC 27A4	NM_005094	<p>Solute carrier family 27 member 4 (fatty acid transporter) Translocation of long chain fatty acid across plasma membrane Principal fatty acid transporter in small intestinal enterocytes Required for fat absorption in early embryogenesis.</p>
FXD3	NM_00597	<p>FXD domain containing ion transport regulators 3 Induce channel activity in experimental expression systems May function as a chloride channel or chloride regulator Induce a hyperpolarization activated chloride current when expressed in oocytes May be a modulator capable of activating endogenous oocyte channels Over-expression was seen in pancreatic cancer, may contribute to the proliferation activity of this malignancy (Kayed et al 2006).</p>
MUC 7	S83198	<p>Mucin 7 secreted (praline rich lacrimal) Histatin-1 precursor = salivary proteins that are considered to be major precursors of protective proteinaceous structure on tooth surface (enamel pellicle) Mucins = secreted by mucous gland, forms mucus when mixed with water A glycoprotein (protein and carbohydrate).</p>
RGS7	AF090116	<p>Regulator of g-protein signalling 7 Increase gptase activity of g protein alpha subunits thereby driving them into inactive gdp- bound form – this inhibits signal transduction. RGS: responsible for the rapid turnoff of G-protein-coupled receptor signalling pathways. “G protein” = guanine nucleotide binding proteins, belongs to GTPase group. Signal by exchange of GDP for GTP.</p>
EDF1	AB002282	<p>Endothelial differentiation-related factor 1 This gene encodes a protein that may regulate endothelial cell differentiation. It has been postulated that the protein functions as a bridging molecule that interconnects regulatory proteins and the basal transcriptional machinery, thereby modulating the transcription of genes involved in endothelial differentiation. Transcriptional coactivator stimulating nr5a1 and ligand-dependent nr1h3/lxra and pparg transcriptional activities. enhances the DNA-binding activity of atf1, atf2, creb1 and nr5a1.</p>

GLP2R	NM_004246	<p>Glucagon-like peptide 2 receptor The GLP2 receptor (GLP2R) is a G protein-coupled receptor superfamily member closely related to the glucagon receptor and GLP1 receptor. this is a receptor for glucagon-like peptide 2. the activity of this receptor is mediated by g proteins which activate adenylyl cyclase. GLP2 prevents intestinal hypoplasia resulting from total parenteral nutrition.</p>
THSD1	NM_018676	<p>Thrombospondin, type i, domain containing 1 The protein encoded by this gene contains a type 1 thrombospondin domain, which is found in thrombospondin, a number of proteins involved in the complement pathway, as well as extracellular matrix proteins.</p>
TNFSF18	NM_005092	<p>Tumour necrosis factor (ligand) superfamily, member 18 Important for interactions between activated T lymphocytes and endothelial cells and may modulate T lymphocyte survival in peripheral tissues, induction. The protein encoded by this gene is a cytokine that belongs to the tumour necrosis factor (TNF) ligand family.</p>
CCCL13	NM_005408	<p>Chemokine (c-c motif) ligand 13 This gene is one of several Cys-Cys (CC) cytokine genes clustered on the q-arm of chromosome 17. Cytokines are a family of secreted proteins involved in immunoregulatory and inflammatory processes. The CC cytokines are proteins characterized by two adjacent cysteines. The CC cytokines are proteins characterized by two adjacent cysteines. The cytokine encoded by this gene displays chemotactic activity for monocytes, lymphocytes, basophils and eosinophils, but not neutrophils.</p>
LTB	NM_002341	<p>lymphotoxin beta (tnf superfamily, member 3) Cytokine that binds to LTBR/TNFRSF3. May play a specific role in immune response regulation. Provides the membrane anchor for the attachment of the heterotrimeric complex to the cell surface. Isoform 2 is probably non-functional. FLS are involved in multiple stages of the inflammatory process, including the recruitment and retention of lymphocytes in the synovial microenvironment.</p>

PGDS	NM_014485	Prostaglandin-D synthase is a sigma class glutathione-S-transferase family member. The enzyme catalyzes the conversion of PGH2 to PGD2 and plays a role in the production of prostanooids in the immune system and mast cells. The presence of this enzyme can be used to identify the differentiation stage of human megakaryocytes.
SVIL	AF035278	Supervillin This gene encodes a bipartite protein with distinct amino- and carboxy-terminal domains. The amino-terminus contains nuclear localization signals and the carboxy-terminus contains numerous consecutive sequences with extensive similarity to proteins in the gelsolin family of actin-binding proteins, which cap, nucleate, and/or sever actin filaments. The gene product is tightly associated with both actin filaments and plasma membranes, suggesting a role as a high-affinity link between the actin cytoskeleton and the membrane. Its function may include recruitment of actin and other cytoskeletal proteins into specialized structures at the plasma membrane and in the nuclei of growing cells. Two transcript variants encoding different isoforms of supervillin have been described.
NAP1L1	NM_004537, NM_139207	Nucleosome assembly protein 1-like 1 This gene encodes a member of the nucleosome assembly protein (NAP) family. This protein participates in DNA replication and may play a role in modulating chromatin formation and contribute to the regulation of cell proliferation. Alternative splicing of this gene results in several transcript variants; however, not all have been fully described.