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Development of management recommendations to assist in advisories around seafood safety during toxic bloom events in the Gippsland Lakes

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Abbreviations

b.w	Body Weight – the measured weight of the fish whole
CAPIM	Centre for Aquatic Pollution Identification and Management
d.w.	Dry weight - a measurement of the mass of tissues when completely dried i.e. all its constituents excluding water.
DEWLP	Department of Environment, Water, Land and Planning
DHHS	Department of Health and Human Services
ELISA	Enzyme-linked immunosorbent assay; a means of assessing microcystin and nodularin concentrations using antibodies which specifically bind to structural elements of the compounds (generally the unique Adda moiety).
EPA	Environmental Protection Agency
FRDC	Fisheries Research Development Corporation.
LC-MS/MS	Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS).
MC-LR	A hepatotoxic cyclic heptapeptide produced by many types of cyanobacteria.
NATA	National Association of Testing Authorities
N. spumigena	Filamentous cyanobacteria (blue-green algae) that is found in brackish water and produces the toxin nodularin.
NOD-R	A hepatotoxic cyclic pentapeptide produced by the toxic filamentous cyanobacteria (blue-green algae) *Nodularia spumigena*.
VR-Fish	Victorian Recreational Fishing Peak Body
VFA	Victorian Fisheries Authority
w.w	Wet Weight - a measurement of the mass of tissues un-dried i.e. all its constituents including water.
WHO	World Health Organisation

Executive Summary

Over the last 4 years, scientists from the Centre for Aquatic Pollution Identification and Management (CAPIM) have been leading a research program to better understand the risks to seafood safety during toxic cyanobacterial blooms in the Gippsland Lakes and best practices for monitoring and managing these risks. The program has successfully generated a number of recommendations to assist in providing advice around seafood safety and to deal with restrictions around harvesting during blooms not only in the Gippsland Lakes, but on a national scale.

The safety of seafood products as a food source is of great importance from both a public health and economic viewpoint. The occurrence of cyanobacterial blooms in fresh, estuarine and coastal waters can lead to the accumulation of toxins in seafood species, with worldwide concern regarding the health risks associated with consumption of seafood caught during toxic cyanobacterial blooms.

Nodularin is a cyclic pentapeptide hepatotoxin produced by the cyanobacterium *Nodularia spumigena*, which forms blooms predominantly in estuarine and coastal systems, but also saline inland lakes. The Gippsland Lakes, situated in south-eastern Victoria, are one of Australia's largest lake systems supporting a range of recreational and commercial activities, including fishing. Over the last few decades the lakes have experienced an increase in the frequency and intensity of cyanobacterial blooms, with seven major blooms of *N. spumigena* since 1995. Prior to this, only one significant bloom had been documented in the previous 20 years. In 1999 and more recently 2011-12 and 2012-13 toxic blooms of *N. spumigena* led to restrictions on the harvesting and sale of shellfish, prawns and un-filleted finfish for periods up to 6 months due to the presence of nodularin in seafood tissues. Significant economic losses have been incurred by the fishing and tourism industry in the region, during the restricted periods, and by government agencies through the costs of monitoring the blooms and providing associated advice around seafood safety.

Following the lengthy restrictions, around harvesting of seafood, the commercial and recreational fishing sectors in the Gippsland Lakes have expressed concerns regarding the monitoring protocols, notably around the decision-making processes for harvest closures and re-opening. However, the lack of information on nodularin uptake, tissue distribution and elimination in finfish has been identified as a significant knowledge gap, by government, hampering the ability to provide appropriate advice regarding safe seafood harvesting and consumption during toxic blooms and in development of scientifically sound monitoring protocols. In order to quantitatively assess the risks that cyanotoxins in finfish pose to human health during blooms and develop scientifically sound, robust monitoring and management protocols, an understanding of uptake, accumulation and tissue distribution in commercially and recreationally relevant species is required.

This study was undertaken as two main components: (1) to assess nodularin uptake and depuration in commercially and recreationally relevant finfish species for the Gippsland Lakes; and (2) to review and compare the current monitoring protocols with those used nationally and internationally for managing risks to seafood safety from cyanotoxins and marine biotoxins.

The objectives of the project were threefold:

1. Determine uptake, elimination and tissue distribution of nodularin in commercially and recreationally relevant finfish species under laboratory and field conditions
2. Review current algal bloom response plan for the Gippsland Lakes and those used in monitoring programs in Australia and around the world.
3. Provide sampling and risk management recommendations, based on scientific and research findings from objectives 1 and 2, to deal with fishing closures and re-opening during bloom events.

A review of national and international monitoring and management programs for biotoxins identified that worldwide there is a lack of adequate data on cyanotoxins in seafood to be able to complete a detailed food safety risk evaluation around consumption of finfish during cyanobacterial blooms. While for marine biotoxins there are established internationally accepted health alert guidelines and guidance protocols for managing seafood safety risks, for cyanotoxins there are no internationally accepted health guideline values or guidance protocols. Currently there are only four countries with documented health alert guidance values for cyanotoxins in finfish and other seafood products, and of those only Denmark and the Gippsland Lakes, Australia have documented protocols for monitoring and managing food safety risks for cyanotoxins in seafood. There is an identified need at a national and international level to evaluate the risks posed by cyanotoxins in seafood and develop monitoring and management systems to minimise the risks to human health and reduce economic impacts for industry and governments.

While the Gippsland Lakes were identified as taking a lead in the development of a protocol to manage food safety risks posed to seafood during toxic cyanobacterial blooms, it was identified that there were a number of elements of the monitoring and management protocol that need to be addressed. In particular, there is a need to develop and implement a more comprehensive incident response plan, with the need for a funding agreement to be determined and documented to ensure funds area available to deploy for sampling and toxin analysis in the event of a toxic bloom. Industry need to play a greater role in the monitoring and management of seafood safety. Clear definition of the roles and responsibilities of government agencies and industry therefore need to be defined. This would also assist to determine equitable funding contributions. Lastly there needs to be continued research on cyanotoxins in seafood in order to allow for a thorough, scientifically robust, food safety risk evaluation to be undertaken on the risk of consuming finfish caught during cyanobacterial blooms and to feed into monitoring and management programs.

The experimental component of the program successfully assessed the uptake, accumulation, tissue distribution and elimination of nodularin in Black Bream (*Acanthopagrus butcheri*) and Southern Sand Flathead (*Platycephalus bassensis*), identifying that there were species-specific and dose dependant differences in nodularin uptake, accumulation and elimination, however not tissue distribution. Key outcomes from the experimental research were that a 'gut and gill' policy should be incorporated into the Gippsland Lakes protocol, as this significantly reduces risk of exposure to nodularin toxin, while allowing for market access for commercial industry during harvesting restrictions. As such, the use of Black Bream as a sentinel species in the current Gippsland Lakes protocol needs reassessment. Until this, advisories should be based on nodularin concentrations in a wider range of species. Lastly, all decisions around harvesting restricting should only be made based on the measurement of nodularin toxin in finfish tissues, particularly the muscle tissue and tissues that would be removed if gutted and gilled.

Recommendations

Based on the outcomes from the experimental research and Gippsland Lakes response plan review, a number of recommendations are provided which would assist in advisories and monitoring and management of seafood safety in the Gippsland Lakes during toxic blooms. These include:

- Develop and implement a comprehensive response plan that is based on internationally respected risk assessment principles and a scientifically sound management framework.
- Develop an appropriate cost sharing agreement so as funding will be available each year in the event of a bloom. Funding needs to be available to be deployed for sampling and toxin analysis.
- Undertake further research into uptake, tissue distribution and elimination of nodularin under field conditions in a greater number of finfish species of commercial and recreational significance to better understand risks and select an appropriate sentinel species
- Undertake further laboratory and field assessment for other toxins to fully evaluate food safety risks.
- Investigate other methods which could help in monitoring of toxins during blooms

Keywords

Black Bream, *Acanthopagrus butcheri*, Southern Sand Flathead, *Platycephalus bassensis*, Cyanobacterial blooms, *Nodularia spumigena*, seafood safety, Gippsland Lakes, Incident response plans, nodularin, toxin accumulation

Introduction

Cyanobacterial blooms and seafood safety

Cyanobacterial blooms are not a new phenomenon and have plagued humans for centuries. However, the increasing frequency and intensity of blooms and the variety of species and habitats in which they are occurring, has led to a growing awareness of the environmental, public health and economic impacts of these events. Increased eutrophication, climate change and altered hydrological patterns are expected to intensify the occurrences of cyanobacterial blooms posing further risk to society (O'Neil et al 2012; Sotton et al 2015). This risk is associated with the hazardous secondary metabolites, known as cyanotoxins, which are produced by many cyanobacterial species. These cyanotoxins pose a risk to human and animal health, primarily through direct exposure or consumption of contaminated water, but also through consumption of contaminated finfish and shellfish, a pathway which is often underappreciated, but potentially major (Ibelings and Chorus, 2007; Bartrum et al 1999).

A cyanobacteria species of concern is *Nodularia spumigena*. This species is a filamentous form that blooms most often in slightly saline to brackish waters, such as saline inland lakes, estuaries and coastal lakes and embayments around the world (Sivonen and Jones 1999; Van Buynder et al 2001; Kankaanpaa et al 2002; Sotton et al 2015), with some reports of its occurrence in freshwater lakes (Akcaalan et al 2009; Kaloudis et al 2013; Baker and Humpage 1994). Typically, *N. spumigena* is restricted to the temperate regions of the continent (Francis 1878), however, there have been reports in recent years of blooms in the tropical lakes of Australia (Stewart et al 2012; McGregor et al 2012). Globally, major blooms of *N. spumigena* have been recorded in the Baltic Sea, North Sea coastal lakes and basins, Lake Ellesmere in New Zealand, in Pyramid Lake in the USA, and South Africa (Moisander and Paerl 2000; Stal et al. 2003). In Australia, *N. spumigena* blooms have been reported in the Peel-Harvey Estuary, Western Australia, Lake Alexandrina in South Australia, Orielton Lagoon, Tasmania, Lake Corangamite in southwest Victoria, in a recreational ski lake in south-east Queensland and in the Gippsland Lakes in southeast Victoria (Francis 1878; Huber 1985; Codd et al. 1994; Jones et al. 1994; Blackburn et al. 1996; Stewart et al 2012; McGregor et al 2012; Vanbuynder et al 2001; Eaglesham et al 2002).

Due to the production of the cyclic pentapeptide hepatotoxin nodularin, blooms of *N. spumigena* are generally toxic. The structure and biological activity of nodularin is similar to that of the cyclic heptapeptides called microcystins, the most frequently encountered and best recognized group of toxins produced by freshwater cyanobacteria (Akcaalan et al., 2009; Mazur-Marzec et al., 2009). Like microcystins, nodularin inhibits protein phosphatases 1 (PP-1), 2A (PP-2A) and 3 (PP-3), which leads to functional disturbance and structural disruption of the liver (Eriksson et al 1988; Yoshizawa et al 1990; Honkanan et al 1991). Nodularin is also reported to act as a tumor promoter and is a suspected carcinogen (Ohta et al 1994). Although no human fatalities have been attributed to nodularin intoxication, it has been associated with stock and domestic animal poisoning events (Nehring 1993; Harding et al 1995; Van Halderen et al 1995). The accumulation of nodularin into seafood species, including finfish, is well documented, notably in the Baltic Sea (Sipia et al 2001a, 2001b; Persson et al 2009; Mazur-Marzec et al 2007; Kankaanpaa et al 2002; Kankaanpaa et al 2005) but also in New Zealand (Dolamore et al 2017) and Australia (Eaglesham et al 2002; Falconer et al 1992; Van Buynder et al 2001; Stewart et al 2012; Department of Health 2014). Finfish may uptake nodularin via two main routes: absorption via the gills and/or skin or through the gastrointestinal tract (Ibelings and Chorus 2007). During toxic blooms, nodularin toxin is generally contained within the cells and mainly released into the water as the cells die and lyse (Myers, 2008). Fish are therefore more likely to be exposed to nodularin through ingestion of toxic cells or contaminated food, and to a lesser extent through dissolved toxin. After ingestion, nodularin is transported into the blood through the bile acid transport system and is predominately concentrated in the liver, however can accumulate in other tissues and organs (Vourinen et al 2009; Rezaitabar et al 2017). The safety of seafood products is of utmost importance from both a human health and economic viewpoint. Nodularin and other cyanotoxins can induce illness

if contaminated seafood is consumed. This is not only a problem for commercially harvested seafood, but for recreational fishers as well, especially for those where it may be for subsistence fishing.

Commercial and recreational fishing in the Gippsland Lakes

The Gippsland Lakes, situated in south-eastern Victoria, are the largest estuarine coastal lagoon system in Australia. This system consists of three main interconnected lakes and a number of surrounding marshes and lagoons which are the receiving waters of six major rivers with a total catchment area of over 20,000km^2 (EPA 2009). The lakes are connected to Bass Strait by a permanent narrow opening (Lakes Entrance) at the east, and represent a unique aquatic ecosystem not only of ecological significance, but as a significant fishery supporting both commercial and recreational value to the region. The commercial fishery is currently made up of ten Gippsland Lakes Fishing Access Licences which harvest a range of species for human consumption, ten Gippsland Lakes (bait fishery) Access Licences and two Eel Fishery Access Licences which collect eels for human consumption (DEDJTR 2016). The commercial fishery is divided into 13 different areas across the lakes (Figure 1; DEDJTR 2016), with commercial catch comprising primarily of Black Bream, Anchovy (*Engraulis australis*), Sea Mullet (*Mugil cephalus*) and Silver Trevally (*Pseudocaranx georgianus*). Other species taken in this fishery include Dusky Flathead (*Platycephalus fuscus*), Carp (*Cyprinus carpio*), King George Whiting (*Sillaginodes punctatus*), Eastern River Garfish (*Hyporhamphus regularis*), Yellow-eye Mullet (*Aldrichetta forsteri*), Luderick (*Girella tricuspidata*), Australian Salmon (*Arripis trutta*), Estuary Perch (*Macquaria colonorum*), Tailor (*Pomatomus saltatrix*) and Leartherjacket (*Oligoplites saurus*) (Conron et al 2016; Department of Primary Industries 2012). The Eel fishery is based on two species being the Short- and Long- Finned Eels (*Anguilla australis* and *A. reinhardtii*, respectively; DEDJTR 2016). The Gippsland Lakes are also a highly valued destination for recreational fishers, with an estimated economic contribution to the Victorian economy of $381 million (DEDTJR 2016). Recreational fishers predominantly target Black Bream, Dusky Flathead, Silver Trevally and Yellow-eye Mullet, with catches for some of these species being equal to or exceeding that of the commercial sector on an annual basis (DEDJTR 2016; Conron et al 2016).

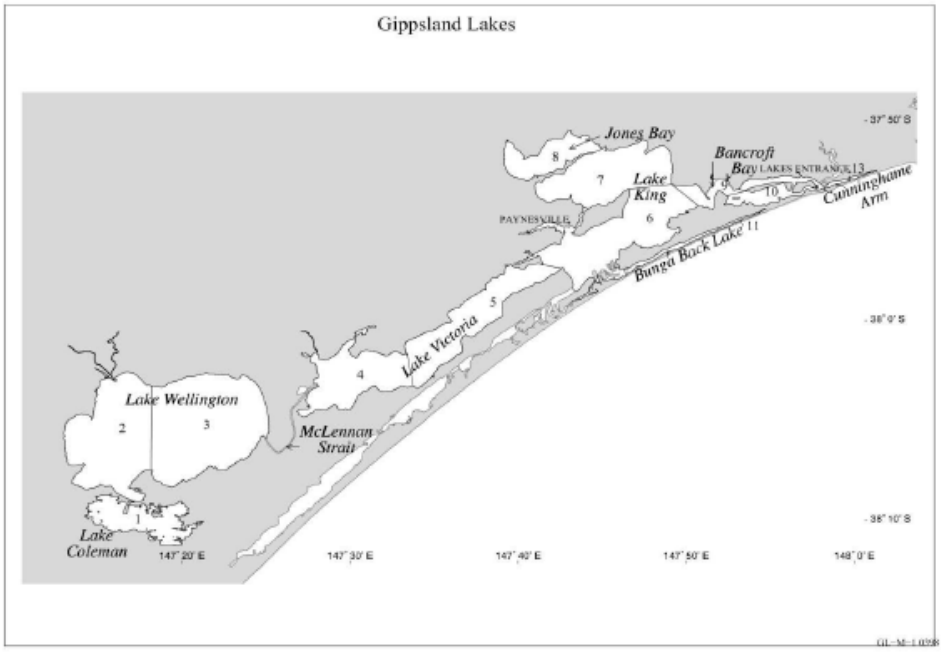

Figure 1: Map of the Gippsland Lakes commercial fishing areas. Source: DEDJTR 2016

Nodularia spumigena blooms in the Gippsland Lakes

Over the last few decades the Gippsland Lakes have experienced an increase in the frequency and intensity of cyanobacterial blooms, with twelve major blooms since 1985 (Day et al. 2011). *Nodularia spumigena* blooms in 2001 and 2002 resulted in nodularin accumulation in naturally occurring prawns ranging from 220-22,430 µg/kg d.w. in the viscera and 5-143 µg/kg d.w. in muscle and mussel samples contained between 40 - 2725 µg/kg d.w. nodularin (Van Buynder et al 2001; Eagelsham et al., 2002). While in fish liver samples (including Black Bream, Leatherjacket, Mullet and Trevally) nodularin concentrations in the range 35-450 µg/kg d.w. were reported and up to 2.5 µg/kg in muscle (Eaglesham et al 2002; Van buynder et al 2001). During blooms in 2012-13 nodularin was detected in whole fish samples up to 37 µg/kg and in mussels up to 39.7 µg/kg, however there was no toxin detected in gilled and gutted fish (Department of Health Victoria website, last updated 21st May 2014).

Seafood safety in the Gippsland Lakes is of great importance from both a public health and economic viewpoint. In 2001-02 and more recently 2011-12 and 2012-13 toxic blooms of *N. spumigena* led to closures of the Gippsland Lakes Estuarine fishery due to the presence of nodularin in seafood tissues (VBIFA 2013). During the 2011-12 bloom harvesting of finfish was permitted, following an initial closure, providing fish were gilled and gutted prior to sale. This requirement lasted for four months (VBIFA 2013). Blooms result in significant economic losses to both the fishing and tourism industries in the region and high financial cost for government agencies monitoring the blooms. For instance the blooms in 2008, resulted in a net impact (as lost revenue and impact on jobs) estimated at $18 million (MWH 2014). Following the 2011-12 and 2012-13 blooms the commercial and recreational fishing sectors expressed concerns regarding the monitoring and management of the fishery during toxic blooms and subsequent economic impacts to the industry and region. This concern was further identified in a seafood industry risk assessment conducted in 2013, which ranked finfish and cephalopod fisheries in the Gippsland Lakes as "high risk" based on contamination by biotoxins resulting in extended shutdowns of the finfish industry and subsequent high economic impacts for industry relative to other seafood categories (MWH 2014).

Current protocols for seafood monitoring in the Gippsland Lakes

The current protocol for "*managing risks to seafood safety from blue-green algal blooms in the Gippsland Lakes*" (Anonymous, 2011) was designed to provide a response capability for ensuring seafood safety during toxic blooms in the Lakes. It involves a multi-agency approach between various government organizations, including Department of Health, DEWLP, PrimeSafe, Fisheries Victoria, EPA Victoria, Parks Victoria and local councils. In short, the protocol is based on weekly seafood sampling of mussels, prawns and a sentinel finfish species, the Black Bream, at various sites once the bloom has been identified as toxin producing. If levels of toxins in these seafood reach health alert levels set by a scientific advisory committee on algal toxins in seafood (Mulvena et al., 2012; Table 1), the Department of Health advise the general public through media releases and signage and PrimeSafe (Victoria's statutory authority responsible for regulation of commercial seafood safety) regarding the collection and sale of seafood from the lakes. PrimeSafe then advise the commercial fisherman that they need to cease commercial harvest and sale of prawns and whether they need to gut and gill finfish prior to sale until levels of toxin in the tissues have declined to below the health alert levels (Anonymous, 2011).

Whilst this protocol has been implemented during blooms since 2011 a number of issues have been identified as requiring further research in order to provide confidence in design and execution. More specifically, issues identified include:

1. Weekly sampling of seafood is intensive and costly
2. Advisory periods have been lengthy due to issues in sampling and analysis

3. Provision of advisories is given based on nodularin concentrations in a sentinel species, the Black Bream, with little scientific understanding of uptake and depuration rates for toxins in all species harvested.
4. The gut and gill advisory is time consuming and costly for commercial operators
5. There is no secured funding for event response during toxic blooms
6. There is a lack of certified laboratories in Australia for the routine analysis of cyanobacterial toxins

The lack of information on nodularin uptake and depuration in seafood has been regarded as a significant knowledge gap, as it hampers the ability to provide appropriate advice to commercial and recreational fishers regarding seafood harvesting and consumption during toxic blooms. Furthermore, this lack of knowledge makes it difficult for such organisations to monitor blooms effectively through development of cost effective and timely sampling protocols. The need for improvement in the current incident management procedure for algal blooms has also been identified by industry in a food safety risk assessment of the seafood industry in Victoria together with a need for ongoing input as new knowledge is gained (MWH 2014).

Table 1: Health Guideline values for Nodularin and Microcystin toxins in seafood for Victoria (From Mulvenna et al. 2012).

Toxin	Health guideline value (µg/kg whole organism)		
	Fish	Prawns	Mussels or Molluscs
Microcystin-LR^ or equivalent toxins, i.e. Nodularin	24	32	51

^ due to lack of suitable standards and adequate comparative toxicity data, the recommendation of the Australian Drinking Water Guidelines is to treat all microcystins as having equivalent toxicity to microcystin-LR. The guideline value therefore represents the sum value for all detectable microcystins and nodularin

Objectives

The objectives of this study were formulated in consultation with the commercial fishing industry from the Gippsland Lakes, Seafood Industry Victoria, and the Victorian Department of Health and Human Services. They were further supported by VRFish and the Sydney Fish Market. They were:

Objective 1:

Determine uptake, elimination and tissue distribution of nodularin in commercially and recreationally relevant species under laboratory and field conditions

Objective 2:

Review current algal bloom response plan for the Gippsland Lakes and those used in monitoring programs in Australia and around the world.

Objective 3:

Provide sampling and risk management recommendations, based on scientific and research findings from objectives 1 and 2, to deal with fishing closures and re-opening during bloom events.

As there were no *N. spumigena* blooms in the Gippsland Lakes during the course of the project, field assessments were not undertaken as part of Objective 1. However, further laboratory experiments were implemented to assess nodularin uptake, accumulation, tissue distribution and elimination in species of commercial and recreational significance.

Methods

Full details of the methods for the objectives are provided in Appendices A to C. However, summaries of the methodology employed for each of the objectives are detailed below.

Objective 1: Determination of elimination and tissue distributions of nodularin in commercially and recreationally relevant species under laboratory and field conditions.

The focus of objective one was to examine the uptake, tissue distribution and depuration of nodularin in the finfish species currently used as a sentinel during toxic algal blooms in the Gippsland lakes, Black Bream, under laboratory conditions and during a *N. spumigena* bloom. The use of Black Bream as a sentinel to provide finfish advisories during toxic blooms is based on the commercial and recreational importance of the species together with detection of the highest concentrations of nodularin in Black Bream compared to all other fish species during a bloom in the Gippsland lakes in 2002 (Eaglesham et al 2002), its potential to accumulate nodularin to a greater degree than other finfish due to its dietary preferences for shellfish and prawns, which are known to accumulate toxins, and potential to spend the majority of time within areas experiencing blooms compared to other fish species (Anonymous 2011).

A further aim was to assess the tissue distribution of nodularin in other commercially relevant finfish species during the *N. spumigena* bloom in order to compare to that in Black Bream and make an assessment of the validity of using Black Bream as a sentinel species. Unfortunately, during the period of the project there was no *N. spumigena* bloom in the Gippsland Lakes, or elsewhere in Victoria, where nodularin accumulation, tissue distribution and elimination could be assessed. Objective 1 was therefore undertaken as two separate laboratory studies which assessed uptake and depuration of nodularin in Black Bream, and the commercially and recreationally relevant finfish species the Southern Sand Flathead.

Laboratory experiment 1: Investigation of nodularin tissue distribution and elimination in the Gippsland Lakes sentinel species, Black Bream.

The uptake, accumulation and tissue distribution of nodularin was assessed in the Black Bream. Full details of the experimental methodology, sample processing, toxin extraction and analysis are provided in Appendix A, however are also summarised below and in Table 2.

Experimental Fish

Forty-seven mature Black Bream were obtained from Fisheries Victoria breeding stock for these experiments. These fish had been held in captivity by Fisheries Victoria for over 10 years.

Nodularin slurry

A non-axenic strain of *N. spumigena*, originally isolated from field samples collected from the Gippsland Lakes (March 2003, J. Myers) was grown in batch culture. The culture was maintained in MLA media (Bolch and Blackburn 1996) at 21 ± 1^{o}C under constant illumination (cool white fluorescent lamps) at the CAPIM laboratory in the School of BioSciences, University of Melbourne Parkville campus. *Nodularia spumigena* cells were harvested, via filtration, from batch cultures to prepare nodularin contaminated slurry. A subsample of the filtered biomass was analysed to determine the nodularin content, while the remainder was stored at -80^{o}C for later use in the preparation of toxic slurry. Nodularin concentrations in the cell concentrate were determined by the commercial laboratory Advanced Analytical using liquid chromatography mass spectrometry (LC-MS) and in house using enzyme-linked immunosorbent assay (ELISA) to allow comparison of the two methods for nodularin detection. The toxin concentration of the *N. spumigena* filtrate was 9.52 µg/g as determined by ELISA

and 13.81µg/g determined by LC-MS. At this point we did not have enough cell biomass containing toxin to undertake the dosing. Therefore, culturing and harvesting was continued over a further 6 week period to collect enough cell biomass. A subsample of the final cell biomass collected was again analysed for nodularin content, however only ELISA was used, as both the ELISA and LC-MS method produced comparable results during the first analysis of cell concentrate, and time and sample processing cost did not permit LC-MS analysis a second time. The toxin concentration of the new *N. spumigena* cell biomass was determined to be 0.5mg/g. This concentration was in line with that reported for Australian strains of *N. spumigena* (0.73mg/g to 5mg/g (Jones et al 1994; Platt 2005) and is similar to toxin production determined for this isolate of 0.1-0.6 mg/g (Myers, 2008). Using this value as a guide, the slurry for oral gavage was prepared.

The slurry mixture was composed of the laboratory cultured *N. spumigena* cell biomass, ground fish pellets (Marine 45/20 dinking 4mm pellets, Ridley Aqua Feed) and ultra-pure water. Two *N. spumigena*-containing slurry's were prepared by mixing the ground fish pellets and water with an appropriate mass of *N. spumigena* biomass to produce nodularin concentrations in the slurry's appropriate to deliver the nominal doses of 50 µg/kg b.w. and 200 µg/kg b.w. to fish, while allowing for similar volumes of slurry to be injected into fish stomachs for both treatment levels. The control slurry consisted of the ground fish pellets mixed with ultra-pure water, but without *N. spumigena* biomass. For details of the ratios of the components added to produce the toxic and control slurry's for Black Bream exposures see Table 1 in Appendix A.

Experimental procedure

Nodularin was dosed orally as a single delivery of the slurry directly into the stomachs of fish to achieve nominal doses of either 50 or 200 µg nodularin/kg b.w.. Control fish were dosed in the same way as nodularin-exposed fish, with control slurry. These doses were based on levels of nodularin reported in prawns during *N. spumigena* blooms by Eaglesham et al (2002), which would equate to providing a fish consuming these prawns with a nominal nodularin dose of 221 µg nodularin /kg w.w..

Immediately prior to dosing fish were anesthetized, one at a time, in aerated seawater containing AQUI-S (AQUI-S New Zealand Ltd). Under anaesthesia fish were weighed and measured and then slurry was delivered into the stomach via a flexible polypropylene tube attached to a 50-mL syringe. The amount to be delivered to each individual was determined on the basis of fish weight and a table that was compiled beforehand (See Table 2 in Appendix A). The syringe was weighed before and after injection to obtain the exact amount of slurry delivered. After treatment fish were placed in a 500-L resin tank and once they had recovered, they were returned to their appropriate treatment tank.

The experiment commenced on the 4th June 2014 (day 0), when all fish were oral gavaged with a single dose of the appropriate slurry. During the experimental period, fish were fed uncontaminated commercial fish pellets three times per week. At pre-determined time periods (1, 2, 7, 14 and 20 days) post gavage, three fish from each nodularin treatment were sampled. Control fish were sampled on days 1 and 20.

At each sampling point, fish were euthanized one at a time, and then the spinal cord severed. Each fish was weighed and the total and fork lengths measured. The body cavity was opened, the fish was sexed and the liver, a muscle sample (taken from behind the left pectoral fin) and gill sample were removed, weighed and kept for nodularin analysis. The experiment was conducted under animal ethics permit number AEC SETP12 0088, Department of Environment and Primary Industries.

Nodularin determination

Frozen liver, gill and muscle tissue samples, together with *N. spumigena* biomass spiked slurry samples and control slurry samples (ca. 1 g) were sent to the commercial laboratory Advanced Analytical for nodularin extraction and analysis using LC-MS. The detection limit for nodularin in fish tissues was 20 µg/kg.

Nodularia spumigena biomass spiked slurry and control slurry were also assessed for nodularin concentration in house using ELISA. Samples were extracted in methanol, washed with hexane and cleaned up with Solid Phase Extraction (SPE). Eluted extracts were then dried under an air stream and reconstituted in methanol for analysis. The extracts were analysed using a direct competitive commercial ELISA kit (Abraxis Microcystins-ADDA ELISA, Abraxis LLC, USA) calibrated with 0.1, 0.5, 1.0, 5.0, 10.0 and 50.0 µg/L nodularin solutions. Each sample was screened with 1:10 and 1:100 dilutions. The analyses were performed according to the manufacturer's instructions with a microplate washer (Wellwash Microplate Strip washer, Thermo Scientific Pty Ltd) to wash plates. The absorbance at 450nm in each well was read by a Multiskan EX microplate reader (MTX Lab Systems, Inc) within 10 minutes of adding the stop solution. In order to verify the accuracy and precision of the ELISA measurements, check standards (i.e. nodularin standards run as samples) were run on each ELISA plate during each ELISA test. The ratio of nominal concentrations and result values averaged 106% (range 41 - 288%; n = 10). This result indicates the ELISA was on average slightly over estimating results (by 6% on average).

Table 2: Experimental design for assessing uptake, tissue distribution and elimination of nodularin in *Acanthopagrus butcheri* (Black Bream) following a single oral dose.

Species	***Acanthopagrus butcheri* (Black Bream)**
Number of fish	11 control, 18 per treatment
Treatments	55 µg nodularin /kg b.w. 200 µg nodularin /kg b.w.
Control	Uncontaminated slurry 0 µg nodularin /kg b.w.
Sampling time post gavage (days)	1, 2, 7, 14, 20, 30
Tissues examined	Muscle, liver, gill

Experiment 2: Laboratory investigation of nodularin uptake, tissue distribution and depuration in commercially and recreationally relevant finfish species.

The uptake, accumulation and tissue distribution of nodularin was assessed in Black Bream and Southern Sand Flathead. These species were chosen based on their importance as commercial and recreational species in the Gippsland Lakes and as they represent different feeding groups, which would therefore be exposed to cyanobacterial toxins through different ways. Black Bream is one of the most important recreational and commercial species in the Gippsland Lakes. Recent estimates indicate that Black Bream constitute 37% of the total commercial catch (Conron 2016). Given the popularity of recreational fishing in the region, recreational catch is considered likely to equal or to exceed that of the commercial sector (DEDJTR 2016). Black Bream are opportunistic omnivores, consuming a wide range of prey, including sessile, burrowing, benthic and pelagic species such as mussels, barnacles, tubeworms, crabs, bloodworms, squirtworms, ghost shrimp, cockles, prawns, amphipods, copepods, small fish (i.e.: gobies, hardyheads and anchovies) and plant material including algae (Norris et al 2002; Sarre et al 2000). Dusky Flathead (*Platycephalus fuscus*) are a common species in the Gippsland Lakes, forming a small part of commercial catch but are more significant as a recreational species. In this study, we used the closely related species *Platycephalus bassensis* (Southern Sand Flathead) (Department of Primary Industries 2012). This was due to ease of capture of this species within close vicinity of Victorian Marine Science Consortium (VMSC) laboratory facilities. Flathead are carnivores, feeding primarily on fish, prawns, squid and also large benthic crustaceans. They are considered ambush predators, hiding from their prey by burying in the sediment (Perry et al 1995). Full

details of the experimental methodology, sample processing, toxin extraction and analysis are provided in Appendix B, with a summary provided below and in Table 3.

Table 3: Experimental design for assessing uptake, accumulation, tissue distribution and elimination of nodularin in *Acanthopagrus butcheri* (Black Bream) and *Platycephalus bassensis* (Southern Sand Flathead) following a single oral dose.

Species	*Acanthopagrus butcheri* (Black Bream)	*Platycephalus bassensis* (Southern Sand Flathead)
Number of fish	13 control, 21 per treatment	12 control, 16 per treatment
Treatments	869 µg nodularin /kg b.w.	106 µg nodularin /kg b.w 776 µg nodularin /kg b.w.
Control	Uncontaminated slurry (0 µg nodularin /kg b.w.)	Uncontaminated slurry (0 µg nodularin /kg b.w.)
Sampling time post gavage (days)	1, 2, 7, 14, 20	1, 2, 7, 14, 20
Tissues examined	Muscle, liver, gut*	Muscle, liver, gut*

* Guts included tissues that would be removed upon gutting a fish; heart, kidney, intestine, stomach and gall bladder, herein denoted as "gut".

Experimental fish

Black Bream were caught by seine net from Swan Bay, a coastal embayment near Queenscliff in Victoria during September and October 2016, while Southern Sand Flathead were caught by rod and line from Port Phillip Bay, Victoria during October 2016.

Nodularin slurry

A non-axenic strain of *N. spumigena*, originally isolated from field samples collected from the Gippsland Lakes (March 2003, J. Myers) was grown in batch culture. The culture was maintained in MLA media (Bolch and Blackburn 1996) at 21 ± 1^{o}C under constant illumination (cool white fluorescent lamps) at the CAPIM laboratory in the School of BioSciences, University of Melbourne Parkville campus. *N. spumigena* cells were harvested, via centrifugation, from batch cultures to prepare nodularin contaminated slurry. Once a significant amount of *N. spumigena* biomass had been collected, the frozen biomass was freeze dried. A subsample of the freeze-dried biomass was sent to colleagues at Cawthron Institute (Nelson, New Zealand) for analytical determination of nodularin, while the remainder was stored at -80^{o}C for later use in the preparation of toxic slurry.

The average nodularin (as Nod-R) content of the cyanobacterial biomass, as determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS), was 700 µg/g. None of the microcystin variants analysed were detected in the sample (< 0.5 µg/g). Using this value as a guide, the *N. spumigena*-containing slurry for oral gavage was prepared.

The slurry mixture was composed of the laboratory cultured and freeze-dried *N. spumigena* biomass and eastern king prawn (*Melicertus plebejus*) homogenate. The prawn homogenate consisted of ground eastern king prawns and ultra-pure water. Two *N. spumigena*-containing slurry's were prepared by mixing the prawn homogenate with an appropriate mass of freeze dried *N. spumigena* biomass to produce a calculated nodularin concentration of either 3 or 10 µg/mL. These calculated concentrations were determined to be appropriate to deliver the nominal doses of 50 µg/kg b.w. and 200 µg/kg b.w. to fish, while allowing for similar volumes of slurry to be injected into fish stomachs for both treatment levels. The control slurry consisted of the prawn homogenate without freeze-dried *N. spumigena* biomass. For details of the volumes of prawn homogenate and freeze dried *N. spumigena* biomass

added to produce the toxic slurry's for Black Bream and sand flathead exposures see Appendix B Table 2.

Experimental procedure

Nodularin was dosed orally as a single delivery of the slurry directly into the stomachs of fish to achieve nominal doses of either 50 or 200 µg nodularin/kg fish for Southern Sand Flathead and 200 µg nodularin/kg fish for Black Bream. Control fish were dosed in the same way as nodularin-exposed fish; however the slurry contained only prawn homogenate. The nominal does given to fish were determined based on those used in similar studies in the wider scientific literature (Platt 2005; Vourinen et al 2009; Kankaanpaa et al 2002) and calculation of the amount of nodularin fish may be exposed to if consuming prawns during blooms in the Gippsland Lakes.

Immediately prior to dosing, fish were anesthetized one at a time in aerated seawater containing AQUI-S (AQUI-S New Zealand Ltd). Under anaesthesia fish were weighed and measured and then the appropriate slurry was then delivered into the stomach via a flexible tube attached to a syringe. The amount to be delivered to each individual was determined on the basis of fish weight and a table that was compiled beforehand (see Appendix B, Table s 3 and 4). The syringe was weighed before and after injection to obtain the exact amount of slurry delivered. Following gavage, fish were placed in a holding tank to recover, thereafter they were returned to their appropriate treatment tank. Figure 2 shows the dosing procedure in Black Bream and the nodularin slurry present in the gut of Southern Sand Flathead 24hrs post dosing.

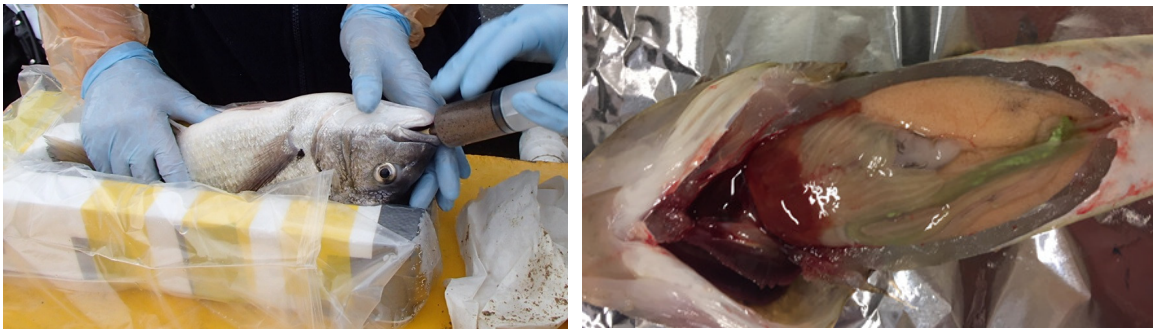

Figure 2: Left: Dosing of Black Bream with control slurry; Right: Southern Sand Flathead showing the toxic *N. spumigena* slurry in the intestine 24hr post dosing.

The experiment started on the 19th October 2016 for Southern Sand Flathead and the 1st November 2016 for Black Bream (day 0), when all fish received a single dose of appropriate slurry via oral gavage. During the experimental period, fish were fed uncontaminated chopped raw prawns three times per week. For both experiments, three exposed fish were sampled 1, 2, 7, 14 and 20 days post gavage. Control fish were sampled on days 1 and 20 for both species. For Black Bream, an additional set of control fish were sampled on day 14.

At each sampling point, fish were euthanized one at a time, and then the spinal cord severed. Each fish was weighed and the total and fork lengths measured. The body cavity was opened, the fish was sexed and the liver, a muscle sample (taken from behind the left pectoral fin) and guts (which included tissues that would be removed upon gutting a fish; heart, kidney, intestine, stomach and gall bladder, herein denoted as "gut") were removed, weighed and kept for nodularin analysis. The experiment was conducted under animal ethics permit number AEC 1613834.1 (University of Melbourne).

Nodularin determination

Frozen liver, guts, muscle tissue samples and *N. spumigena* biomass spiked slurry samples and control slurry samples (ca. 1 g) were extracted in methanol, then washed with hexane and cleaned up by solid phase extraction (SPE). Eluted sample extracts were then dried under a compressed air stream. Dried sample extracts were sent to colleagues at Cawthron Institute, New Zealand, where they were reconstituted and toxin concentrations determined using LC-MS/MS analysis.

Toxin variants assessed included the nodularin variants Nod-R and desmethyl-nodularin-R, and microcystin variants MC-LR and dmMC-LR. No microcystin variants were detected in fish tissue or slurry samples. Nodularin in experimental fish tissues and food slurry is reported as the combination of Nod-R and dmNod-R variants. The detection limit in each sample matrix (liver, muscle or guts) was calculated using the analytical detection limit of 0.05 ng/mL, assuming 1 g of sample was used, incorporating the average mass spectrometer suppression/enhancement effects (for that sample matrix) and the average analyte recovery level observed (for that sample matrix). These are shown in Table 4.

Table 4: Calculated detection limits in the assessed sample matrices, after incorporation of average mass spectrometer ionisation suppression and enhancement effects for nodularin (combination of nodularin-R and desmethyl-nodularin-R) and recovery levels of desmethyl-microcystin-LR.

Fish	Sample Matrix	Matrix Specific Detection Limit (µg/kg)
Black Bream	Food	0.07
	Muscle	0.05
	Liver	0.19
	Gut*	0.27
Southern Sand Flathead	Food	0.07
	Muscle	0.04
	Liver	0.12
	Gut*	0.19

* Guts included tissues that would be removed upon gutting a fish; heart, kidney, intestine, stomach and gall bladder, herein denoted as "gut".

Assessment of recovery and suppression effects

In order to assess the levels of nodularin recovery from the different sample matrices (liver, muscle and guts) and any mass spectrometer suppression/enhancement effects spiking experiments were undertaken. To assess loss of nodularin during sample extraction and clean-up (i.e., nodularin recovery) samples of muscle, liver and gut tissues from Black Bream and Southern Sand Flathead were fortified with nodularin standard prior to extraction. Control samples (unfortified tissues) and dried aliquots of the nodularin fortification material were also prepared to determine the level in the fortification material. Samples were extracted as per the method briefly described above (detailed methods are in Appendix B). These were also sent to Cawthron and analysed using LC-MS/MS.

To assess the different tissue extracts for mass spectrometer suppression/enhancement effects, resuspended extracts of control samples were fortified with a known concentration of Nod-R. These samples were then analysed by the LC/MS-MS method.

Objective 2: Review of current seafood monitoring program for the Gippsland Lakes and those used in seafood monitoring programs in Australia and world-wide

The review of current seafood monitoring programs for the Gippsland Lakes and those used in seafood monitoring programs in Australia and world-wide was undertaken as two separate sections. Firstly, a review of cyanotoxin regulation, management and monitoring in seafood in Australia and overseas was completed. Literature included in the review was sourced from searches of scientific literature using the database Web of Science, review of bibliographies of identified scientific papers, searching of grey literature and the internet using key words such as cyanotoxin monitoring, cyanobacterial bloom management, cyanotoxins in seafood, seafood safety during cyanobacterial blooms, blue-green algal toxins in fish, coupled with inquiries with government agencies involved in the monitoring and management of seafood safety in Australia; i.e. DEWLP, Department of Human Services, State fisheries authorities.

Secondly an evaluation of the Gippsland Lakes "Protocol for managing risks to seafood safety from blue green algal toxins in the Gippsland Lakes" in comparison to other Australian State marine biotoxin management plan documents was undertaken. Information was collected from internet searches and contact with State biotoxin program managers. Sources of information used to conduct the review included: State Shellfish Biotoxin Management Plans and/or monitoring program outlines (see Table 5); Response protocols and operational procedures for Harmful Algal blooms (recreational); Relevant State legislation, and follow-up contact with Shellfish Quality Assurance Program managers via telephone and/or email for clarification and further details.

Table 5: Marine biotoxin management plans from various Australian states used in the review.

State	Marine Biotoxin Management Plan Document title	Year last updated	Author
Victoria	Victorian Marine Biotoxin Management Plan Third Edition	March 2009	Ecowise Environmental
New South Wales	Marine Biotoxin Management Plan NSW Shellfish Program	September 2014	NSW Food Authority
Queensland	Department of Primary Industries and Fisheries Aquaculture Procedure Manual Biotoxin Contingency Plan For Moreton Bay Oyster Industry	2005	Department of Primary Industries and Fisheries, Queensland Government
	Queensland Shellfish Water Assurance Monitoring Program Aquaculture Procedure Manual FAMPM002		
Western Australia	Marine Biotoxin Monitoring and Management Plan 2015	2015	Department of Health, Government of Western Australia
South Australia	South Australian Shellfish Quality Assurance Program Marine Biotoxin Management Plan Version16	September 2014	Biosecurity SA, Government of South Australia.
Tasmania	Tasmanian Shellfish Quality Assurance Program Biotoxin Management Plan Version 3	June 2014	Department of Health and Human Services, Tasmanian Government

In order to provide comparison between practices in Australian state marine biotoxin programs and that of the Gippsland Lakes incident response plan information was summarised in common format to highlight key elements. The subheadings for comparison were as follows:

- Program administration
- Phytoplankton and biotoxin monitoring
- Sampling procedures
- Closure and opening protocols
- Notification procedures
- Triggers for review

At the end of the comparison strengths and weaknesses of the Gippsland Lakes protocol were assessed and comments provided to assist in the process of improvements.

Objective 3: Provide sampling and risk management recommendations, based on scientific and research findings from objectives 1 and 2, to deal with fishing closures and re-opening during bloom events.

The provision of sampling and risk management recommendations to deal with fishing closures and re-opening during blooms was undertaken as two components. Firstly, the scientific and research findings from objectives 1 and 2 were summarised and compared in order to identify risk management recommendations around monitoring and management of seafood safety during toxic *N. spumigena* blooms in the Gippsland Lakes. Secondly meetings were held with government representatives involved in seafood monitoring in the Gippsland Lakes and the commercial fishing industry to disseminate the results of the study, discuss recommendations and develop a work plan to implement strategies into current protocols.

Results

Objective 1: Determination of nodularin accumulation, elimination and tissue distributions in commercially and recreationally relevant species under laboratory and field conditions.

Experiment 1:

These experiments investigating uptake and elimination of nodularin in Black Bream were unsuccessful due to analytical issues, which ultimately led to lower than expected doses of nodularin being delivered to fish and little to no toxin uptake (nodularin dose was 2-12 µg/kg b.w. instead of 50-200 µg/kg b.w). Further details of the analytical issues can be found in Appendix A, while a summary of the results are provided below.

There was no uptake of nodularin toxin observed in any Black Bream tissues during this experiment. This result was unexpected based on the nominal concentrations expected to be dosed into the fish. Further investigation of these results showed that the measured concentrations of nodularin in the *N. spumigena* contaminated slurry's, used for gavaging fish, were orders of magnitude less than the nominal concentrations (Table 6). Based on actual measured concentrations of nodularin in the slurry's, rather than receiving doses of 50 and 200 µg nodularin /kg b.w., fish received a dose of between 2-5 µg/Kg b.w. and 8-12 µg/Kg b.w. for the 50 and 200 µg/kg b.w. doses respectively. These concentrations would not be expected to result in uptake into the fish (Myers et al 2010), which is consistent with the observations made during this study.

Table 6: Nominal and measured concentrations of nodularin in fish food slurry (as determined by ELISA (n=3) and LC-MS (n=2)).

Dose in fish (µg/kg)	Expected Nodularin concentration (µg/Kg)	Measured Concentrations	
		LC-MS (CV%) (µg/Kg)	ELISA (CV%) (µg/Kg)
Control (0)	0	0	0
50	167	6 (0)	15.5 (28.5)
200	667	27 (5.4)	39.2 (32.8)

Experiment 2:

Experiment 2 was successful in dosing two recreationally and commercially relevant fish species, Black Bream and Southern Sand Flathead, at nodularin levels which they could encounter in their food sources during toxic blooms in the Gippsland Lakes and therefore providing knowledge on the uptake, accumulation, tissue distribution and depuration of nodularin in these species.

Species-specific differences were observed in the uptake, accumulation and elimination of nodularin following a single oral dose, however not for tissue distribution. In both Black Bream and Southern Sand Flathead the primary tissue of nodularin accumulation was the liver (ca. 80% of toxin), with only a small amount accumulated into the muscle tissue (ca. 2.3 % for Black Bream and 4.5 % in Southern Sand Flathead). Around 14% of nodularin was detected in the guts. However, as the gut included tissues that would be removed upon gutting of a fish (i.e.: heart, kidney, intestine, stomach and gall bladder) the toxin concentrations detected will be a culmination of the toxic slurry dosed into the fish stomachs and toxins being removed by the fish.

Uptake of nodularin differed between the two species. Nodularin uptake in Southern Sand Flathead was 1.41% of the dosed amount, while in Black Bream was only 0.53%. Concentrations of nodularin in the liver of Southern Sand Flathead were an average 2.6 times greater than those detected in Black Bream livers, even though they received a slightly lower dose of nodularin (Table 7). Even greater differences were observed in concentrations of nodularin in muscle tissues between species, with an average 8.6 times more nodularin being detected in Southern Sand Flathead muscle compared to that in Black Bream (Table 7).

Both Black Bream and Southern Sand Flathead accumulated nodularin to levels that exceeded the Victorian health alert guideline value of 24 µg nodularin/kg for whole fish (Mulvenna et al 2012). However, exceedance was due to the concentrations of toxin in the liver tissues of both species (maximum liver concentrations: Black Bream 24.54 µg/kg w.w; Southern Sand Flathead 35.92 µg/kg w.w.; Table 7). Concentrations of nodularin in muscle tissues were well below recommended health alert levels (maximum muscle concentrations: Black Bream 0.62 µg/kg w.w; Southern Sand Flathead 4.35 µg/kg w.w.; Table 7).

Table 7: Concentrations of nodularin (as a combination of nodularin-R and desmethyl-nodularin-R) detected in muscle and liver tissues of Black Bream and Southern Sand Flathead following feeding with *Nodularia spumigena* contaminated slurry. * Mulvena et al (2012). N = 21 for Black Bream. N = 16 for Southern Sand Flathead.

Species	Dose administered (µg/kg b.w.)	Tissue	Nodularin concentration (µg/kg w.w.)	
			Max.	Mean (±S.E.)^
Black Bream	869	Liver	24.54	4.11 (1.14)
		Muscle	0.62	0.07 (0.03)
		Nodularin uptake (as a % of dose given)	3.17	0.53 (0.15)
Southern Sand Flathead	776	Liver	35.92	10.84 (2.23)
		Muscle	4.35	0.6 (0.32)
		Nodularin uptake (as a % of dose given)	4.5	1.41 (0.34)
***Health Alert Guideline for Fin-fish (whole fish)**			24 µg/kg w.w.	

^mean is the concentration of nodularin in fish tissues sampled 1, 2, 7, 14 and 20 days post gavage.

Accumulation of nodularin into liver and muscle tissues of Black Bream and Southern Sand Flathead occurred within 24-hrs of exposure; however the accumulation rates and patterns differed thereafter (Figure 3). Southern Sand Flathead rapidly accumulated nodularin into their livers, reaching peak concentrations 24rs post exposure, while in Black Bream nodularin accumulation was much slower reaching peak concentrations at 14 days post exposure. In muscle tissues the opposite was observed with nodularin accumulation occurring more rapidly in Black Bream, reaching peak concentrations 2 days post exposure, while in Southern Sand Flathead it was 7 days post exposure before concentrations in muscle tissues peaked.

Depuration of nodularin from Black Bream tissues occurred slowly from the muscle, while rapidly from the liver. In Southern Sand Flathead nodularin was rapidly depurated from the muscle, however slowly from the liver. By 20 days post exposure no nodularin was detected in muscle tissues of both fish species and low concentrations were detected in the livers (Figure 3).

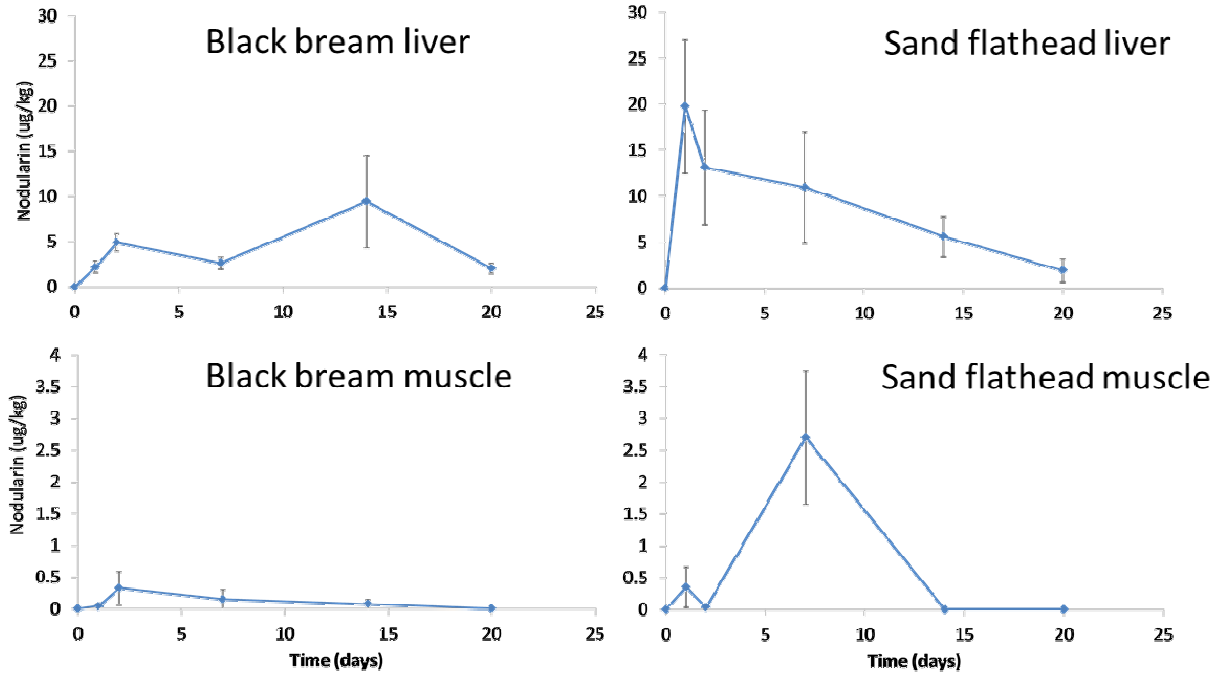

Figure 3: Accumulation and elimination of nodularin (as a combination of nodularin-R and desmethyl-nodularin-R) in Black Bream and Southern Sand Flathead tissues following exposure to average doses of 869 µg/kg b.w. and 776 µg/kg b.w. respectively. Data are the mean (Black Bream n = 4; Southern Sand Flathead n = 3) and error bars = ± standard error.

Dose-dependent differences in nodularin uptake, accumulation and depuration were also observed in the Southern Sand Flathead, however not in tissue distribution. The primary tissue for nodularin accumulation was the liver, regardless of the concentration of nodularin given to fish. Nodularin uptake, as a percentage of the dose given was greater in fish exposed to a lower dose (1.91% for fish dosed at 106 µg/kg b.w. compared to 1.41% for fish dosed at 776 µg/kg b.w.), however a higher dose resulted in higher nodularin concentrations in fish tissues (Table 8). Southern Sand Flathead that received a dose of 106 µg/kg b.w. accumulated an average 1.76 µg/kg w.w. and 0.11 µg/kg w.w. nodularin into their liver and muscle tissues, respectively, concentrations which were ca. 5.5 times lower than those accumulated into fish dosed at 776 µg/kg b.w (average nodularin concentrations in liver and muscle 10.84 µg/kg w.w. and 0.6 µg/kg w.w., respectively; Table 8).

Unlike the rapid accumulation of nodularin into livers of fish dosed at 776 µg/kg b.w., accumulation of nodularin in livers of fish exposed to 106 µg/kg b.w. was much slower, peaking 14 days post exposure (Figure 4). In muscle tissues, maximum nodularin accumulation occurred 7 days post exposure in fish given a dose of 776 µg/kg b.w., but not until 20 days post exposure in fish dosed at 106 µg/kg b.w. (Figure 4).

While in fish dosed at 776 µg/kg b.w. nodularin elimination was observed and little to no toxin was detected in liver and muscle tissues by day 20, in fish given a dose of 106 µg/kg b.w. nodularin was still detected in both tissues 20 days post exposure and little elimination had occurred (Figure 4).

Table 8: Concentrations of nodularin (as a combination of nodularin-R and desmethyl-nodularin-R) detected in muscle and liver tissues of Southern Sand Flathead following feeding with *Nodularia spumigena* contaminated slurry at two dose levels. * Mulvena et al (2012). N = 16 fish in each treatment for Southern Sand Flathead.

Species	Dose administered (µg/kg b.w.)	Tissue	Nodularin concentration (µg/kg w.w.)	
			Max.	Mean (±S.E.)^
Southern Sand Flathead	776	Liver	35.92	10.84 (2.23)
		Muscle	4.35	0.6 (0.32)
		Nodularin uptake (as a % of dose given)	4.5	1.41 (0.34)
	106	Liver	8.76	1.76 (0.52)
		Muscle	0.9	0.11 (0.06)
		Nodularin uptake (as a % of dose given)	7.45	1.91 (0.49)
***Health Alert Guideline for Fin-fish (whole fish)**			24 µg/kg w.w.	

^mean is the concentration of nodularin in fish tissues sampled 1, 2, 7, 14 and 20 days post gavage.

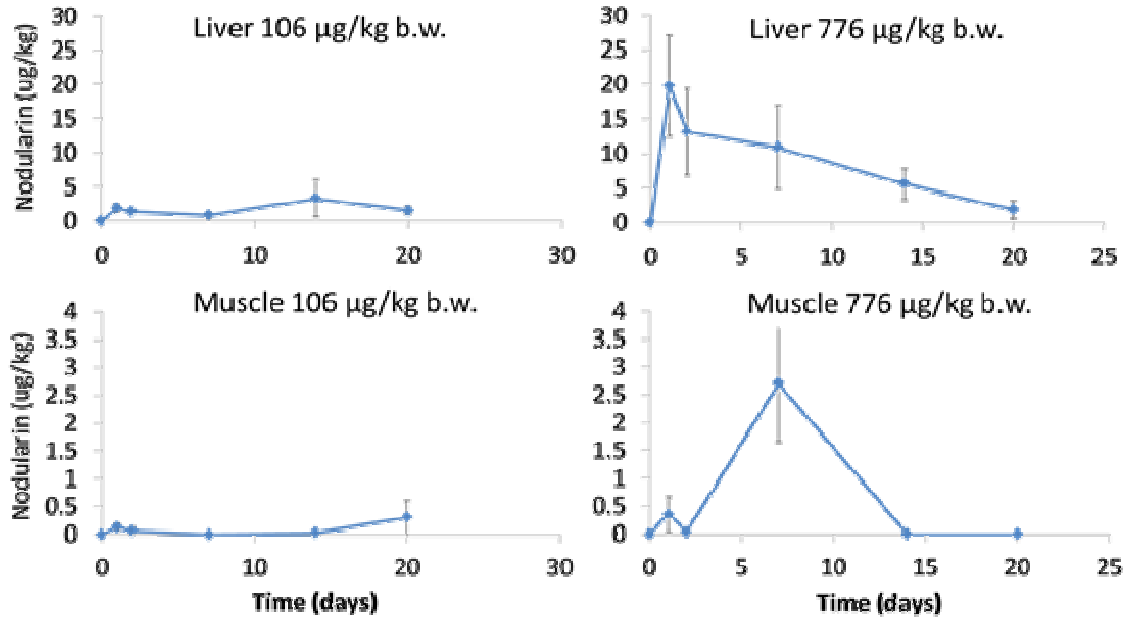

Figure 4: Accumulation and elimination of nodularin (as a combination of nodularin-R and desmethyl-nodularin-R) in Southern Sand Flathead tissues following exposure to average doses of 106 µg/kg b.w. or 776 µg/kg b.w.. Data are the mean (n = 3) and error bars = ± standard error.

Analytical considerations

Determining and particularly quantifying the presence of cyanotoxins in seafood requires appropriate analysis of the toxin concentrations in tissues. This is not always straightforward with differences in sample processing, e.g.: extraction procedures, use of sample clean-up, and determination methods (HPLC, LC-MS/MS, ELISA, PPIA), all impacting the final toxin quantification. High accuracy is required for reliable results, especially when concentrations are to be used in risk assessment and the

provision of advice around seafood safety. The experiments conducted as part of Objective 1 have shown the importance of good quality assurance and quality control in analytical methods to obtaining high quality and reliable data. Experiment 1, undertaken to assess nodularin uptake and depuration in Black Bream, was unsuccessful due to analytical issues. During these experiments an analytical technique known as ELISA was employed to determine the nodularin concentrations in algal cell biomass. This method of analysis resulted in an over estimation of the nodularin concentration in the cell biomass, which ultimately led to an under dosing of nodularin into the toxic slurry given to fish. It was discovered during these initial experiments that there are a growing number of reports indicating that the ELISA kit used in the study provided unreliable estimates of toxin concentrations. As a result, the use of ELISA was not recommended and we sought the use of an LC-MS-MS method for follow up experiments.

While LC-MS-MS is considered one of the most reliable methods for the determination for nodularin, as part of experiment 2, it was identified that there still needs to be an appropriate level of QA/QC maintained. A number of experiments and steps were included in the sample processing and analytical determination methods in order to assess the reliability and quality of the data obtained. These included spike recoveries, to assess sample loss during processing, and fortification of extracted samples to assess mass spectrometer suppression/enhancement effects (see Appendix B for full details of these processes). Severe mass spectrometry suppression effects were observed for nodularin-R, during QA/QC assessments. The level of suppression/enhancement was dependant mostly on tissue type, with smallest effects observed in muscle tissue and gut samples the greatest, but to some degree on fish species. Mass spectrometer suppression effects for nodularin were in the range of 8-12%, 12-55% and 80-92% for muscle, liver and gut tissues respectively. Previous levels of mass spectrometer enhancement effects (e.g.: +20% to +30%) have been detected using the LC-MS/MS method used during this study in muscle samples from New Zealand shortfin eels (*Anguilla australis*; Dolamore et al 2017). Similar studies published in the wider literature have also reported mass spectrometer suppression for nodularin of 38% for fish liver samples (Karlssonn et al 2005) and -10 to +40% for liver and muscle samples of roach and flounder (Sipia et al 2006). Cadel-six et al (2014) reported low mass spectrometer effects for microcystin in intestines (+1%) and muscle (+15%) tissues of rainbow trout, however higher levels for livers (+23%).

Recovery experiments are important quality assurance and quality control, allowing assessment of losses of toxin during sample extraction and processing. Recovery of nodularin from tissues in the current study ranged 56-82% for liver tissues, 48-80% for muscle and 61-113% for gut. These are in the range of recoveries published in the wider literature for nodularin in liver (28-84%) and muscle (40-84.9%) (Stewart et al 2012; Van buynder et al 2001; Karlsson et al 2003; Sipia et al 2007). While the nodularin recoveries were in the range of those reported in the wider literature, our study found that they varied significantly between species and tissues. Due to this variability, together with high mass spectrometer suppression effects observed for nodularin, we employed an internal standard, dmMC-LR, into every sample. This level of QA/QC has not been reported in the scientific literature, in fact very few studies report any sort of recovery assessments, let alone account for them in provision of their results. Recovery of dmMC-LR, the toxin used as an internal standard in the current study, ranged from 25-30% in livers, 59-66% in muscle. Unfortunately, dmMC-LR could not be measured in the gut samples as the sample matrix severely affected the chromatography for this compound. Recovery of dmMC-LR from fish tissues has been reported to range from 28-75% in liver and 35-76% in muscle in the wider literature (Ni et al 2017; Karjalainen et al 2008; Cadel-six et al 2014; Stewart et al 2012).

High variability in nodularin tissue concentrations was observed between individual fish. This has been frequently reported in studies investigating cyanotoxin uptake in fish under field conditions (Karjalainen et al 2008; Kankaanpaa et al 2005; Sipia et al 2006; Jia et al 2014; Acou et al 2008), and has been observed in laboratory studies as well (Paakkonen et al 2008; Cazenave et al 2005). Variability in toxin concentrations between individuals could be due to differences in metabolism and feeding habits (Jia et al 2014), or in studies where not reported and accounted for, due to differences in recovery between tissues and species. It could also be due to an inadequate number of samples. For the

current study, it is unlikely it's related to methods, as a high level of QA/QC was applied during sample processing and analysis; or due to differences in feeding habits of fish as the fish were all administered a dose in the same manner. It is most likely due to differences in metabolic rates of individual fish and/or low sample numbers.

The sample processing and analytical QA/QC conducted as part of this study indicates the need to ensure appropriate controls are included in sample processing and analysis steps in order to have high reliability data. Otherwise over- or under-estimations of the concentrations of toxins in tissues could occur, which could have significant implications for providing advisories regarding seafood safety. At minimum recovery experiments should be conducted on each tissue matrix being examined and run with each sample batch. The inclusion of an internal standard in every sample is the only way to fully evaluate toxin loss during processing and provide the highest level of reliability in data obtained. Further addition of a greater number of replicates would potentially reduce variability in results. The results from the present study demonstrate why performing the analysis without any quality control measures is not recommended.

Objective 2: Review of current seafood monitoring program for the Gippsland Lakes and those used in seafood monitoring programs in Australia and world-wide

Section 1: A review of cyanotoxin regulation, management and monitoring in seafood in Australia and overseas

Section 1 of the literature review provided an understudying of current regulation, management and monitoring applied both within Australia and worldwide for cyanotoxins in seafood. A summary of the results from this review are provided below, while the full review is provided in Appendix C.

In Australia and worldwide there are far fewer regulations for exposure via food as compared to exposure via drinking water and recreation. The primary focus for exposure risks to algal toxins are based on marine seafood, where there are well established protocols implemented to monitor and manage risks both in Australia and globally. Key to being able to assess and manage the risks of cyanotoxins to human health from consumption of seafood is the development of guidelines, standards and monitoring and management procedures. These standardised guidelines or procedures address the concentrations of cyanobacteria or their toxins that should not be exceeded and generally provide immediate, short-term actions to take if the concentrations are exceeded in order to prevent or minimize exposure to harmful cyanobacteria or their toxins. In Australia there are no national guidelines on safe levels of cyanotoxins in freshwater seafood at a national level (with the exception of saxitoxins in bivalve shellfish). The State of Victoria, however, have developed health alert levels, which are the basis to provide advice around commercial and recreationally harvested fish, shellfish, mussels and molluscs safety during cyanobacterial blooms (Table 9). The State of New South Wales (NSW) has adopted the Victorian Health alert trigger values as an interim measure to provide advice around seafood safety during cyanobacterial blooms in NSW (NSW Food Authority 2012). Similarly, the international development of guidelines and procedures to manage risks of cyanotoxins in freshwater seafood is limited. Only three countries have developed and apply guideline values for cyanobacterial toxins in seafood, the USA, Denmark and France (Table 10).

Unlike marine biotoxins, most countries, including Australia, do not have protocols in place to manage risks from cyanotoxins in freshwater seafood. Well established frameworks are available to guide government agencies and industry in the development and undertaking of monitoring programs that will meet legislative requirements, limit risks from marine biotoxins and provide confidence in marine seafood products during toxic blooms. Denmark is the only country to routinely monitor and manage risks from cyanotoxins in shellfish (Christoffersen and Warming 2012). While in Australia, Victoria is the only state to have a contingency plan in place to monitor cyanotoxins in seafood during toxic blooms in a lake frequented by cyanobacterial blooms and being a significant commercial and

recreational fishery (Stewart et al. 2012). There is growing awareness of the risk from cyanobacterial toxins, with an increasing number of health authority reports identifying that there is evidence from around the world to suggest that risk exists and that without monitoring programs the risk is potentially significant (Scottish Government 2012; Higman et al. 2014). However, in most countries, including Australia the risks posed by cyanotoxins in seafood are not fully understood and routine or event based monitoring to provide advice around safety of consumption during toxic bloom events is not conducted or occurs in limited cases.

Table 9: Health Guideline values for cyanobacterial toxins in seafood for Victoria (From Mulvenna et al. 2012).

Toxin	Health guideline value (µg/kg whole organism)		
	Fish	Prawns	Mussels or Molluscs
Microcystin-LR^ or equivalent toxins, i.e. Nodularin	24	32	51
Cylindrospermopsin and deoxy-cylindrospermopsin	18	24	39
Saxitoxins	800	800	800

^ due to lack of suitable standards and adequate comparative toxicity data, the recommendation of the Australian Drinking Water Guidelines is to treat all microcystins as having equivalent toxicity to microcystin-LR. The guideline value therefore represents the sum value for all detectable microcystins and nodularin

Table 10: Guidance values and other regulations or recommendations for managing cyanotoxins in seafood (adapted from Ibelings et al 2014).

Country	Management framework	Toxin	Guideline or trigger values	Reference
Australia (Victoria & NSW)	Guidelines applied in regulation of commercial and recreational harvest	Microcystins and Nodularin	24-51 µg/kg whole organism	Mulvenna et al. 2012
		Cylindrospermopsin	18-39 µg/kg whole organism	
		Saxitoxins	800 µg/kg whole organism	
USA (California)	Guidelines applied on a voluntary basis by local, regional, State and tribal entities	Microcystins	10 µg/kg w.w.	Butler et al., 2012
		Cylindrospermopsin	70 µg/kg w.w	
		Anatoxin-a	5000 µg/kg w.w	
USA (Ohio)	Guideline to do not eat fish.	Microcystins	28µg/kg fish fillet	Illinois EPA 2012
USA (Illinois, Kansas)	Guidelines to only eat fish if gutted and gilled. Do not eat shellfish.	Microcystins	20µg/L in water	Illinois EPA 2012
Denmark	European regulations on food hygiene and control of products of animal origin	No specific guideline values for cyanotoxins. No harvesting of shellfish if algal toxins of any type detected in water or flesh.		Christoffersen and Warming 2012
France	French Agency for Food Safety opinion	Microcystins	Exposure to toxins in water and fish: 5.6 µg/kg (adult) 1.4µg/kg (child) Exposure via fish consumption only: 28µg/kg (adult) 7µg/kg (child)	Arnich 2012
		Saxitoxins	800 µg/kg	

Section 2: Review of current monitoring program for providing seafood safety in the Gippsland Lakes and comparison with marine biotoxin management programs in Australia

This section of the review provided a comparison of the current protocol applied in the Gippsland Lakes to provide seafood safety with those applied across Australia and internationally for marine biotoxin monitoring and management. It provided an understanding of the nationally and internationally recognised practices for seafood monitoring programs which allowed identification of a number of key strengths and weaknesses in the Gippsland Lakes incident response plan and therefore provision of recommendations to improve the programs design and implementation. These results from this review are provided in summary below, however for the full evaluation see Appendix C.

Key strengths of the protocol include the derivation and application of health alert guidelines to base advice around seafood safety during blooms and the existing legislative powers to restrict or prevent the harvesting of seafood if levels of toxins in tissues exceed health alert values and pose a threat to public health are in place. A further strength to the program is the surveillance strategy being based on routine phytoplankton monitoring combined with cyanotoxin testing in seafood. When potentially toxic algal species are present above a specified "threshold levels" in water, the levels of toxins in seafood are determined; with toxin concentrations in seafood tissues being the trigger for providing advice around seafood harvesting.

A number of key weaknesses were identified in the Gippsland Lakes incident response plan. One in particular, considered as being significant to implementation and conduction of a comprehensive plan, is the lack of funding for event based monitoring. This has been identified in a number of food safety risk assessments of the seafood industry in Victoria (MWH 2014). Funding for event response has been an ongoing issue over the years, with government and industry all trying to relinquish this responsibility. There are a number of stakeholders involved in bloom response and the provision of seafood safety in the Gippsland Lakes. These include government agencies, recreational and commercial industry. The funding of an incident response and monitoring should not be the responsibility of one agency or industry. The costs of the program need to be shared by all users, enhancing the coverage of monitoring information, and reducing the direct cost for any one stakeholder. There needs to be commitment and support from all stakeholders, and in particular between health agencies and industry, but not excluding recreational and tourism industries.

A further key weakness identified in the current protocol is the lack of objective scientific data for the use of Black Bream as a representative sentinel species for all species harvested in the Gippsland Lakes. When using a sentinel species to provide information regarding the toxin risk of other nearby species, it is imperative that the indicator species be relatively sensitive and accumulates toxins more efficiently than other species during algal bloom events (McLeod, 2014). However, a potential problem with indicator species is that uptake and elimination rates of toxins may vary between species, and spatial variation in occurrence of toxin producing algal blooms can occur over very small geographical scales leading to differences in exposure between species. In view of these potential issues, it is necessary to evaluate the appropriateness of particular species as indicators through assessment of a range of species toxin uptake and depuration rates (McLeod, 2014). Regulatory monitoring can greatly benefit if toxin uptake and depuration patterns are known for each species and each toxin, however without this information the risk of differences in species accumulation rates leading to incorrect advisories are high. Scientific research is needed to support a basis for choosing a sentinel species. It is recommended that research in the use of sentinel species or samplers be undertaken to strengthen the scientific basis for policy decisions regarding which indicator species are appropriate to use in the event based monitoring program.

Aside from the lack of funding and the issues with the sentinel species, comparison of the Gippsland Lakes incident response protocol with Australian state marine biotoxin monitoring and management plans, which are developed based on guidance from the ASQAP (2009) operations manual, indicated that there was a number of other key elements and considerable details concerning cyanotoxins monitoring and management lacking. In particular details in relation to the resources managed under the protocol, the roles and responsibilities of industry in the protocol, sampling sites, sample collection and transport methods, phytoplankton triggers used to initiate seafood sampling, closure and opening criteria (and guidelines for their application), methods of communication and notification, laboratories for phytoplankton and toxin analysis, sample turnaround times were either missing or not clearly documented. Ideally, the incident response plan should be a stand-alone document that contains all necessary information to enable appropriate cyanotoxin contingency arrangements to proceed efficiently.

The following provides more detail around the general issues identified with the current incident management plan.

- **What are the seafood resources managed under the incident response plan?** - There needs to be information included in the plan that details the scope of the resources managed; e.g. recreational gathering and commercial wild harvest. Further details of the area that will be managed by the protocol and seafood species need to be documented.
- **The roles and responsibilities of stakeholders** - In order to provide a co-ordinated and efficient response, roles and responsibilities of all stakeholders need to be defined and documented. In particular, the role of industry is not documented in the current protocol. This has been an issue in the Gippsland Lakes protocol identified as far back as 1996 (Norman, 1996).
- **Communication and notification section is unclear** - Clear and open communication networks need to be established and documented in the response plan. Notification protocols are provided but no actual contact details are listed for any individual or agency and details of the methods of communication/notification are not provided (e.g. sms, phone, fax, email). Details of proformas for laboratory requests and notification templates for advisories and public warning signs should be provided together with timeframes for providing appropriate notifications.
- **Lack of detail regarding analytical services** - There are no details of suitably qualitied laboratories for processing and analysis of phytoplankton and cyanotoxins documented in the protocol. A list of the appropriately accredited (e.g. National Association of Testing Authorities (NATA) as a minimum) laboratories, their methods of analysis, phytoplankton identification and enumeration and toxins analysis capabilities and detection limits and sample turnaround times needs to be provided in the protocol. Contact details and any special sample requirements should also be detailed.
- **Lack of detail regarding sample collection and handling** - Sample collection and handling details need to be better defined to ensure consistency in sampling methods and provide quality assurance and quality control in their collection, processing, analysis and reporting. The current methods documented are ad hoc and would lead to inefficiencies in sample collection and analysis. Documented procedures need to be included, such as, who will collect samples, when, how, where and what samples will be collected. Details of how the samples are to be handled and transported once collected so as to preserve them for analysis needs to be described.
- **Phytoplankton monitoring methods are not described** - Details of the phytoplankton monitoring program used to provide early warning of the onset of a toxic cyanobacterial bloom in the Lakes and to initiate seafood monitoring needs to be provided. Phytoplankton trigger levels used to initiate seafood sampling needs to be defined together with sample frequency and methods (e.g. defining how samples are collected and processed).
- **Criteria for provision of advisories around seafood harvesting are not readily apparent and guidelines for their application are mixed with incident notification procedures** - The criteria for providing an advisory to restrict harvesting of seafood during a toxic bloom are currently not readily apparent. A clear outline of the criteria used to provide advisories and the mechanisms for providing advisories needs to be documented. Advisories to restrict harvesting of commercial and recreational seafood in the Gippsland Lakes are based on a single criterion in the current plan: "A health guideline value for toxin in any one or more of prawns, mussels or fish is reached". At this point the Department of Health will issue advice to PrimeSafe of a need to inform commercial licensees to cease harvesting. Considerations for providing advisories based on criteria around cell concentrations of a toxic algal species exceeding action levels, and the cell concentration levels that could initiate a closure pending the results of toxin testing of seafood tissues should be included in the protocol. Further closure criteria should be added based on the reporting of human illness fitting case definitions for hepatotoxin or neurotoxin exposure. Case definitions for hepatotoxin and neurotoxin exposure should be

included in the protocols as well. Procedures for mechanisms to provide advisories should also be clearly documented and timeframes around providing advisories outlined.

- **Complementary re-opening criteria matching the closure criteria are required** - The current details on lifting advisories require some amendment. It needs to be stated that the lifting of advisories can only occur when certain toxin criteria are satisfied. For all toxins, concentrations should be below the relevant regulatory limit in two consecutive samples collected over a minimum period time; e.g. 14 days. Additional criteria should be provided based on the absence or reduction in the abundance of the causative toxic algal species to cell concentrations below prescribed threshold or 'action' levels, and criteria based on the absence of any seafood poisoning reported since the date of the first "clear" sample. Guidelines concerning the application of the criteria should also be documented.
- **Annual reviews of the protocol are required** - The Gippsland Lakes protocol assessed in the review was dated from September 2011. Annual reviews are needed to re-assess the phytoplankton and biotoxin monitoring strategy, to evaluate the efficacy of management procedures and inter-agency communications during the most recent toxic bloom events and incorporate any important variations made to standard operating procedures. Any changes should be documented as an addendum to the Management Plan and formally inserted into the Plan during annual reviews.

Objective 3: Provide sampling and risk management recommendations, based on scientific and research findings from objectives 1 and 2, to deal with fishing closures and re-opening during bloom events.

Sampling and risk management recommendations, determined based on scientific and research findings from objectives 1 and 2 are based around the incident response plan, market access during harvesting restrictions and further research. These are detailed below.

Recommendations around the incident response plan

Develop an agreement for incident response funding

It is recommended that an appropriate cost sharing agreement be developed between government agencies and commercial and recreational industries, that will allow for provision of adequate resources to cover seafood testing, monitoring and management in the event of a toxic bloom. Funds need to be available so that they can be rapidly deployed in the event of a bloom. Clearly defining the roles and responsibilities of government agencies and industry, both commercial and recreational, could assist in determining equitable funding contributions.

Adopt a clear and comprehensive algal bloom incident management plan for seafood

There is a need to adopt a clear and comprehensive algal bloom response plan to provide seafood safety during toxic algal blooms in the Gippsland Lakes. The incident response plan should have industry support, scientific input and direction from State and local Government agencies. Recreational and commercial industry members should be encouraged to play an active role in the development and implementation of the plan. The plan needs to be a stand-alone document that contains all necessary information to enable immediate deployment of cyanotoxin contingency arrangements if required.

There are well-established biotoxin programs for marine shellfish which provide guidance for the monitoring, management and response to toxic marine algal blooms. Each Australian State and Territory that allows commercial shellfish harvest has a biotoxin management documenting the procedures for routine and incident response monitoring and management. State plans developed based on guidance from the Australian Shellfish Quality Assurance Program Manual (the Manual). This

manual comprises the procedures and administrative practices that, if adhered to, enable food safety programs to comply with the Food Standards Australia New Zealand Food Standards Code and Export Orders, including the schedule to Standard 4.2.1 of the Food Standards Code. The ASQAP manual is further noted in the Food Standards Code as the "National guideline for managing risks in the harvesting, relaying, depuration and wet storage of shellfish". It is recommended that in development and adoption of a clear and comprehensive incident response plan for the Gippsland Lakes that the concepts and procedures outlined in the most recent ASQAP manual and Australian state marine biotoxin monitoring and management plans be followed. This will make sure the plan complies with the appropriate food standards codes and that key control measures are in place and can be assessed for reliability.

Specific recommendations addressing weaknesses identified in the Gippsland Lakes response plan following review with national and international marine biotoxin management plans are detailed below:

Seafood resources - Details of the scope of the resources managed; e.g. recreational gathering and commercial wild harvest, the species (commercial and recreational), and areas that are managed under the protocol needs to be document in the response plan.

Roles and responsibilities - The roles and responsibilities of stakeholders (government agencies and industry) needs to be clearly defined and written in the plan. At the very least the role of industry needs to be documented in the current protocol.

Communication - Clear and open communication networks need to be established and written into the response plan. Contact details for representatives from government agencies, commercial and recreational industry and any other relevant parties need to be provided in the plan and kept up-to-date. Notification protocols needs to be clearly identified and documented together with provision of notification templates for advisories and public warning signs. Details of the management and storage of data on phytoplankton monitoring, cyanotoxin testing, related environmental data and any case investigations need to be written into the plan

Laboratories and analytical services – Details of the suitably qualitied laboratories for processing and analysis of phytoplankton and cyanotoxins need to be written in the protocol. The methods of analysis, phytoplankton identification and enumeration and toxins analysis capabilities and detection limits, sample turnaround times and reporting requirements need to be documented in the protocol. Contact details and any special sample requirements need to be detailed.

It is recommended that agreements be put in place with an appropriately qualified laboratory in Australia that can perform testing for all cyanotoxins covered by the protocol. The laboratory should be at minimum NATA accredited for the methods of analysis required in the response plan. Currently there are no laboratories in Victoria with the capabilities for cyanotoxins testing in seafood. In developing an agreement consideration should be made regarding how samples may be transported to that laboratory. It may be that agreements with freight companies also need to be made.

Sample collection and handling - Sample collection and handling details need to be better defined to ensure consistency, quality assurance and quality control in their collection. Clear details of sample collection and handling procedures including who collects samples, when, how, where and what samples will be collected need to be written in the protocol. Handling and transport procedures need to be clearly detailed. If a freight company is to be used for sample transport contact details need to be documented in the protocol.

Phytoplankton monitoring methods - Details of the phytoplankton monitoring program used to provide early warning of the onset of a toxic cyanobacterial bloom in the lakes and to initiate seafood monitoring need to be documented in the protocol. Phytoplankton trigger levels used to initiate seafood sampling, together with sampling frequency and methods need to be written in the protocol.

Closure and opening criteria – The plan needs to include clear definitions of closure and re-opening criteria for all cyanotoxins covered by the plan. Both sets of criteria and guidelines for their application need to be clearly documented in the plan. Currently criteria for provision of advisories around seafood harvesting and guidelines for their application are not readily apparent.

Protocol review - The Gippsland Lakes incident response plan for seafood should be kept up to date and reviewed annually to ensure it is effective and reflects current operating procedures. It is recommended that following an incident an audited of the plan be undertaken and any new knowledge or identified improvements be incorporated. The updated plan should then be sent out to all stakeholders.

Recommendations around market access during harvesting restrictions

Retain the 'gut and gill' policy, but incorporate details around its implementation into the incident response plan - The scientific literature and laboratory study conducted during this project suggest that removal of gut tissues significantly reduces risk of exposure to nodularin concentrations in exceedance of Health Alert Levels. Marketable tissues of finfish during blooms therefore could include muscle, as long as the internal organs are removed and the gutted fish is washed in clean water before sale. It is recommended that a gut a gill policy for finfish be included in the incident response plan. However, as there is a lack of scientifically robust data on the accumulation, tissue distribution and elimination of nodularin in the range of species harvested from the Gippsland Lakes, or else-where in Australia, and there is potential for nodularin accumulation to be greater under extended exposure, it is recommended that decisions to implement a gut and gill policy be based on analytical confirmation of toxin levels in fish tissues.

Investigate other available market opportunities during blooms - In NSW, the commercial industry divert catches to bait during toxic cyanobacterial blooms (NSW Food Authority 2012; 2017). It is recommended that an investigation into other potential market options for continued sale of fish during blooms, such as diversion of catch to bait, be undertaken. This could provide options for market access during periods where harvesting restrictions are enforced.

Develop an education campaign - Education and understanding of cyanotoxin management in relation to seafood is vitally important in promotion of safe seafood during toxic blooms. It is recommended that educational material be developed, that can be included in media communications during toxic blooms and marketing to inform consumers about the safety of commercially harvested seafood, and how it undergoes toxin testing prior to sale. This would provide greater confidence in commercial product.

Recommendations around Research

Further investigations around sentinel species - In order to choose an appropriate sentinel species, we require further understanding of the levels of nodularin, in a wider range of commercially and recreationally relevant fish samples for the Gippsland Lakes. Currently two papers exist in the scientific literature on the accumulation of nodularin in seafood during *N. spumigena* blooms in the Gippsland Lakes (Eaglesham et al 2002; Van Buynder et al 2001). These papers provide limited information on the levels of nodularin in finfish species during blooms in 1999-2001. The current study has provided further knowledge of nodularin accumulation and depuration in two species of significance, however there are over 16 key finfish species of commercial and recreational relevance in this region (Conron et al 2016; Department of Primary Industries 2012). Studies in the wider literature have shown the accumulation of nodularin to elevated levels exceeding health alert levels in eels (Dolamore et al 2017) and mullet (Stewart et al 2012) which show that you need an understanding of all species before risk can be fully evaluated.

Both laboratory and field studies are needed to understand the mechanisms of nodularin toxicity in fish and risks to human health. Larger, higher-frequency sample sets, spanning multiple years and

investigating multiple fish species are recommended before having confidence in the choice of sentinel species. As much information as possible should be collected during a toxic bloom event, and should include not only phytoplankton and toxin monitoring data, but also environmental data.

Research into the management of algal blooms – The best way to reduce the occurrence of nodularin in seafood is to control the development of blooms, as less or no nodularin would be accumulated into seafood. While *N. spumigena* is a natural component of phytoplankton communities in the Gippsland Lakes, and there are anecdotal reports of cyanobacterial blooms in the Gippsland Lakes prior to 1886 (Holland et al 2013); nevertheless, their frequency and intensity has increased over the last few decades. It is well established that anthropogenic enrichment of water bodies with nutrients is associated with increases in bloom occurrences worldwide (O'Neil et al 2012; Sotton et al 2015). A more sustainable approach to reducing the risks of exposure to cyanotoxins would be to control anthropogenic inputs. It is therefore recommended that there be support for further research in managing and predicting the occurrence of algal blooms.

Discussion

The objectives of this project were threefold: (1) to assess nodularin uptake and elimination in commercially and recreationally relevant finfish species for the Gippsland Lakes, (2) to review and compare the current monitoring protocols with those used nationally and internationally, and (3) to develop management recommendations to deal with harvesting opening/closing during toxic cyanobacterial blooms.

In addressing the first objective, two commercially and recreationally relevant finfish species, Southern Sand Flathead and Black Bream, were examined under laboratory conditions to answer questions relating to:

- Nodularin elimination;
- Time required to reduce nodularin levels in tissues to below health alert values;
- Nodularin tissue concentrations and distributions in other commercially relevant fish species;
- Recommendations relating to the marketable parts of the fish during blooms; and
- Management recommendations for sampling regimes and appropriate species as a sentinel during blooms.

These experiments were successful in assessing these parameters. In particular the studies found:

Nodularin uptake, accumulation and elimination in Black Bream and other commercially and recreationally relevant finfish:

In terms of nodularin uptake, accumulation and elimination, this was species-dependant. Black Bream and Southern Sand Flathead both accumulated nodularin into their body tissues following a single oral dose; however, uptake was approximately 3 times greater in Southern Sand Flathead compared to Black Bream. Nodularin accumulation occurred within 24-hrs of exposure in both species, however uptake in Black Bream was rapid into muscle tissues but slower into liver tissue. In contrast, in Southern Sand Flathead uptake was rapid into liver tissue, while slower into muscle tissues.

Elimination of nodularin also differed between species, with rapid removal occurring from the muscle tissue of Black Bream and the liver tissue in Southern Sand Flathead, while slow removal was observed from muscle of Southern Sand Flathead and livers of Black Bream.

Exceedance of Health Alert Trigger Values:

In both species tissue concentrations reached and exceeded health alert trigger values which are applied in the Gippsland Lakes during blooms as a basis to provide advice around seafood harvesting (Mulvenna et al 2012). In Black Bream, it took 14 days for nodularin concentrations to reach health alert values; while in Southern Sand Flathead nodularin concentrations exceeded health alert values 24-hr post exposure and remained above health alert values after 7 days. Black Bream eliminated nodularin faster than Southern Sand Flathead with concentrations reduced below health alert values 7 days after. While in Southern Sand Flathead it took 14 days to reduce nodularin concentrations to below health alert values once they were exceeded.

Tissue distribution of nodularin in commercially and recreationally relevant species:

While nodularin is clearly transported into various organs and tissues in fish, following uptake, the primary organ of accumulation in most species is the liver (Ibelings and Chorus 2007; Sotton et al 2015). In Black Bream and Southern Sand Flathead ca. 80% of accumulated nodularin was detected in liver tissue, with 2-4.5% accumulated in muscle tissue. This is in line with observations for various fish species in both field and laboratory studies where reports of 80-100% accumulated toxin is detected in

the liver and between 0.1-18% in muscle tissues (Sipia et al 2001a; Kankaanpaa et al 2002; Sipia et al 2006; Cazenave et al 2005; Mazur-Marzec et al 2007).

Marketable parts of fish during blooms:

As a measure to control the risks of exposure to nodularin contaminated seafood during toxic blooms in the Gippsland Lakes, restrictions have been enforced around the harvest and sale of finfish unless it is gutted and gilled, when health alert trigger levels have been exceeded. Further recreational fishers have been advised to remove internal contents and thoroughly wash fillets prior to consumption. The results of this study support this measure, indicating that the removal of gut organs (including kidney, intestine, gall bladder, heart, gonads) could be a potentially effective control measure. In the current study, Black Bream and Southern Sand Flathead were exposed to nodularin doses (average range 106-869 µg/kg b.w.) in the range of concentrations which they may encounter in their food sources during *N. spumigena* blooms in the Gippsland Lakes (nodularin detected in prawns in range 56 to 22,430 µg/kg d.w and mussels 31 to 2,500 µg/kg d.w, Van Buynder et al 2002; Eaglesham et al 2002; Department of Health Victoria, 2014). Provided with a single oral dose, nodularin was detected in Southern Sand Flathead and Black Bream at levels exceeding the Victorian health alert guideline of 24 µg nodularin/kg whole fish (Black Bream average maximum total nodularin detected 24.9 µg/kg w.w., Southern Sand Flathead 36 µg/kg w.w.), indicating potential for these species to accumulate nodularin to levels that may pose risk to human consumers. These results are consistent with reports for concentrations of nodularin detected in tissues of Black Bream during blooms in2011-12 in the Gippsland Lakes, where nodularin concentrations in whole fish exceeded health alert guidelines (24 samples of 36; detected nodularin concentration ranged 16-203 µg/kg w.w., Department of Health Victoria 2014). The exceedances of the health alert guidelines observed in fish during the current study where due to the accumulation of nodularin in the liver, with concentrations in muscle tissue never reaching health alert guideline values (maximum concentrations in muscle of 0.62 µg/kg w.w. and 4.35 µg/kg w.w. in Black Bream and Southern Sand Flathead, respectively). This data corresponds with field data on Black Bream sampled during cyanobacterial blooms in the Gippsland Lakes, whereby concentrations of nodularin in gut and gilled fish have been well below health alert guidelines (nodularin concentrations ranged from below detection limits to 7.5 µg/kg) (Poon 2012; NSW Food Authority 2017; Van buynder et al 2001). As such, the current studies data and that collected from the field during blooms supports removing those parts of the fish in which the toxin primarily accumulates – such as liver and guts prior to processing and consumption to significantly reduce risks of exposure.

Under prolonged exposure, as in during cyanobacterial booms extending long periods, the accumulation potential into tissues could be greater than observed in the current study, with reports in the literature of cyanotoxin concentrations in fish tissues exceeding health alert levels during blooms (Amrani et al 2014; Bukaveckas et al 2017; Hauser-Davis et al 2015). Therefore, it would be recommended that during a bloom assessment of any potential health risks and subsequent advisories be based around monitoring of tissue concentrations.

Further investigations under both laboratory and field conditions are recommended to better understand the differences between species and to confirm that all species of commercial and recreational interest in the Gippsland Lakes are primarily accumulating toxins into the liver.

Management recommendations for sampling regimes and appropriate species as a sentinel during blooms.

Monitoring and surveillance of seafood safety during blooms in the Gippsland Lakes is currently based on testing of product (i.e. seafood) for concentrations of toxins. Assessment of toxin concentrations is undertaken in a number of seafood species, including:

- Eastern king prawns (*Melicertus plebejus*)
- School prawns (*Metaoenaeus macleayi*)

- Black Bream (*Acanthopagrus butcheri*)
- Blue mussels (*Mytilus edulis*)

Whole tissue samples for each species are tested for toxin levels, while in finfish gutted and gilled samples are also analysed. It is recommended that any advice around harvesting is based on verification of toxin concentrations in finfish tissues, as is currently undertaken in the Gippsland protocol.

During *N. spumigena* blooms in 2002, Black Bream were found to accumulate the highest concentrations of nodularin of the fish species sampled and tested (Eaglesham 2002). It was proposed that Black Bream would accumulate cyanobacterial toxins to a greater degree than other fish species as their diet includes mussels and prawns, which are also known to accumulate toxins. Additionally, Black Bream are resident in the Lakes during summer, while other finfish species may move between the marine environment and the Lakes. As a result, Black Bream would be more likely to spend a greater amount of time in bloom affected areas and were proposed as a sentinel species in the monitoring program (Anonymous 2011).

The results obtained in the current study challenge the suitability of the use of Black Bream as an early-warning indicator to inform public health decision-making relating to seafood safety in the Gippsland Lakes during toxic blooms. The study showed that of the two species, Southern Sand Flathead were found to pose a higher seafood risk, with uptake of available nodularin being three times greater than that observed for Black Bream. Nodularin tissue concentrations were on average between 2.6- 8.6 times higher in Southern Sand Flathead compared to Black Bream. The accumulation of nodularin was much more rapid into Southern Sand Flathead, while elimination was also generally slower.

Due to the current lack of understanding of nodularin accumulation, tissue distribution and elimination in the large range of finfish species currently harvested by commercial and recreational fishers in the Gippsland Lakes it is recommended that monitoring and advisories during toxic bloom events not be based on one species as a sentinel, but that a range of the commercially and recreationally relevant species be tested. Results from the current study also showed that there can be high variation in toxin accumulation between individual fish. A statistically determined number of samples should therefore be taken for each species to allow a robust evaluation of toxin accumulation and tissue distribution in different species.

It is recommended that further laboratory and field investigations into the uptake, accumulation, tissue distribution and toxin elimination be undertaken on a wider range of commercially and recreationally relevant species for the Gippsland Lakes in order to verify the use of a sentinel species.

There were a number of quality control procedures identified during the current investigations that should be included in protocols for the analysis and determination of cyanotoxins in fish tissues, in order to obtain accurate, reliable and consistent data. Without these quality controls there may be on over- or under-estimation of the concentrations of toxins in tissues which could have significant implications for providing advisories regarding seafood safety. The following are recommendations relating to quality control in sample analysis:

- At minimum, recovery experiments should be conducted on each tissue matrix and species being examined and run with each sample batch.

- If funding allows, include an internal standard in every sample prior to extraction. This is the only way to fully evaluate any toxin losses during processing and provide the highest level of reliability in data obtained. Ideally, this internal standard would be a ‘heavy’ version of the analyte of interest (i.e. the same compound with isotopes incorporated to shift the mass of the compound by several Daltons to allow simultaneous, but specific, detection of both compounds by mass spectrometry).

- Details of quality control performance are provided in reports.

The second objective of this study was to review the current protocol used in the Gippsland Lakes to provide incident response during toxic algal blooms with those used in monitoring programs in Australia and worldwide. This review identified that the monitoring and management of seafood safety during cyanobacterial blooms is limited worldwide, with only a handful of countries that have criteria around safe levels of toxins in seafood and monitoring programs in place to provide incident response during blooms events.

The last objective of the project was to provide a number of recommendations around sampling and risk management based on the outcomes of the first two objectives. Recommendations arising from the project to assist in advisories around seafood safety during toxic blooms in the Gippsland Lakes include:

- Develop an agreement for incident response funding
- Develop and adopt a clear and comprehensive algal bloom incident management plan for seafood
- Retain the gut and gill policy, but incorporate details around its implementation into the incident response plan
- Investigate other available market opportunities for industry during blooms
- Develop an education campaign around commercially harvested seafood
- Further investigations around uptake, accumulation and elimination of nodularin toxins in finfish to assist in sampling regimes and to verify a sentinel species
- Research into the management of algal blooms

Conclusion

The overall aims of the project have been achieved, with a number of recommendations to assist in advisories around seafood safety in the Gippsland Lakes during toxic algal blooms being provided.

More specifically we have:

- Succeeded in determining the uptake, accumulation, tissue distribution and elimination of nodularin in two commercially and recreationally relevant finfish species for the Gippsland Lakes, Black Bream and Southern Sand Flathead. Southern Sand Flathead posed a greater risk to consumers in the fact that they accumulated higher concentrations of nodularin into their tissues and at a greater rate than that of Black Bream. Elimination of nodularin toxin from the tissue of Southern Sand Flathead was also slower than that observed for Black Bream. While both species accumulated nodularin to levels exceeding the health alert guidelines applied in the Gippsland Lakes for finfish (24 µg/kg in whole fish), this was due to the accumulation of nodularin in liver tissues, and never muscle tissue. Advice to gut and gill fish is therefore likely to reduce the risk of toxin exposure. It is, however, recommended that advice around seafood safety be made based on verification of levels of toxins in fish.

  The safety of seafood as a food source in the Gippsland Lakes is of great importance not only for human health, but also from an economic perspective for industry. In choosing a sentinel species to monitor and manage seafood risks during blooms the species needs to be representative of all species and therefore provide early detection of risks together with an indication of when it is safe to harvest whole fish following a bloom. The results of the current study suggest that the use of Black Bream as a sentinel may need reassessment. Further data is needed, from both laboratory and field studies during blooms, on the accumulation, tissue distribution and elimination of nodularin in a wider number of the finfish species commercially and recreationally harvested in the Gippsland Lakes to inform an appropriate sentinel species.

- Reviewed current algal bloom response plan for the Gippsland Lakes and those used in monitoring programs in Australia and overseas and have been able to identify that, while the Gippsland Lakes are one of the only programs for monitoring and management of seafood safety during cyanobacterial blooms in the world, there are a number of improvements that could be made to the procedural and administrative protocols to make it a clearer and more comprehensive protocol. In particular there needs to be an agreement made around the funding of the program and details around the following need to be clearly outlined in a more comprehensive protocol:

  - The resources managed under the protocol,
  - The roles and responsibilities of industry in the protocol,
  - Sampling sites, sample collection and transport methods,
  - Phytoplankton triggers used to initiate seafood sampling,
  - Closure and opening criteria (and guidelines for their application),
  - Methods of communication and notification,
  - Details of laboratories for phytoplankton and toxin analysis, sample turnaround times, analysis methods

- We have provided a number of recommendations based on the outcomes of the objectives which if implemented will assist in improving the monitoring and management of seafood safety in the Gippsland Lakes during toxic algal blooms.

Implications

- This project has been able to identify a number of recommendations around the procedural and administrative protocols for monitoring and management of seafood safety during blooms in the Gippsland Lakes – in particular related to finfish, which could result in improved monitoring and management. However, whether improvements are made will be dependent on the uptake of the recommendations by government and industry.

- The data on nodularin tissue distribution in two commercially and recreationally relevant finfish species provides greater confidence in gill and gut advice made during *Nodularia spumigena* blooms. This also allows market access during blooms for commercial fishers.

- The data on elimination times for nodularin from Black Bream and Southern Sand Flathead tissues provides greater confidence in the currently applied criteria of requiring toxin levels in seafood samples to be below health alert guidelines for two successive weeks before being able to allow harvesting of whole fish for sale.

- The differences in uptake, accumulation of nodularin between Southern Sand Flathead and Black Bream indicate further research is required to provide a scientific basis for the choice of species to be used as a sentinel species in the monitoring program. The results suggest that Southern Sand Flathead pose a greater risk due to higher nodularin accumulation and uptake rates.

- Uptake of the recommendations to develop a funding agreement and comprehensive incident response plan for the Gippsland Lakes would provide greater confidence in administration and procedures by all stakeholders, and therefore provide greater confidence in the safety of seafood during blooms.

- The review of current programs identified a number of key elements which will benefit the development of a comprehensive monitoring and management plan for incident response to provide seafood safety during blooms.

Recommendations

- At the industry level, broadcast the information about findings of the project and provide reports generated as part of the project. Encourage industry to start discussions with government agencies to develop and adopt a comprehensive incident response plan and work to develop agreements for program funding arrangements.

- At the recreational fishing level, broadcast information about the findings of the project and how it may impact recreational fishers, further about the roles they could play in the development and implementation of a comprehensive seafood incident response plan.

- At the government level, provide reports and manuscripts generated as part of this project and encourage discussions amongst stakeholders (including recreational and commercial fishers) on how they may start to develop and adopt a comprehensive incident response plan adopt other recommendations provided based on this research.

- At the scientific community level, publish the manuscript on the experimental research and associated outcomes from objective 1 - investigation of nodularin uptake, accumulation, tissue distribution and elimination from Black Bream and Southern Sand Flathead in peer-reviewed scientific journal.

- Work with industry and government to plan how may undertake further research to provide a better understanding of seafood risks by investigating nodularin and other cyanotoxin transfer to seafood.

Further development

- There is a need to develop and implement a comprehensive incident response plan for seafood monitoring during toxic blooms, to ensure seafood is harvested in a safe manner, following internationally respected risk assessment principles and a scientifically sound management framework. The plan needs to provide clear guidance on the administrative and procedural protocols, be robust and meet best practice principles. The ASQAP manual and state biotoxin monitoring plans would be good models to provide guidance in development of a comprehensive plan for Gippsland Lakes. A national protocol would be more beneficial. SafeFish could lead development of a national protocol or funding from FRDC could support development of or inclusion of cyanotoxin protocols in ASQAP.

  Development of a comprehensive plan requires involvement from all key stakeholders: including commercial and recreational fishing representatives (SIV, VR-Fish, commercial licence holders), and government agencies such as DEWLP, DHHS, VFA, EPA, Primesafe. The roles and responsibilities of all stakeholders need to be well defined and documented.

- The government, industry and recreational sectors need to develop a cost sharing agreement so as funding will be available each year in the event of a bloom. Funding needs to be available to be deployed in the event of a toxic bloom for sampling and toxin analysis. This has been an issue in past bloom events and hinders the ability of providing adequate sampling to provide data for advisories around harvest closures and reopening. Clearly defining roles and responsibilities of all stakeholders can assist in determining the basis of equitable funding arrangements.

- Develop clear and open communication networks at local, state and national level to allow sharing of data, knowledge and experience. Further to promote openness between parties. This could be done through the form of workshops to discuss specific topics. Clear communication networks will ensure everyone involved in cyanotoxin monitoring and management are up-to

date with the latest research, will help ensure research isn't duplicated and assist in development of co-ordinated and focused research which addresses the key knowledge gaps that will help improve monitoring and management programs.

- Provide continued support and funding for research that underpins scientifically robust monitoring and management programs. Specifically, further research is needed to support a basis for choosing a sentinel species. It is recommended that research in the use of sentinel species or samplers be undertaken to strengthen the scientific basis for policy decisions regarding which indicator species are appropriate to use in the event based monitoring program.

Research recommendations include:

Collection of larger, high frequency data sets during blooms and laboratory assessment of cyanotoxin uptake, tissue distribution and elimination.

The current study was limited to laboratory investigations of uptake, accumulation, tissue distribution and elimination of nodularin in two finfish species. Larger, higher frequency sample sets collected over multiple toxic blooms and investigating a greater range of species of commercial and recreational significance are needed to provide a better understanding of species specific and exposure specific differences in nodularin uptake, accumulation, tissue distribution and elimination.

Further, there needs to be research undertaken on the other cyanotoxins listed in the protocol and their uptake, accumulation, tissue distribution and elimination under field and laboratory conditions in order to complete a thorough, scientifically robust risk assessment of seafood safety during cyanobacterial blooms and choose an appropriate species as a sentinel.

Investigation of passive samplers to monitor toxin levels:

International programs for marine biotoxins have employed the use of passive samplers (SPATT bags) and/or bags of mussels deployed at set sampling sites as sentinels for occurrence of toxins in shellfish. The use of these types of sentinels could be investigated as sampling tools during times advisories are in place. Research into the use of these in place of weekly seafood sampling, once advisories are in place, could reduce costs associated with sample collection and handling while toxins are remaining at levels exceeding health guidelines. When these samplers indicate dropping toxin levels, seafood samples could then be collected and analysed for toxins in order to lift advisories.

Extension and Adoption

The research undertaken as part of objective 1, determining nodularin uptake, accumulation, tissue distribution and elimination in commercially and recreationally relevant finfish species, has been completed and the data generated will be submitted to a peer-reviewed scientific journal. This will be publicly available. This research has also been presented at a national conference and local workshop.

All reports from this project have been provided to SIV, DELWP, EPA Victoria, DOH, and commercial fishers in Gippsland Lakes. They are also available from CAPIM at any time. Details of forms of communication to promote extension and adoption of the information generated as part of this project are provided in Table 11 below.

Table 11: Communication promoting extension and adoption of project 2013/217 findings

Communication Type	Description	Audience	Person Responsible	completed
Meeting	Meeting with commercial fishers at Gippsland Lakes Co-op to describe the project	Commercial fishers in Gippsland Lakes	Jackie Myers Vincent Pettigrove	February 2014
Pamphlet	Information brochure outlining project given to commercial fishers, and at Co-op in Lakes Entrance.	Recreational and commercial fishers, community in Gippsland Lakes	Jackie Myers	February 2014
Meeting	To inform of and discuss project	Gippsland Coastal Board	Jackie Myers Vincent Pettigrove	February 2014
Meeting	To inform of and discuss project	DEPI Bairnsdale and Queenscliff	Jackie Myers Vincent Pettigrove	February 2014
Workshop attendance	Attend ASQAAC workshop in Hobart and meet with industry and government involved in biotoxin monitoring and management. Discuss project, specifically objective 2 the review.	Government and industry	Jackie Myers	October 2014
Phone/email	Discuss biotoxin management in Australia, review the report of objective 2.	Alison Turnbull (SARDI)	Jackie Myers	Various times during 2014-2016
Emails/phone discussions	With government personal involved in biotoxin monitoring and management in Australia to inform the review.	Government personal including: Swan River Trust Laurie Jeremiah Anthony Costigan Pradeepa Adihetty John Mercer Andrew Clarke Anthony Zammit	Jackie Myers	During 2015-16

		Clinton Wilkinson Tracey Stamp Rachael Poon		
Meeting	With researchers at Cawthron Institute, New Zealand. Toured facility where they monitor biotoxins in seafood and undertake algal monitoring. Discussed current project and developed a collaboration for objective 1 experiments	Scientists: Tim Harwood Susie Wood Jonathan Puddick	Jackie Myers	August 2015
Presentation	SETAC Australasia conference, Nelson New Zealand. Present results from the review of monitoring and management programs	Scientists, industry, government	Jackie Myers	August 2015
Presentation	SETAC Australasia conference, Gold Coast. Present results from the uptake, tissue distribution and elimination of nodularin in finfish.	Scientists, industry, government	Jackie Myers	September 2017
Presentation	CAPIM Research summit, Melbourne. Present results from the uptake, tissue distribution and elimination of nodularin in finfish.	Scientists, industry, government, community groups	Jackie Myers	August 2017
Presentation	EPA Victoria	Government, Scientists	Jackie Myers	December 2017
Email	Reports related to milestones 2 and 3 sent to stakeholders. Ask to have meetings regarding project outcomes.	SIV, DEWLP, EPA, DHHS, commercial fishers in Gippsland Lakes, VR Fish	Jackie Myers	August 2017
Email	Invite to presentation at the CAPIM summit.	SIV, DEWLP, EPA, DHHS, commercial fishers in Gippsland Lakes, VR-Fish	Jackie Myers	August 2017
Meeting	With SIV to discuss project outcomes and plan for communication of results to commercial fishers.	SIV	Jackie Myers	September 2017

Project coverage

- An article around the safety of seafood during *N. spumigena* blooms, based on results of experimental research conducted as part of objective 1 was published in the CAPIM newsletter and available on the CAPIM webpage.
- A number of twitter tweets were sent to inform CAPIM followers of the presentations at the CAPIM research summit on August 25th 2017. These included details of the talk on seafood safety during toxic algal blooms by Dr Jackie Myers.

Project materials developed

- Manuscript describing the uptake, accumulation, tissue distribution and elimination of nodularin in two commercially and recreationally relevant finfish species, is in the process of preparation. This will be submitted to a scientific journal for publication.

- A number of technical reports were prepared as part of objectives 1, 2 and 3. These have been provided to FRDC as part of milestones 1 to 4 and include:

    - Myers J.H., Long S., Hassell K., and Pettigrove P. (2014) Determination of nodularin elimination and tissue distribution in Black Bream under laboratory conditions. Centre for Aquatic Pollution Identification and Management, Technical Report October 2014, University of Melbourne, Victoria, Australia.

    - Myers J.H. (2017) A review of protocols for managing the risks to seafood safety from cyanobacterial toxins: A Gippsland Lakes Perspective. Centre for Aquatic Pollution Identification and Management, Technical Report No. 82, University of Melbourne, Victoria, Australia.

    - Myers J.H., Puddick J., Wood S., and Pettigrove V. (2017) Nodularin uptake, accumulation and tissue distribution in commercially and recreationally relevant fin-fish species under laboratory conditions. Centre for Aquatic Pollution Identification and Management, Technical Report No. 83, University of Melbourne, Victoria, Australia.

    - Myers J.H. and Pettigrove V. (2017) Progress report: FRDC Project 2013/217: Sampling and risk management recommendations to provide seafood safety during cyanobacterial blooms in the Gippsland Lakes, Victoria, Australia. Centre for Aquatic Pollution Identification and Management, Technical Report No. 84, University of Melbourne, Victoria, Australia.

- A Factsheet "Developing Recommendations to Assist Advisories Regarding Seafood Safety during Toxic Bloom Events: Information for Commercial Fishing Operators" was produced at the start of the project and disseminated to commercial fishing operators in the Gippsland Lakes. (See Appendix F).

- The research components of objectives 1 and 2 have been presented at two national conferences and a Victorian workshop:

    - Myers J.H. (2015) Providing seafood safety during cyanobacterial blooms: A review of current approaches. Society of Ecotoxicology and Chemistry (SETAC) Australasian Conference, Nelson, New Zealand, August 2015, oral presentation.

    - Myers J.H., Puddick J., Wood S. and Pettigrove V. (2017) Are those fish safe to eat? Accumulation of nodularin in commercial and recreationally relevant fish species. CAPIM Research Summit, August 25th, Bio21 Institute, University of Melbourne, oral presentation.

    - Myers J.H., Puddick J., Wood S. and Pettigrove V. (2017) Are those fish safe to eat? Accumulation of nodularin in commercial and recreationally relevant fish species. Society of Ecotoxicology and Chemistry (SETAC) Australasian Conference, Gold Coast, September 2017, oral presentation.

Appendices

Appendix A: Objective 1 Report 1: Determination of nodularin elimination and tissue distribution in black bream under laboratory conditions.

Determination of nodularin elimination and tissue distribution in black bream under laboratory conditions

Jackie Myers, Sara Long, Kathyrn Hassell and Vincent Pettigrove
October 2014

FRDC Project No **2013/217**

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Acknowledgments

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The authors would like to thank Dr Elizabeth Morris for providing comments on this report and Rhianna Boyle, Rebecca Reid, Michael Shipley, Erin Cummings for assistance with fish maintenance, sample processing and algal culture.

Glossary

Nodularia spumigena:	filamentous cyanobacteria (blue-green algae) that produces the toxin nodularin.
Nodularin:	a hepatotoxic and cyclic pentapeptide isolated from toxic filamentous cyanobacteria(blue-green algae) *Nodularia spumigena*
ELISA	enzyme-linked immunosorbent assay; a technique of high specificity and selectivity used for confirmation of presence and quantification of analytes. ELISA is based on antigen-antibody interaction.
LC-MS	Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS).

Executive Summary

Cyanobacteria, also known as blue-green algae, are a naturally occurring component of freshwater, marine and estuarine environments. If conditions permit some cyanobacteria species have the ability to form dense blooms. Not only do these blooms degrade the ecosystem and interfere with water quality, the cyanobacterial toxins present a hazard to human health if sufficient levels are ingested in water or food, are inhaled or come in direct dermal contact. Recently there has been an apparent increase in the occurrence of cyanobacterial blooms, due mostly to increased eutrophication.

Gippsland Lakes, one of Australia's largest lake systems, situated in south-eastern Victoria support a range of recreational and commercial activities, including fishing. They provide about one-third of the state commercial fishing catch and are also home to a significant recreational fishery. In the last decade the lakes have experienced an increase in the frequency and intensity of cyanobacterial blooms, with seven major blooms of *Nodularia spumigena* since 1995, after only one significant bloom in the previous 20 years. In 1999 and more recently 2011-12 and 2012-13 toxic blooms of N. spumigena have led to restrictions on the harvesting and sale of shellfish, prawns and un-filleted fin-fish due to the presence of nodularin toxin in seafood tissues for periods up to 6 months. Significant economic losses have been incurred by the fishing and tourism industry in the region during the restricted periods and government agencies through costs of monitoring the blooms and providing advice around seafood safety

Due to the closures the commercial and recreational fishing sectors have expressed concerns about the monitoring process for toxins in seafood during blooms and the health alert levels used by the Department of Health to provide advisories. The lack of information on nodularin uptake and elimination in seafood is regarded as a significant stumbling as it hampers the ability to provide appropriate advice regarding harvesting and consumption of seafood during blue-green blooms. Furthermore, this lack of information also makes it difficult to monitor blooms effectively, and as such there is a very clear need to develop cost effective and timely sampling protocols.

The current study, as part of a larger to provide management recommendations to deal with fishing closers and re-opening events during bloom events in the Gippsland Lakes, was to determine tissue distributions and elimination rates of nodularin in a commercially and recreationally relevant fish species (black bream, *Acanthopagrus butcheri*) under controlled laboratory conditions. Further to assess biomarker responses and histology of various organs to determine the biological effects of nodularin in black bream.

The results of the study showed that there was no uptake of nodularin toxin by fish exposed to nominal doses of 50µg/Kg or 200µg/kg nodularin. This result was unexpected based on the nominal concentrations provided to the fish. Comparison of nominal nodularin doses to actual measured nodularin concentrations in the toxic food indicated that the slurries used to dose the fish had significantly less nodularin than expected based on the nominal dose. The concentrations of nodularin in the spiked food slurries (2-12 µg/Kg) would not be expected to result in uptake into the fish (Myers et al 2010).

From the results of actual measured concentrations of nodularin in spiked slurries using LC-MS it is likely that the ELISA method may have over-estimated the nodularin content of the *N. spumigena* cell concentrate, which lead to an under dosing of the slurries provided to fish. While the results have not allowed the provision of information on tissue distributions and elimination rates of nodularin from fish tissues due to the over estimation of nodularin in *N. spumigena* cell concentrate used for spiking into fish food, the study did provide positive outcomes relating to the development and validation of an LC-MS method at a commercial laboratory in Australia, where samples can be sent in the event of a bloom with an office in Melbourne that can receive the samples. The study also allowed the development and validation of an oral gavage method in fish. We were able to successfully dose fish directly into the stomach and maintain this does within the fish stomach. Following anaesthetic and the procedures of insertion of the tube to provide the slurry fish showed good recovery, with no mortalities due to the procedures undertaken.

It is recommend from this work that abraxis ELISA kits not be used as a basis for decisions around toxin levels, with any future studies using LC-MS methods for analysis of nodularin toxin prior to any dosing. The use of ELISA could be as a screening technique of samples after dosing, and with a commercial ELISA kit that has been well validated.

Introduction

The Gippsland Lakes are one of Australia's largest lake systems, supporting a wide range of recreational and commercial activities, including fishing. Over the last decade these lakes have experienced an increase in the frequency and intensity of cyanobacterial blooms, with seven major blooms occurring since 1995, which contrasts only one significant bloom in the previous 20 years. The Gippsland Lakes blooms are frequently toxic due to the presence of *Nodularia spumigena* a cyanobacterium, which produces nodularin, a cyclic pentapeptide hepatotoxin.

The bioaccumulation of nodularin into seafood species is well documented from *N. spumigena* blooms, notably in the Baltic Sea (Sipia et al 2001a, 2001b) but also in Australia, in the Peel Harvey Estuary and the Gippsland Lakes (Falconer et al 1992; Van Buynder et al 2001). Laboratory studies have also documented the accumulation of nodularin into various seafood species including fish, prawns, mussels and clams (Paakkonen et al 2008; Kankaanpaa et al 2002; Vourinen et al 2009; Lehtonen et al 2003; Kankaanpaa et al 2007), documenting tissue distributions, however there appears to be no studies specifically obtaining uptake and elimination rates of nodularin (Myers et al 2010). Field and laboratory studies indicate that nodularin accumulates primarily in the liver of fish, with accumulation of nodularin into fish muscle not always being evident. Field studies during blooms of *N. spumigena* in the Baltic Sea, Gulf of Bothnia, Swedish waters (Oresund) and the Gulf of Finland have reported the occurrence of nodularin in livers of various fish species (flounders, Baltic herring, salmon, roach) up to 557 ug/kg d.w. with concentrations in muscle tissues generally not detected (Sipia et al 2002; Sipia et al 2006; Pearsson et al 2009). When measured, nodularin concentrations in fish muscle were at generally at much lower concentrations than liver (up to 73 ug/kg d.w.) (Sipia et al 2006; Persson et al 2009). The few laboratory studies investigating tissue distributions of nodularin in various fish species (flounder, black bream, sea trout) have confirmed field observations of nodularin compartmentalisation into the livers, and to a lesser extent other tissues (Platt 2005; Kankaanpaa et al 2002; Vourinen et al 2009; Myers et al 2010). While field and laboratory studies indicate the primary site of nodularin accumulation is the liver in the fish species that have been investigated, the results indicate that nodularin concentrations in tissues vary depending on fish species. For instance Sipia et al (2007) investigated nodularin concentrations in three-spined stickleback (*Gasterosteus aculeatus*) and herring (*Clupea harengus*) during dense *N. spumigena* blooms in the northern Baltic Sea in 2002-2003. The average nodularin content in livers of stickleback was 430 mg kg-1 d.w. while in herring livers the average nodularin concentration was 75 mg kg-1 d.w.. This study indicates the importance of understanding tissue distributions and elimination rates in different fish species.

Nodularin acts as a hepatotoxin. Once ingested, it is absorbed across the ileum in the intestine and transported to the liver, where it is concentrated via bile acid carriers and taken up by the hepatocytes (Carmicheal 2001; Hunter 1998). Nodularin is a potent inhibitor of protein phosphatases types 1 and 2A, enzymes crucial to cell growth and tumour suppression (Hunter 1998). Nodularin binds to the catalytic subunits of the protein phosphatase enzymes thereby inhibiting their activity (Hunter 1998). Neurotoxic effects have also been suggested after an inhibition of acetylcholinesterase (AchE) following high doses of nodularin was observed in clams (Lehtonen et al 2003). Experimental exposures to microcystins and nodularin have also shown instances of liver lesions, haemorrhages, the occurrence of apoptotic cells, oxidative stress and changes in enzyme activities in organs such as gills, livers and muscle tissues of fish, clams and mussels (Mezhoud et al 2008; Kankaanpaa et al 2007; Lentonen et al 2003). Molecular indicators, or biomarkers and organ histology are valuable tools to assess this type of damage and help understand the biological effects of toxins and may provide new tools for early detection of fish exposed to algal toxins (Lehtonen et al 2003; Mezhoud et al 2008; Kankaanpaa et al 2007).

Nodularin has been previously detected using enzyme-linked immunosorbant assays (ELISA), protein phosphatase (PP) inhibition assay, liquid chromatography/mass spectrometry (LC-MS), Hi-

Performance liquid chromatography (HPLC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry. The method used most commonly used for regulatory analysis of hepatotoxins in that of LC-MS or HPLC (Johnson 2010). These techniques require expensive laboratory instruments and require experienced personal to operate them, making processing and analysis costs quite high. Immunological methods, such as ELISA and the protein phosphatase (PP) inhibition assay are therefore becoming more commonly applied for screening of samples with regulatory LC-MS methods used for final toxin quantification (Johnson 2010; Sipia et al 2001; Karjalainen et al 2008;Kankaanpaa et al 2002Sipia et al 2006; Marzur-Marzec et al 2006; Soares et al 2004). ELISA has the advantage of being rather a simple and rapid method, and has previously achieved valid results for nodularin in various seafood tissues, zooplankton, water and cyanobacterial cells (Karjalinen *et al* 2008; Myers et al 2010; Myers 2008; Kankaanpaa et al 2007).

In 1999, and more recently in 2011-12 and 2012-13, toxic blooms of *N. spumigena* led to restrictions on the harvesting and sale of shellfish, prawns and finfish for periods up to six months, due to the presence of nodularin toxin in seafood tissues above regulatory limits (*Pers. comm*. Gippsland Lakes commercial fisherman and Victorian Department Health). *Nodularia spumigena* blooms in 2001 and 2002 in the Gippsland Lakes resulted in nodularin bioaccumulation in naturally occurring prawns ranging from 6.4 – 22 mg/kg dry weight d.w. in the viscera and 22-143 µg/kg d.w. in prawn muscle; while whole mussel samples contained between 40 and 2725 µg/kg d.w. nodularin (Van Buynder et al 2001; Eagelsham et al., 2002). In fish liver samples (including black bream, leatherjacket, mullet and trevally) nodularin concentrations in the range 35-450 µg/kg d.w. were documented, with no nodularin detected in fish flesh (Eaglesham et al 2002; Van buynder et al 2001). During blooms in 2012-13 nodularin was detected in whole fish samples at concentrations up to 37µg/kg and in mussels up to 39.7µg/kg, however there was no toxin detected in gilled and gutted fish (Department of Health Victoria, 2014).

The Gippsland Lakes contributes one third of the State's commercial fishing catch and are also a significant recreational fishery. The occurrence of toxic algal blooms in the Gippsland lakes has led to significant economic losses to both the fishing and tourism industries in this region and has a high financial cost to government agencies monitoring the blooms. In recent years there have been concerns raised regarding the monitoring processes for seafood during toxic blooms, with the lack of information on tissue distribution and elimination of nodularin in sentinel species used to determine seafood risks during a bloom identified as impeding the ability to provide appropriate advice to commercial and recreational fishers regarding harvesting and consumption during toxic blooms.

Current monitoring and health alert advisories for seafood consumption during Nodularia spumigena blooms are based on information on toxin occurrence in seafood samples obtained randomly over the years and scientific research on the freshwater blue-green algal toxin microcystin. The current monitoring programme lacks scientific data on nodularin movement into and out of seafood, and world-wide very little information exists regarding nodularin uptake and elimination in fish species. To provide a clearer understanding of the risks to seafood safety during blooms; allow appropriate public health responses, and provide risk management strategies to minimize the impacts of advisories on sale and consumption of seafood to the commercial fishing sector, recreational fishers and the tourism industry an understanding of toxin tissue distribution and elimination in fish species used as a sentinel is needed.

Objectives

This study is part of a larger investigation into monitoring processes for toxic algal blooms in the Gippsland Lakes, Australia and other sites around the world-wide. The larger investigation also includes field investigations of nodularin elimination in commercially and recreationally relevant fish species during toxic blooms (FRDC project 2013/217). The objective of this component was to determine tissue distributions and elimination rates of nodularin in a commercially and recreationally relevant fish species (black bream, *Acanthopagrus butcheri*) under controlled laboratory conditions.

This information will increase the capacity to provide advice around sampling protocols for fish during blooms and to determine which tissues of the fish are safe for consumption during bloom events. The specific objectives of this study were:

- To determine uptake, tissue distribution and elimination rates of nodularin toxin in black bream following oral exposure.
- To determine if there were any concentration-dependent differences in uptake, tissue distribution, elimination, histology and biomarker responses from nodularin exposure.

Method

Experimental Fish

Black bream (*Acanthopagrus butcheri*) were obtained from Fisheries Victoria breeding stock. These fish had been in captivity for over 10 years. They were originally collected from two site sin Victoria, Swan Bay and Lake Tyers. Fish were maintained in one 5000L poly tank supplied with flow through seawater and aerated via air stones. All holding and experimental tanks were situated under a large outdoor covered roof and received natural light and photoperiods. Prior to experimentation fish were fed pellets (Marine 45/20 dinking 4mm pellets, Ridley Aqua Feed), three days a week. One week prior to the start of the experiment fish were randomly allocated into their treatment groups and separated into three 5000L flow through poly tanks, each supplied with air via air stones. Eleven fish were controls, and there were 18 fish in each of the treatment groups. Fish were maintained and all experiments were conducted at the Victorian Marine Science Consortium (VMSC), Department of Environment and Primary Industries Centre at Queenscliff, Victoria.

Nodularia spumigena culture and toxin production

A non-axenic strain of *N. spumigena*, originally isolated from field samples collected from the Gippsland Lakes (March 2003, J. Myers) was grown in batch cultures. The culture was maintained in MLA media (Bolch and Blackburn 1996) at 21 ± 1^{o}C under constant illumination (cool white fluorescent lamps) at the CAPIM laboratory in the Department of Zoology, University of Melbourne Parkville campus.

N. spumigena cells were harvested from batch cultures to obtain toxic cells for spiking into the food slurry for dosing. Batch cultures were filtered through GF/A filters (45mm, Whatman) and the cells retained on the filters were collected into jars by rinsing with MLA media. This resulted in a concentrate of *N. spumigena* cells. The *N. spumigena* concentrate was then extracted to determine the nodularin concentration for spiking of the food slurry. *N. spumigena* concentrate (20mL) was filtered through pre-weighed GF/A filters, the filters then reweighed and placed into centrifuge tubes and frozen (-80^{o}C) prior to analysis.

Nodularin concentrations in the cell concentrate were determined using both liquid chromatography mass spectrometry (LC-MS) and enzyme-linked immunosorbent assay (ELISA) to allow comparison of the two methods for nodularin detection. For nodularin determination using LC-MS, two filter samples containing cells were sent to the commercial laboratory, Advanced Analytical were they were extracted and analysed. For nodularin determination using ELISA, samples were extracted and processed at the CAPIM Laboratory, Zoology Department, The University of Melbourne as described below.

For ELISA analysis, nodularin was extracted from the filter papers by placing thawed and re-frozen (three times) filters in methanol:water (10 mL; 75:25; v:v) and kept in the dark at 4^{o}C overnight. The submerged filters were sonicated (15 min; Bransonic 220, SmithKline Co.), centrifuged (15 min, 4,000 rpm; Rotofix 32, Hettich, Germany), and the supernatant separated from the filter-pellet. The filter-pellet was then re-extracted in methanol:water (10 mL; 75:25; v:v), sonicated (15 mins; Bransonic 220, SmithKline Co.), centrifuged (15 mins, 10,000 rpm; Rotofix 32, Hettich, Germany) and the supernatant again separated from the filter-pellet. The combined supernatants were diluted with deionised water to provide a methanol concentration <10%. The extracts were analysed using a direct competitive commercial ELISA kit (Abraxis Microcystins-ADDA ELISA, Abraxis LLC, USA) calibrated with 0.1, 0.5, 1.0, 5.0, 10.0 and 50.0 µg/L nodularin solutions. The analyses were performed according to the manufacturer's instructions. The absorbance at 450nm in each well was read using a Synergy 2 microplate reader (Biotek Instruments, USA) within 10 mins of adding the stop solution.

The toxin concentration of the *N. spumigena* concentrate was 9.52 µg/g as determined by ELISA and 13.81µg/g determined by LC-MS. The amount of *N. spumigena* concentrate we had was approximately 500g which equated to a total nodularin toxin of 4760µg. To undertake the dosing we required approximately 6000µg of nodularin (based on doses of 50µg/Kg and 200µg/Kg and the fish weights). Therefore we did not have enough nodularin to spike the slurry, and the experiment was delayed while more *N. spumigena* was cultured to obtain appropriate amount of toxin.

Nodularia spumigena cultures were prepared and harvested weekly as described above. Cultures were grown and harvested over a 6 week period to collect a large enough *N. spumigena* cell concentrate for food spiking. A sample of this concentrate was extracted as described above and analysed using ELISA, as both the ELISA and LC-MS method produced comparable results during the first analysis of *N. spumigena* cell concentrate, and time and sample processing cost did not permit LC-MS analysis a second time. The toxin concentration of the new *N. spumigena* concentrate was estimated from ELISA to be 0.5mg/g. This concentration is in line with that reported for Australian strains of *N. spumigena* (0.73mg/g to 5mg/g (Jones et al 1994; Platt 2005) and is similar to toxin production determined for this isolate of 0.1-0.6 mg/g (Myers, 2008). Using this value as a guide, the slurry for oral gavage of whole cyanobacteria was made as described below.

Preparation of the *N. spumigena* slurry

The dosing slurry for the black bream composed of *Nodularia spumigena* cell concentrate, ground fish pellets (Marine 45/20 dinking 4mm pellets, Ridley Aqua Feed) and water. Based on the methods detailed in the scientific literature, trials of slurry preparation and post-mortem food delivery into the stomachs of black bream, a suitable mixture of ground fish pellets and water was determined to be 200g ground pellets to 600mL water. Fish pellets were ground with a coffee grinder (150 Watt, Homemaker Australia) and mixed with the appropriate amount of cell concentrate and distilled water to produce two food slurries to give nominal doses of 50 and 200 µg nodularin/kg of fish. These doses were based on those used by Myers et al (2009) in selective feeding studies with black bream. Myers et al (2009) exposure concentrations were determined based on toxin concentrations reported in prawns sampled from the Gippsland Lakes during a *N. spumigena* bloom (Eaglesham et al 2002) which equated to providing fish with a nominal nodularin dose of 221µg nodularin/kg fish. Control food was mixed in the same ratio as nodularin spiked food; however distilled water was used instead of cell concentrate. Table 1 details the volumes of fish food, water and *N. spumigena* concentrate that were added to each slurry treatment.

Table 1: Slurry mixtures for control, low (50µg/kg) and high (200µg/kg) dose nodularin treatments.

Dose	Fish meal (g)	Distilled water (mL)	*N. spumigena* concentrate (mL)
Control	200	600	0
Low (50µg/kg)	200	598	2.0
High (200µg/kg)	267	789	11

Laboratory experiment: Exposure and sampling

For estimation of the correct feeding tube delivery depth, post-mortem measurements of the body length and length from the tip of the snout to the stomach of five black bream, purchased from local fish supplier were made. This allowed estimation of tubing depths in bream of different lengths. The black bream in captivity were divided into three groups: a group of control fish (11 specimen) and two groups of 18 fish to be exposed to nodularin.

Nodularin was dosed orally as a single delivery of the slurry directly into the stomachs of fish to give nominal doses of either 50 or 200 µg nodularin/kg fish. Control fish were dosed in the same way as

nodularin exposed fish; however the slurry contained no *N. spumigena* cells. Immediately prior to dosing fish were anesthetized one at a time in aerated seawater containing AQUI-S (AQUI-S New Zealand Ltd). Under anesthesia fish were weighed and measured, then checked for the presence of an internal microchip tag using a microchip scanner (Trovan portable microchip scanner, Trovan Ltd). Tagged fish had the numbers recorded to assist with later identification of specific individuals. The slurry containing nodularin, as *N. spumigena* cells, was then delivered into the stomachs of the experimental fish, while the slurry without *N. spumigena* cells was delivered into the stomachs of the control fish. The amount to be delivered to each individual was determined on the basis of fish weight and a table that was compiled beforehand (See Table 2). The slurry was drawn into a 50-ml syringe fitted with a specified length piece of flexible polypropylene tube and the food was delivered directly into the stomach. The syringe was weighed before and after injection to obtain the exact amount of slurry delivered. After treatment bream were put in a 500L resin tank and once they had recovered, they were returned to their appropriate treatment tank.

Table 2: Dose, in mL, to be administered to Black bream based on weight of the fish (g)

fish wet weight (g)	Dose in mL of slurry to administer to bream
500-550	15.75
551-600	17.25
601-650	18.75
651-700	20.25
701-750	21.75
751-800	23.25
801-850	24.75
851-900	26.25
901-950	27.75
951-1000	29.25
1001-1050	30.75
1051-1100	32.25
1101-1150	33.75
1151-1200	35.25
1201-1250	36.75
1251-1300	38.25
1301-1350	39.75
1351-1400	41.25
1401-1450	42.75
1451-1500	44.25

The experiment commenced on the 4th June 2014 (day 0), when all fish were oral gavaged with appropriate slurry. The size of bream and the mean doses of nodularin slurry or control slurry are listed in Table 1. Bream were sampled on day 1, 2, 7, 14, 20 and 30 post gavage. Bream were euthanized in Aquis-S (Aquis-S New Zealand Ltd), the tag was read, the fish was weighed, and the total length was measured. Three gill filaments were removed, one preserved for histology, one for biomarker analysis and one for hepatotoxin analysis. After opening of the body cavity the gallbladder was removed and bile was transferred with a 1-ml syringe into an Eppendorf vial, placed on ice

together with the gallbladder which was placed into an Eppendorf vial and maintained on ice for hepatotoxin analysis. After removal of the liver, the liver was weighed and a piece of the liver (always the same area in the middle of the organ) was fixed for histology. The remaining liver was macerated and a small portion (approximately 100g) was stored in a labelled Eppendorf vial for biomarker analysis on dry ice and the remaining liver was wrapped in aluminium foil, placed on ice in order to be used for hepatotoxin analysis. The stomach and intestine were removed and a piece preserved for histology, the remainder wrapped in aluminium foil for hepatotoxin analysis. The heart and brain were also removed and a sample taken for biomarker analysis and the remainder was used for hepatotoxin analysis. A flesh sample was taken for biomarker and hepatotoxin analysis. The remainder of the fish carcass was wrapped in aluminium foil and frozen at -20^{o}C. Otoliths were removed for potential fish aging. The experiment was conducted under animal ethics permit number AEC SETP12 0088, Department of Environment and Primary Industries.

Samples for Toxin Analysis by LC-MS

Liver, muscle and gill samples were extracted and analysed by the commercial laboratory, Advanced Analytical. Samples were maintained frozen at -80^{o}C prior to being transferred to Advanced Analytical Laboratory in Melbourne. Samples were sent by Advanced Analytical Melbourne office to their chemistry laboratories in Sydney for sample extraction and analysis using LC-MS.

Toxin extraction and Analysis by ELISA

All solvents (methanol, hexane and water) in this study were of analytical or chromatographic grade (Sigma-Aldrich, GmbH; Merck Pty Australia). Ultrapure deionized water was produced by Milli-Q Plus equipment (Millipore, Bedford, MA, USA). Samples that were to be analysed by ELISA included heart, bile, gall bladder, brain, stomach and intestine. Samples of spiked food were also analysed by ELISA to allow comparison of this technique to LC-MS results.

Spiked and control food samples (2g) as well as *N. spumigena* cell concentrate (2mL) were placed into 50mL centrifuge tubes with methanol:water (10 mL; 75:25; v:v) and kept in the dark at 4^{o}C overnight. The samples were then sonicated (15 min; Bransonic 220, Smith Kline Co.), centrifuged (15 min, 4,000 rpm; Rotofix 32, Hettich, Germany), and the supernatant collected. The pellet was then re-extracted in methanol:water (10 mL; 75:25; v:v), sonicated (15 mins; Bransonic 220, Smith Kline Co.), centrifuged (15 mins, 10,000 rpm; Rotofix 32, Hettich, Germany) and the supernatant again separated from the pellet. The combined supernatants (food samples only) were shaken twice with hexane (25 mL) and diluted with deionised water to provide a methanol:water concentration < 10% v/v. The sample was then filtered through a GF/C filter (Whatman International, United Kingdom), before being loaded onto a pre-conditioned (5mL methanol followed by 5mL deionised water) SPE cartridge (Strata-X 500mg, 6mL syringe, Phenomenex, Australia). The cartridge was washed with MilliQ water (5 mL) followed by methanol (5mL, 10%), and then the nodularin eluted with methanol (5mL). The samples were evaporated under a stream of air, and finally resuspended in methanol:water (1mL; 10:90; v:v) for ELISA analysis.

The extracts were analysed using a direct competitive commercial ELISA kit (Abraxis Microcystins-ADDA ELISA, Abraxis LLC, USA) calibrated with 0.1, 0.5, 1.0, 5.0, 10.0 and 50.0 µg/L nodularin solutions. Each sample was screened with 1:10 and 1:100 dilutions. The analyses were performed according to the manufacturer's instructions with a microplate washer (Wellwash Microplate Strip washer, Thermo Scientific Pty Ltd) to wash plates. The absorbance at 450nm in each well was read by a Multiskan EX microplate reader (MTX Lab Systems, Inc) within 10 minutes of adding the stop solution.

Results

Food spiking

Nominal and actual concentrations in the spiked fish food slurries, as determined by ELISA and LC-MS, are shown in Table 3. The LC-MS results indicated that there was 6µg/Kg nodularin in the low dose fish food slurry and 27µg/Kg nodularin in the high dose food slurry. ELISA results were slightly higher than those detected using LC-MS with 15.5µg/Kg nodularin detected in the low dose food and 39.2µg/Kg nodularin in the high dose food.

Table 3: Nominal and measured concentrations of nodularin in fish food slurry (as determined by ELISA (n=3) and LC-MS (n=2)).

Dose in fish (µg/kg)	Expected Nodularin concentration (µg/Kg)	LC-MS (CV%) (µg/Kg)	ELISA (CV%) (µg/Kg)
Control (0)	0	0	0
50	167	6(0)	15.5(28.5)
200	667	27(5.4)	39.2 (32.8)

In order to verify the accuracy and precision of the ELISA measurements, check standards (i.e. nodularin standards run as samples) were run on each ELISA plate during each ELISA test. The ratio of nominal concentrations and result values averaged 106% (range 41 - 288%; n = 10). This result indicates the ELISA was on average slightly over estimating results (by 6% on average).

Nodularin uptake and elimination from Black bream

Liver, muscle tissue and gill samples from Black bream sampled on days 1, 2, 7, 14 and 20 together with control fish from day 1, were analysed by LC-MS. The results indicated that there was no nodularin present in tissues of control of fish gavaged with nodularin at either concentration, with all results being less than the method detection limit of 20µg/Kg. As the results of nodularin analysis in fish livers, muscle and gill samples from LC-MS showed no uptake of nodularin toxin and assessment of spiked food using both LC-MS and ELISA indicated lower than expected nodularin concentrations no further samples were processed using ELISA.

Discussion

The objective of the present study was to determine the tissue distributions and elimination rates of nodularin from the commercially relevant fish species, *Acanthopagrus butcheri* (Black bream) after oral doses of nodularin contaminated food. The occurrence of nodularin toxin in muscle and liver tissues of wild caught fish during algal blooms has been well documented (Sipia et al 2006; Karjalainen et al 2008; Mazur-Marzec et al 2006; Sipia et al 2001; Eaglesham et al 2002; Vanbuynder et al 2001; Department of Health Victoria 2014), however laboratory investigations to understand the tissue distribution and elimination rates of nodularin from fish after defined exposure are limited (Myers et al 2010).

The results of the current study showed that there was no uptake of nodularin toxin by fish exposed to nominal doses of 50µg/Kg or 200µg/kg nodularin. This result was unexpected based on the nominal concentrations dosed into the fish. However, comparison of nominal nodularin doses to actual measured nodularin concentrations in the toxic food indicated that the slurries used to dose the fish had significantly less nodularin than expected based on the nominal dose. Based on actual measured concentrations (LC-MS and ELISA) of nodularin in food slurries fish received a dose of between 2-5 µg/Kg for the low dose and 8-12 µg/Kg for the high dose. These concentrations of nodularin in the spiked food slurries (2-12 µg/Kg) would not be expected to result in uptake into the fish (Myers et al 2010), which is consistent with the observations made during this study.

High accuracy in toxin analysis is required for reliable results – notably if data are being used to determine the safety of seafood for human consumption. In the current study we employed two methods for the determination of nodularin in *N. spumigena* cell concentrate. The current regulatory method used for algal toxin monitoring in Australia, LC-MS, and enzyme-linked immunological assay (ELISA). The technique of ELISA is useful for routine screening of water for toxin contamination. In situations where the sample is well characterised in terms of toxin composition, and results are cross-calibrated initially and at periodic intervals against other techniques (LC-MS) it is usually regarded as a reliable measure of total toxin concentration equivalents (Nicholson and Birch 2001). The ELISA method has been used extensively in the scientific literature for screening of algal toxins in water, cyanobacterial cells and seafood matrices (Platt 2005; Soares et al 2004; Lehtonen et al 2003; Vourinen et al 2009; Kankaanpaa et al 2002; Kankaanpaa et al 2001; Kankaanpaa et al 2005; Sipia et al 2001; Karjalainen et al 2008; Sipia et al 2006; Mazur-Marzec et al 2006; Kankaanpaa et al 2007; Myers et al 2010; Paakkonen et al 2008). The use of ELISA to measure toxin concentrations is a considerably simpler, quicker, and less expensive method than LCMS.

The two detection methods, LC-MS and ELISA, produced comparable results in analysis of *N. spumigena* cell concentrate (13.81µg/g for LC-MS and 9.52µg/g ELISA), with the ELISA result being slightly less than that detected using LC-MS. Based on this result the analysis of *N. spumigena* cell concentrates after further culturing and harvesting, in order to determine nodularin concentrations for preparation of toxic food slurries, were undertaken using the ELISA detection method, with samples then frozen for later confirmation using LC-MS. This process is documented throughout the scientific literature, with various studies using ELISA to screen samples, due to it being more rapid and less costly, prior to confirmation of toxin concentrations using LC-MS (Platt 2005; Soares et al 2004; Lehtonen et al 2003; Vourinen et al 2009; Kankaanpaa et al 2002; Kankaanpaa et al 2001; Kankaanpaa et al 2005; Sipia et al 2001; Karjalainen et al 2008; Sipia et al 2006; Mazur-Marzec et al 2006; Kankaanpaa et al 2007; Myers et al 2010). However, from the results of actual measured concentrations of nodularin in spiked slurries using LC-MS it is likely that the ELISA method may have over-estimated the nodularin content of the *N. spumigena* cell concentrate, leading to an under dosing of the slurry provided to fish.

Investigation of scientific literature where ELISA has been used as a screening tool indicates studies where similar results of over-estimation of toxin concentrations as observed in the current study have

been documented. For instance Lehtonen et al (2003) reported the response of commercial ELISA to give a 10-20 fold higher hepatotoxin (nodularin) concentration than that measured with HPLC. Kankaanpaa et al (2002) and (2005) detected total hepatotoxins (nodularin and metabolites) in both sea trout and prawns using commercial ELISA at concentrations of 540 -1200µg/Kg and 800µg/Kg respectively, concentrations that should have been possible to be detected using HPLC, however were not. They reasoned this discrepancy was due to transformation of nodularin to other conjugate forms which could not be detected using HPLC, however these conjugates can still be easily detected using ELISA since their affinity for antibodies is not altered by transformation (Kankaanpaa et al 2002). The results of over estimation described above are consistent with the levels observed during the current study.

More recent investigations into the use of commercial ELISA kits as rapid screening tools for algal toxins confirm that over and under- estimation occurs in ELISA however indicate that of the commercially available kits, some have greater issues than others in relation to false positives or false negatives and over estimation. Eberhart et al (2013) investigated the use of four rapid screening tests for diarrhetic shellfish toxins in shellfish samples from Washington State, and compared them to LC-MS results. One of the methods assessed was the commercial ELISA produced by abraxis. The abraxis commercial ELISA was reported to give five false positives out of 23 samples (>20%) and overestimated the toxin concentration in five samples, while underestimated it in six. The abraxis ELISA kit showed the greatest number of false positives of all methods tested. In another study by Humpage et al (2007) investigating the use of four commercial ELISA kits (Abraxis, Envirologix, Beacon and Strategic diagnostics) and two in house developed ELISA kits for the analysis of nodularin and microcystins in water samples, the abraxis commercial kit was reported to overestimate in all samples. This study was undertaken by two laboratories, one in Australia and one in the US, on a number of samples including toxin standards and natural water samples. For calibrated standards the abraxis ELISA produced results 3-10 times higher than the standards, and for natural samples the abraxis kit detected toxin in 17 of 18 samples, while the other kits detected toxin in an average of 9 samples. The abraxis kit results were consistently 4 -10 times greater than those detected by all other kits. The authors concluded that the Envirologix, Beacon and Strategic Diagnostics kits successfully measured algal toxins accurately, while the abraxas kit overestimated toxin concentrations by a factor of 2.5 or greater 80% of the time, consistently overestimating compared to the other kits throughout the study.

Previously studies undertaken by Myers, 2008 and Myers et al 2010 have achieved good results with ELISA; however this work involved the use of the Envirologix commercial ELISA kit. The current study used the abraxis commercial kits, as used in the study by Humpage et al (2007) as they were the only commercially available kits distributed in Australia during the period of the study.

Conclusions

While the results of this study are inconclusive in terms of tissue distributions and elimination rates of nodularin from fish tissues due to the over estimation of nodularin in *N. spumigena* cell concentrate used for spiking into fish food, the study did identify positive outcomes regarding methodology of dosing fish via oral gavage and the development and validation of a LC-MS method for nodularin. The procedures to provide fish a dose via oral gavage proved to be fairly straight forward, the fish showed good recovery following anaesthetic and the procedures of insertion of the tube to provide the slurry, there was little to no regurgitation of the delivered doses with dissection of the fish post-mortem indicating the doses had been delivered into the stomach. There were no mortalities due to the procedures undertaken. While it was identified a risk that fish may not uptake toxin in our proposal and contingencies were in place to reduce this risk (FRDC project 2013/217), we did not anticipate issues with the commercial ELISA kits that we have used. From this study and the information discovered in the scientific literature regarding abraxis ELISA kits we would not recommend relying on their results to base crucial steps in experimental protocols such as determination of toxin levels

for appropriate dosing and rather in future to have all samples processed using LC-MS unless further validation of ELISA is undertaken or kits that a person on the research team has experience in using are employed. An important outcome from this work has been the development and validation of a commercially available method for nodularin and microsystin in seafood tissues at Advanced Analytical which is available to process seafood and algal samples in the event of toxic bloom for regulatory purposes.

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Appendix B: Objective 1 Report 2: Nodularin uptake, accumulation and tissue distribution in commercially and recreationally relevant fin-fish species under laboratory conditions.

CAPIM Technical Report #83

Nodularin uptake, accumulation and tissue distribution in commercially and recreationally relevant fin-fish species under laboratory conditions

Jackie Myers, Jonathan Puddick, Susie Wood and Vincent Pettigrove

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Glossary

Uptake	The absorption of a substance (in this case nodularin) by an organism and its organs.
Accumulation	The gradual build-up of a substance in an organism and its organs.
Depuration	The process of reducing the concentration of toxins up taken into tissues.
Nodularia spumigena	Filamentous cyanobacteria (blue-green algae) that is found in brackish water and produces the toxin nodularin.
Nodularin (e.g., NOD-R)	A hepatotoxic cyclic pentapeptide produced by the toxic filamentous cyanobacteria (blue-green algae) *Nodularia spumigena*.
Microcystin (e.g., MC-LR)	A hepatotoxic cyclic heptapeptide produced by many types of cyanobacteria.
FRDC	Fisheries Research Development Corporation.
Seafood	Any edible fish, prawns and shellfish collected from fresh, estuarine and marine waters for human consumption.
LC-MS/MS	Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS).
HPLC	High-performance liquid chromatography; a technique for separating compounds according to there physical properties
PPIA	Protein phosphatase inhibition assay; a means of assessing microcystin and nodularin concentrations according to their ability to inhibit protein phosphatase enzymes.
ELISA	Enzyme-linked immunosorbent assay; a means of assessing microcystin and nodularin concentrations using antibodies which specifically bind to structural elements of the compounds (generally the unique Adda moiety).
WHO	World Health Organisation.
CAPIM	Centre for Aquatic Pollution Identification and Management.

Executive Summary

The safety of seafood products as a food source is of great importance from both a public health and economic viewpoint. The occurrence of cyanobacterial blooms in fresh, estuarine and coastal waters can lead to the accumulation of cyanobacterial toxins in seafood species and there is a growing concern regarding the health risks associated with consumption of seafood.

Nodularin is a cyclic pentapeptide hepatotoxin produced by the cyanobacterium *Nodularia spumigena*, which forms blooms in estuarine and coastal systems. The Gippsland Lakes, situated in south-eastern Victoria, are one of Australia's largest lake systems and supports a range of recreational and commercial activities, including fishing. In the last decade the lakes have experienced an increase in the frequency and intensity of cyanobacterial blooms, with seven major blooms of *N. spumigena* since 1995. Prior to this only one significant bloom had been documented in the previous 20 years. In 1999 and more recently 2011-12 and 2012-13 toxic blooms of *N. spumigena* led to restrictions on the harvesting and sale of shellfish, prawns and un-filleted fin-fish for periods up to 6 months due to the presence of nodularin in seafood tissues. Significant economic losses have been incurred by the fishing and tourism industry in the region during the restricted periods and government agencies through the costs of monitoring the blooms and providing associated advice around seafood safety.

Due to the closures the commercial and recreational fishing sectors in the Gippsland Lakes have expressed concerns about the monitoring process for toxins in seafood during blooms. The lack of information on nodularin uptake and depuration in seafood is regarded as a significant knowledge gap, by government, as it hampers the ability to provide appropriate advice regarding harvesting and consumption of seafood during toxic blooms. In order to improve our understanding on the risks of nodularin to seafood safety, studies investigating uptake, accumulation and tissue distribution in commercially and recreationally relevant species are needed.

The present study assessed the uptake, accumulation and tissue distribution of nodularin in two commercially and recreationally relevant fish species. Black Bream (*Acanthopagrus butcheri*) and Southern Sand Flathead (*Platycephalus bassensis*) were exposed via oral gavage to a single dose of prawn slurry contaminated with *N. spumigena* cells containing nodularin (106 µg/kg w.w. or 776-869 µg/kg w.w.). At pre-determined time periods (1, 2, 7, 14 and 20 days) following dosing, fish were killed and various tissues (muscle, liver and gut) were sampled to assess for nodularin accumulation by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Nodularin was detected in liver, muscle and gut tissues of the exposed fish, while no to little nodularin was detected in tissues of control fish. The primary organ of nodularin accumulation was the liver (ca. 80% in both species), followed by the muscle (ca. 3.5%), with ca. 15% remaining in the gut. The concentration of nodularin accumulated by fish varied between species, tissue and by dose administered. Southern Sand Flathead accumulated the highest levels of toxin into both liver and muscle tissues of the two species (Southern Sand Flathead: max. 35.92 µg/kg w.w. and 4.35 µg/kg w.w., for liver and muscle respectively; Black Bream max. 24.54 µg/kg w.w. and 0.62 µg/kg w.w. for liver and muscle, respectively), when administered a similar dose. In Southern Sand Flathead administered different nodularin doses, fish that received the higher dose (776 µg/kg b.w.) had significantly higher nodularin concentrations in tissues than those administered the low dose (106 µg/kg b. w.). Accumulation and depuration patterns in fish also differed between species and administered dose. Southern Sand Flathead absorbed nodularin rapidly into livers with concentrations peaking 24 hrs post gavage, and thereafter they began to remove nodularin from this tissue. In muscle, nodularin was absorbed more slowly; peaking 7 days post gavage before being removed. The opposite was observed in Black Bream. Nodularin was absorbed rapidly into the muscle over the first 24 hrs, thereafter it was removed and in livers nodularin was absorbed more gradually, peaking at 14 days post gavage before concentrations being removed. Accumulation of nodularin was greater in Southern Sand Flathead compared to Black

Bream with an average 1.41% of the initially administered dose accumulated into Southern Sand Flathead tissues compared to 0.53% in Black Bream.

In Southern Sand Flathead administered a low dose of nodularin (106 µg/kg b.w.) a different accumulation pattern to that observed in fish administered a high dose (776 µg/kg b.w.) was observed. Fish gradually absorbed low levels of nodularin into both liver and muscle tissues (although greater in liver), with concentrations peaking at 14 and 20 days post gavage in each tissue, respectively. In fish from both treatment levels, the concentration peak observed in muscle tissues occurred six days following that observed in liver tissues. In terms of the percent of nodularin accumulated into fish relative to the dose administered, fish given a lower dose accumulated an average of 1.91%, while those at the higher dose an average of 1.41%.

Overall, the study found that nodularin uptake, tissue distribution, accumulation and elimination varied between two fish species, different tissues and with the level of dose administered. Black Bream, the species currently used as a sentinel species to provide health advisories in the Gippsland Lakes, accumulated lower concentrations of nodularin into tissues than that of Southern Sand Flathead and differences in accumulation patterns were observed. This result suggests that further research is needed on species of commercial and recreational importance in the Gippsland lakes to ensure the sentinel species will provide not only an early indication of risk, but also indicate safety following decline of blooms.

Levels of nodularin accumulated into fish tissues, following a single oral dose similar to that of which fish would be exposed to under field conditions can result in nodularin concentrations that exceed health alert guidelines (24 µg/kg whole fish). Exceedances were due to the elevated concentrations detected in livers, with concentrations in muscle tissues never reaching health alert levels. Processes to reduce risks from exposure to humans through fish consumption could include gutting and gilling of fish as this would significantly reduce the level of toxin exposure.

To obtain data that is of high reliability it is recommended that spike recoveries be conducted in all tissue matrices and for each species in any study. If possible the use of an internal standard is highly recommended as it significantly reduces the chances of over- or under-estimation in results.

Introduction

Worldwide cyanobacterial blooms are becoming recognised as a significant ecological problem, driven by increased nutrient exports (primarily nitrogen and phosphorus) from anthropogenic sources and increased water temperatures (Myers 2008). Many cyanobacteria species produce hazardous secondary metabolites known as cyanotoxins. These cyanotoxins pose a risk to human and animal health primarily through direct exposure or consumption of contaminated water (Ibelings and chorus, 2007; Bartrum et al 1999). Increased eutrophication, climate change and altered hydrological patterns are expected to intensify cyanobacterial bloom occurrences; this with a growing population and associated needs for water increases the opportunities for human exposures to cyanotoxins (O'Neil et al 2012; Sotton et al 2015). Fish and shellfish living in cyanobacterial contaminated waters can accumulate toxins, representing an underappreciated, but potentially major pathway of exposure.

The cyanobacterium *Nodularia spumigena* forms extensive regular and semi-regular blooms in estuarine and coastal environments around the world (Sivonen and Jones 1999; Van Buynder et al 2001; Kankaanpaa et al 2002; Sotton et al 2015) and can produce the toxin known as nodularin, a cyclic pentapeptide hepatotoxin. The accumulation of nodularin into seafood species, including fin-fish, is well documented during *N. spumigena* blooms, notably in the Baltic Sea (Sipia et al 2001a, 2001b) but also in New Zealand (Dolamore et al 2017) and Australia, in the Peel Harvey Estuary, a recreational lake in Queensland and the Gippsland Lakes (Eaglesham et al 2002; Falconer et al 1992; Van Buynder et al 2001; Stewart et al 2012; Department of Health 2014). Although there have been extensive studies on the accumulation of nodularin in fish under natural conditions (Van Buynder et al 2001; Sipia et al 2001a; Sipia et al 2001b; Eaglesham et al 2002; Karlsson et al 2005; Kankaanpaa et al 2005; Sipia et al 2006; Sipia et al 2007; Mazur-Marzec et al 2007; Karjalainen et al 2008; Stewart et al 2012; Marzur-Marzec et al 2013; Dolamore et al 2017), few studies have investigated the accumulation and tissue distribution of nodularin in fish under controlled laboratory conditions.

The Gippsland Lakes, one of Australia's largest lake systems, situated in south-eastern Victoria, represent a unique aquatic ecosystem of ecological significance as well as hosting beneficial uses including tourism, recreational and commercial fishing. The lakes are home to Victoria's largest fishing port and commercial fishing within the lakes amounts for approximately 200 tonnes of seafood annually and $1.1 million per year (DEDJTR 2016). They are also home to a significant recreational fishery, which on an annual basis may equal or exceed the commercial sector catch for some species (DEDJTR 2016). In the last few decades the lakes have experienced an increase in the frequency and intensity of cyanobacterial blooms, with twelve major blooms since 1985 (Day et al. 2011). In 2001-02 and more recently 2011-12 and 2012-13 toxic blooms of *N. spumigena* led to lengthy restrictions on the harvesting and sale of shellfish, prawns and un-filleted fin-fish due to the presence of nodularin in seafood tissues.

The *N. spumigena* blooms in the Gippsland lakes have resulted in significant economic losses for both the fishing and tourism industries in the region and a high financial cost for government agencies monitoring the blooms. In recent years there have been concerns raised regarding the monitoring processes for seafood during toxic blooms, with the lack of information on nodularin uptake, accumulation and tissue distribution in fin-fish species used to determine seafood risks during a bloom identified as impeding the ability to provide appropriate advice to commercial and recreational fishers regarding harvesting and consumption during toxic blooms.

Current monitoring and advisories provided to recreational and commercial fishers are based on toxin detected in a sentinel species, Black Bream, collected during blooms. This species was chosen as a sentinel based on its importance to commercial and recreational fishers, the detection of elevated levels of nodularin in tissues relative to other fish species during blooms in the Gippsland Lakes during

2002 (Van buynder et al 2002) and as it was thought that Black Bream would be more likely to accumulate cyanobacterial toxins to a greater extent than other species of fin-fish as their diet included shellfish and prawns, which are known to ingest cyanobacteria (Anonymous, 2011). Further, Black Bream are resident in the Gippsland Lakes during summer, whereas other species move between the marine environment and the lakes. As a result Black Bream would spend more time in areas affected by blooms (Anonymous, 2011). The reasoning for use of Black Bream as a sentinel has several drawbacks. Firstly, the results on nodularin concentrations in the 2002 blooms were based on limited numbers of a few species of fish, and often only one individual per species (Vanbuynder et al 2002). Further, high variability in cyanotoxin concentrations between individuals within a species are well documented in field and laboratory studies (Kankaanpaa et al 2005; Cazenave et al 2005; Kankaanpaa et al 2005; Paakkonen et al 2008; Karjalainen et al 2008; Acou et al 2008). The results from the 2002 bloom would therefore not give a statistically sound understanding of the species differences in toxin accumulation. Secondly, there is a growing body of literature on the uptake/accumulation of nodularin, and the closely related microcystins, in field-collected fish which indicates that feeding group or trophic level may influence toxin accumulation, however the results are contradictory (Xie et al 2005; Ibelings et al 2005; Zhang et al 2009; Berry et al 2011; Kopp et al 2013; Jia et al 2014; Rezaitaber et al 2017).

Over 170 species of fish have been recorded within the Gippsland Lakes (EPA 2015). Commercial catch consists primarily of Black Bream (*Acanthopagrus butcheri*), anchovy (*Engraulis australis*), sea mullet (*Mugil cephalus*) and silver trevally (*Pseudocaranx georgianus*). Other species taken in this fishery include dusky flathead (*Platycephalus fuscus*), carp (*Cyprinus carpio*), king george whiting (*Sillaginodes punctatus*), eastern river garfish (*Hyporhamphus regularis*), yellow-eye mullet (*Aldrichetta forsteri*), luderick (*Girella tricuspidata*), Australian salmon (*Arripis trutta*), estuary perch (*Macquaria colonorum*), tailor (*Pomatomus saltatrix*) and leartherjacket (*Oligoplites saurus*)(Conron 2016; Department of Primary Industries 2012). Recreational fishing in the area includes shore and boat-based anglers which frequent the Gippsland Lakes and the estuarine reaches of the inflowing rivers where they predominantly target Black Bream, dusky flathead, silver trevally and yellow-eye mullet. The fishery is most active from spring to autumn (Conron 2016). In order to provide confidence in the sentinel species chosen to provide advisories around fish safety during toxic bloom events an understanding of nodularin uptake and accumulation in various species of importance in the Gippsland Lakes and from different feeding niches and trophic levels are needed.

The highest concentrations of nodularin are typically reported in the liver and gut of fish (see reviews and papers by Jia et al. 2014; Stewart et al. 2012; Berry et al. 2011; Ibelings and Chorus 2007; Myers et al. 2010). While these tissues are not typically eaten and their removal has been shown to significantly lower nodularin exposure (Myers et al. 2010; Ibelings and Chorus 2007; Drobac et al. 2013), elevated concentrations, exceeding tolerable daily intake levels, have been measured in edible portions (muscle) and there are various communities around the world that eat whole fish (Jia et al. 2014; Stewart et al. 2012; Berry et al. 2011; Ibelings and Chorus 2007; Myers et al. 2010; Drobac et al. 2013). Recreational fishers are often advised during cyanobacterial blooms to gut and gill fish prior to consumption and the sale of fin-fish has been allowed if sold as fillets or gutted and gilled fish (NSW Food Authority, 2012; Victorian Bays and Inlets Fisheries Association 2013). In order to understand risks associated with provision of gut and gill recommendations for fish species from the Gippsland Lakes further information on the accumulation and tissue distribution in locally relevant species is required.

Objectives

This study is part of FRDC project 2013/21, Development of Management recommendations to assist in advisories around seafood safety during toxic bloom events in the Gippsland Lakes. The objective of this component was to examine the uptake, accumulation, tissue distribution and depuration of

nodularin in commercially and recreationally relevant fish species experimentally exposed to nodularin through food. A further aim was to assess for any species- or dose-dependant differences in accumulation, tissue distribution and depuration. This information will be incorporated into protocols to manage the risks to seafood safety during toxic blooms to improve fish sampling protocols and determine which tissues of the fish may be safe for consumption.

The specific objectives of this study were:

- To determine uptake, accumulation and tissue distribution of nodularin in Black Bream and Southern Sand Flathead following oral exposure.

- To determine if there were any concentration- and/or species-dependent differences in uptake, accumulation and tissue distribution and depuration following nodularin exposure.

Method

Experimental Fish

The fish species chosen for this study were the Southern Sand Flathead (*Platycephalus bassensis*) and Black Bream (*Acanthopagrus butcheri*). These species were chosen based on their importance as commercial and recreational species and as they represent different feeding groups, which would therefore be exposed to cyanobacterial toxins through different ways. Black Bream is one of the most important recreational and commercial species in the Gippsland Lakes. Recent estimates indicate that Black Bream constitute 37% of the total commercial catch (Conron 2016). Given the popularity of recreational fishing in the region, recreational catch is considered likely to equal or to exceed that of the commercial sector (DEDJTR 2016). Black Bream are opportunistic omnivores, consuming a wide range of prey, including sessile, burrowing, benthic and pelagic species such as mussels, barnacles, tubeworms, crabs, bloodworms, squirtworms, ghost shrimp, cockles, prawns, amphipods, copepods, small fish (i.e.: gobies, hardyheads and anchovies) and plant material including algae (Norris et al 2002; Sarre et al 2000). Dusky flathead (*Platycephalus fuscus*) are a common species in the Gippsland Lakes, forming a small part of commercial catch but are more significant as a recreational species. In this study, we used the closely related species *Platycephalus bassensis* (Southern Sand Flathead) (Department of Primary Industries 2012). This was due to ease of capture of this species within close vicinity of Victorian Marine Science Consortium (VMSC) laboratory facilities. Flathead are carnivores, feeding primarily on fish, prawns, squid and also large benthic crustaceans. They are considered ambush predators, hiding from their prey by burying in the sediment (Perry et al 1995).

Black Bream were caught by seine net from Swan Bay, a coastal embayment near Queenscliff in Victoria during September and October 2016. They were transported (ca. 1.6 km) in a 1000-L polyethylene tank, in brackish water, to the laboratory of the VMSC, Queenscliff, where the feeding experiments were performed. Southern Sand Flathead were caught by rod and line from Port Phillip Bay, Victoria during October 2016. They were transported (ca. 15 km) back to the VMSC laboratory, in 50-L polyethylene tubs, filled with in seawater from the capture site.

At the laboratory Black Bream were transferred to 5000-L dark grey round poly tanks, supplied with flow through seawater and constant aeration (air stones). Southern Sand Flathead were transferred to 1000-L flat bottomed oval poly tanks, maintained under flow through seawater and constant aeration (air stones). Fish were held in acclimation for between 8 and 20 days prior to starting the experiments. During this time they were fed a diet of chopped raw prawns three times a week. All tanks were situated under a large outdoor igloo and received natural light and photoperiods. During acclimation the water temperatures were 14.3^{o}C, while during the experiments average water temperatures were 15.6^{o}C.

Nodularia spumigena culture and toxin production

A non-axenic strain of *N. spumigena*, originally isolated from field samples collected from the Gippsland Lakes (March 2003, J. Myers) was grown in batch cultures. The culture was maintained in MLA media (Bolch and Blackburn 1996) at 21 ± 1^{o}C under constant illumination (cool white fluorescent lamps) at the CAPIM laboratory in the School of BioSciences, University of Melbourne Parkville campus.

N. spumigena cells were harvested from batch cultures to prepare a nodularin contaminated slurry. At fortnightly intervals, *N. spumigena* cultures were centrifuged (15 min, 4,000 × g, Multifuge 3 S-R, Heraeus Germany), the supernatant removed and the *N. spumigena* cell pellets collected and stored at -20^{o}C. Once a significant amount of *N. spumigena* biomass had been collected, the frozen pellets were freeze dried at -50^{o}C (Freezone 2.5, Labconco), then combined and homogenised using a mini

mill (Pulverisette 23, Fritsch Germany). A subsample of the freeze-dried biomass was sent to Cawthron (Nelson, New Zealand) for analytical determination of nodularin-R (Nod-R), while the remainder was stored at -80°C for later use in the preparation of toxic slurry.

Nodularin concentrations in the collected cell biomass were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using a clean metal spatula, the dried sample was weighed out in triplicate (0.20-0.25 g) and the weight was recorded to four decimal places. Each sample was extracted in 80% methanol + 0.1% formic acid (5 mL) in a bath sonicator with ice (30 min). The extracts were clarified by centrifugation (3,200 × g; 10 min) and stored at -20 °C until analysis (within 1 week).

Prior to analysis, sample extracts were diluted 1/100 and 1/1000 in 80% methanol. The extract components were separated on a Waters BEH-C18 Column (50×2.1 mm; 1.7-µm) using a gradient of Milli-Q water to acetonitrile supplemented with 100 mM formic acid and 4 mM ammonia. The eluting compounds were ionised by electrospray ionisation mass spectrometry and analysed using a multiple reaction monitoring method assessing for nodularin as Nodularin-R (Nod-R; the most common nodularin variant) and microcystins (MC-RR, dmMC-RR, didmMC-RR, MC-LR, dmMC-LR, didmMC-LR, MC-AR, MC-YR, MC-FR, MC-WR, MC-RA, MC-RAba, MC-LA, MC-FA, MC-WA, MC-LAba, MC-FAba, MC-WAba, MC-LY, MC-LF, MC-LW). The analytical detection limit for all analytes was 0.2 ng/mL equating to a method detection limit of 0.5 µg/g in the samples (if 0.2 g of cyanobacterial sample was extracted and the sample was diluted 1/100).

The average Nod-R content of the cyanobacterial biomass was 700 µg/g (Table 1). None of the microcystin variants analysed were detected in the sample (< 0.5 µg/g). This concentration is in line with that reported for Australian strains of *N. spumigena* (730 µg/g to 1000 µg/g (Jones et al 1994; Platt 2005) and is similar to toxin concentrations previously determined for this isolate of 100-600 µg/g (Myers, 2008). Using this value as a guide, the *N. spumigena*-containing slurry for oral gavage was prepared as described below.

Table 1: Liquid chromatography-tandem mass spectrometry analysis for nodularin-R (Nod-R) in the *Nodularia spumigena* biomass sample.

Sample Replicate	Sample Weight (g)	Nod-R Content (ug/g)[a]
A	0.2407	720
B	0.2263	680
C	0.2474	710
	Average	700

[a] Values are rounded to two significant figures.

Preparation of the Nodularia spumigena-containing slurry

The mixture to be administered to Black Bream and Southern Sand Flathead was composed of the laboratory cultured and freeze-dried *N. spumigena* biomass and eastern king prawn (*Melicertus plebejus*) homogenate. The prawn homogenate consisted of ground eastern king prawns and ultra-pure water (Milli-Q Plus system; Millipore, Bedford, MA, USA). Based on trials of slurry preparation and post-mortem food delivery into the stomachs of fish, a suitable mixture of ground prawns and water was determined to be 20 g ground whole prawns to 60 mL water. Eastern king prawns were purchased from a local supermarket. Whole prawns were ground with a coffee grinder (150 Watt, Homemaker Australia) and mixed with Milli-Q water. The resulting mixture was then passed through a mesh tea strainer to remove large pieces of prawn which we found blocked the syringe. This produced a homogenate that was thick enough to stay in the fish stomach, but soft enough to be injectable through the gavage syringe.

Two *N. spumigena*-containing slurry's were prepared by mixing the prawn homogenate with an appropriate mass of freeze dried *N. spumigena* biomass to produce a calculated nodularin concentration of 3 and 10 µg/mL. These calculated concentrations were determined to be appropriate to deliver the nominal doses of 50 µg/kg b.w. and 200 µg/kg b.w. to fish, while allowing for similar volumes of slurry to be injected into fish stomachs for both treatment levels. The control slurry consisted of the prawn homogenate without freeze-dried *N. spumigena* biomass. Table 2 details the volumes of prawn homogenate and freeze dried *N. spumigena* biomass added to produce the toxic slurry's for Black Bream and Southern Sand Flathead exposures.

Table 2: Slurry mixture ratios to produce nominal doses for control (0 µg/kg b.w.), low (50 µg/kg b.w.) and high (200 µg/kg b.w.) nodularin treatments for Southern Sand Flathead and control (0 µg/kg b.w.) and high (200 µg/kg b.w.) treatments for Black Bream experiments.

Dose	Toxin Concentration needed (µg/mL)	Prawn homogenate (mL)	*N. spumigena* biomass added (g)
Black Bream			
Control	0	500	0
200 µg/kg	10	800	12
Southern Sand Flathead			
Control	0	120	0
50 µg/kg	3	120	0.6
200 µg/kg	10	120	1.8

Laboratory experiment: Exposure and sampling

For estimation of the correct feeding tube delivery depth, post-mortem measurements of the body length and length from the tip of the snout to the stomach of five Black Bream and five Southern Sand Flathead, purchased from a local fish supplier were made. This allowed estimation of tubing depths in fish of different lengths.

Nodularin was dosed orally as a single delivery of the slurry directly into the stomachs of fish to achieve nominal doses of either 50 or 200 µg nodularin/kg fish for Southern Sand Flathead and 200 µg nodularin/kg fish for Black Bream. Control fish were dosed in the same way as nodularin-exposed fish. Immediately prior to dosing, fish were anesthetized one at a time in aerated seawater containing AQUI-S (AQUI-S New Zealand Ltd). Under anaesthesia fish were weighed and measured. The slurry containing nodularin was then delivered into the stomachs of the experimental fish, while the slurry without *N. spumigena* cells was delivered into the stomachs of the control fish. The amount to be delivered to each individual was determined on the basis of fish weight and a table that was compiled beforehand (see Tables 3 and 4). The slurry was drawn into a 50-mL syringe fitted with a piece of flexible polypropylene tube (of a specified length) and the food was delivered directly into the stomach. The syringe was weighed before and after injection to obtain the exact amount of slurry delivered. After treatment fish were placed in a 500-L resin tank and once they had recovered, they were returned to their appropriate treatment tank. For Southern Sand Flathead experimental tanks consisted of two 5,000-L round poly tanks for the exposed fish and control fish were maintained in a 1,000-L oval poly tank. Black Bream experimental tanks consisted of 5,000-L round poly tanks, one for control fish and one for exposed. All tanks were maintained under flow-through seawater as described previously for acclimation.

Table 3: Dose of *Nodularia spumigena* contaminated or uncontaminated (control) slurry administered to Black Bream based on weight of the fish.

Fish Wet weight (g)	Dose administered to give a nominal 200 µg/kg in fish (mL)
151-200	3.50
201-250	4.50
251-300	5.50
301-350	6.50
351-400	7.50
401-450	8.50
451-500	9.50
501-550	10.50
551-600	11.50
601-650	12.50
651-700	13.50
701-750	14.50
751-800	15.50
801-850	16.50
851-900	17.50
901-950	18.50
951-1000	19.50
1001-1050	20.50
1051-1100	21.50
1101-1150	22.50
1151-1200	23.50
1201-1250	24.50
1251-1300	25.50
1301-1350	26.50
1351-1400	27.50
1401-1450	28.50
1451-1500	29.50
1501-1550	30.50
1551-1600	31.50
1601-1650	32.50
1651-1700	33.50
1701-1750	34.50
1751-1800	35.50

Table 4: Dose of *Nodularia spumigena* contaminated or uncontaminated (control) slurry administered to Southern Sand Flathead based on weight of the fish.

Fish Wet weight (g)	Dose administered to give nominal a 50 µg/kg in fish (mL)	Dose administered to give a nominal 200 µg/kg in fish (mL)
30-40	0.58	0.70
41-50	0.75	0.90
51-60	0.92	1.10
61-70	1.08	1.30
71-80	1.25	1.50
81-90	1.42	1.70
91-100	1.58	1.90
101-110	1.75	2.10
111-120	1.92	2.30
121-130	2.08	2.50
131-140	2.25	2.70
141-150	2.42	2.90
151-160	2.58	3.10
161-170	2.75	3.30
171-180	2.92	3.50
181-190	3.08	3.70
191-200	3.25	3.90

The experiment was started on the 19th October 2017 for Southern Sand Flathead and the 1st November 2017 for Black Bream (day 0), when all fish received a single dose of appropriate slurry via oral gavage. During the experimental period, fish were fed uncontaminated chopped raw prawns three times per week. For both experiments, exposed fish were sampled 1, 2, 7, 14 and 20 days post gavage. Control fish were sampled on days 1 and 20 for both species. For Black Bream, an additional set of control fish were sampled on day 14.

At each sampling point, fish were euthanized one at a time in AQUIS-S (AQUIS-S New Zealand Ltd), then the spinal cord severed. Each fish was weighed and the total and fork lengths were measured. The body cavity was opened, the fish was sexed and various organs were removed. The liver and gut (which included tissues that would be removed upon gutting a fish; heart, kidney, intestine, stomach and gall bladder, herein denoted as "gut") were weighed and kept for Nod-R analysis. A flesh sample was taken from behind the left pectoral fin, for Nod-R analysis. A small section of the liver (always the same area at the top of the organ) was fixed for potential histological analysis. Similarly, the gonads were weighed and fixed for potential histological analysis. The remainder of the fish carcass was wrapped in aluminium foil and frozen at -80°C. All samples were maintained at -80°C until further processing. The experiment was conducted under animal ethics permit number AEC 1613834.1 (University of Melbourne).

Nodularin determination

Toxin extraction and clean-up

All solutions were prepared with analytical reagent grade chemicals and ultrapure water produced by purifying distilled water with a Milli-Q Plus system (Millipore, Bedford, MA, USA).

Frozen liver and gut samples were homogenised using a coffee grinder (150 Watt, Homemaker Australia) prior to extraction. Homogenised liver and gut samples, muscle tissue and *N. spumigena* biomass spiked food samples and control food samples (ca. 1 g) were extracted in 15 mL methanol:water (75:25; v:v) overnight (4^{o}C in the dark), after sonication in a ultrasonic bath (15 min; Bransonic 220, Smith Kline Co.). Prior to sonication and refrigeration all samples were fortified with the microcystin variant desmethyl-microcystin –LR (dmMC-LR; 100 µL, semi-purified extract from *Microcystis* CAWBG617; Cawthron Institute, New Zealand) to allow determination of analyte recovery in each sample (see further details in assessment of recovery and suppression effects below). The extracted samples were centrifuged (15 min, 1,431 x g; Rotofix 32, Hettich, Germany), and the supernatant collected. The pellet was re-extracted in 10 mL methanol:water (75:25; v:v), sonicated (ultrasonic bath, 15 mins; Bransonic 220, Smith Kline Co.), centrifuged (15 min, 1,431 x g; Rotofix 32, Hettich, Germany) and the supernatant collected. The combined supernatants were shaken twice with hexane (25 mL), the methanol fraction collected and diluted with Milli-Q water to provide a methanol:water concentration < 10% v/v. The diluted extract was then filtered through a GF/A filter (Whatman International, United Kingdom), before being loaded onto a pre-conditioned (5 mL methanol followed by 5 mL deionised water) solid-phase extraction cartridge (Strata-X 500 mg, 6 mL syringe, Phenomenex, Australia). The cartridge was washed with Milli-Q water (5 mL) followed by 10% methanol (5 mL; v/v), and then the nodularin/dmMC-LR was eluted with 100% methanol (5 mL). The eluate was evaporated to dryness in a water bath (Heath Lab, Clayson Laboratory Products, New Zealand) at 24^{o}C, under a stream of compressed air, and sent to Cawthron for LC-MS/MS analysis.

Toxin analysis by tandem liquid chromatography-tandem mass spectrometry

Dried extracts were resuspended in 0.5 mL of 80% methanol + 0.1% formic acid and transferred into septum-capped glass vials for toxin analysis by LC-MS/MS. Control samples were divided between two vials and one set was fortified with a known concentration of Nod-R and MC-LR to assess for mass spectrometer suppression or enhancement effects. The extract components (5 µL) were separated on a Waters BEH-C18 Column (50×2.1 mm; 1.7-µm) using a gradient of 10% acetonitrile (ACN) + 100 mM formic acid (FA) + 4 mM ammonia (NH_3; Solvent A) to 95% ACN + 50 mM FA + 2 mM NH_3 (Solvent B). The sample components were loaded on to the column in 0.4 mL/min of 20% B which was maintained for 30 s before increasing linearly to 40%B over 30 s and then increasing linearly to 55%B over a further 3 min. The column was then washed with 99%B for 4 min before equilibrating with 20%B for 1 min prior to the next injection. The eluting compounds were ionised by electrospray ionisation MS in positive mode and analysed using a multiple-reaction monitoring method assessing for the nodularin variants Nod-R (825.4 > 135.1) and desmethyl-nodularin-R (dmNod-R)(811.4 > 135.1), and microcystin variants MC-LR (995.7 > 135.1) and dmMC-LR (981.65 > 135.1). Nodularin in experimental fish tissues and food slurry is reported as the combination of Nod-R and dmNod-R variants.

The analytical limit of quantitation was determined to be 0.05 ng/mL, by assessing the signal-to-noise ratio (S/N) of each of the injections for the 2 ng/mL standard and using the average S/N to calculate the Nod-R concentration which would still provide S/N = 10. The detection limit in each sample matrix was calculated using this analytical detection limit of 0.05 ng/mL, assuming 1 g of sample was used, incorporating the average mass spectrometer suppression/enhancement effects (for that sample matrix) and the average analyte recovery level observed (for that sample matrix).

Assessment of recovery and suppression effects

In order to assess the levels of nodularin recovery from the different sample matrices and any mass spectrometer suppression/enhancement effects spiking experiments were undertaken. To assess loss

of nodularin during sample extraction and clean-up (i.e., nodularin recovery) samples of muscle, liver and gut tissues from Black Bream and Southern Sand Flathead (ca. 0.5-1.5 g) were fortified with nodularin standard (Enzo life sciences, USA) at a one of two levels (0.3 ng/g and 15 ng/g) prior to extraction. Control samples (unfortified tissues) and dried aliquots of the nodularin fortification material were also prepared to determine the level in the fortification material. Samples were extracted as per the method described above, excluding the addition of the dmMC-LR standard. They were then sent to Cawthron and analysed using LC-MS/MS as described above.

To assess the different tissue extracts for mass spectrometer suppression/enhancement effects, resuspended extracts of control samples were fortified with a known concentration of Nod-R. These samples were then analysed by the LC/MS-MS method described above.

Results

Recovery and Liquid chromatography-tandem mass spectrometry suppression effects experiments

Mass spectrometer suppression effects observed for the different tissue matrices and fish species are shown in Table 5. The muscle tissues demonstrated mass spectrometer suppression effects of ca. −20% for both species. The suppression effects for the liver samples were more significant (ca. −60%) in both Black Bream and Southern Sand Flathead, while the gut samples were −50% and −61% (in Black Bream and Southern Sand Flathead fish respectively).

Table 5: Mass spectrometer ionisation suppression for nodularin-R in tissue sample extracts from Black Bream and Southern Sand Flathead.

Sample	Nodularin-R Concentration (ng/mL)			Mass Spectrometer Suppression
	Observed	Background	Expected	
Black Bream – muscle	19.2	0.3	23.6	-20%
Black Bream – liver	9.7	-	23.6	-59%
Black Bream - gut	12.2	0.3	23.6	-50%
Southern Sand Flathead – muscle	17.9	0.3	23.6	-25%
Southern Sand Flathead – liver	8.6	-	23.6	-64%
Southern Sand Flathead - gut	9.3	-	23.6	-61%

When the fortified and control samples were analysed by LC-MS/MS, low levels of nodularin-R was detected in half of the control samples and higher levels were observed in all of the high level fortification samples (15 ng/g; Table 6). When the LC-MS/MS results were compensated for mass spectrometer suppression effects (tissue- and fish-specific), the analyte recovery levels ranged between 48% to 113% recovery. Whilst the recovery levels varied between the different tissues and between the different fish species, recovery levels of the duplicate preparations were relatively consistent. Because of the low recovery levels and mass spectrometer suppression effects observed, the results from the low level fortification samples (0.3 ng/g) were not assessed.

Table 6: Liquid chromatography-tandem mass spectrometry analysis of nodularin-R in tissue sample extracts from Black Bream (BB) and Southern Sand Flathead (SF) to assess analyte recovery levels. ND = Not detected (<0.2 ng/mL).

Sample	Sample Weight (g)	Nodularin-R Conc. (ng/mL)	Nodularin-R Content (ng/g)	Adjusted for MS Suppression (ng/g)	% Recovery
BB - Muscle F1 - Control	1.186	0.3	0.1	0.2	-
BB - Muscle F2 - Control	1.163	0.3	0.1	0.2	-
BB - Muscle F1 - Fortified	1.025	19.8	9.6	12	80%
BB - Muscle F2 - Fortified	1.032	18.2	8.8	11	73%
BB - Liver F1 - Control	1.206	ND	-	-	-
BB - Liver F2 - Control	1.187	ND	-	-	-
BB - Liver F1 - Fortified	1.037	7.2	3.5	8.4	56%
BB - Liver F2 - Fortified	1.087	9.2	4.2	10.3	69%
BB - Gut F1 - Control	1.277	0.3	0.1	0.2	-
BB - Gut F2 - Control	1.003	0.4	0.2	0.4	-
BB - Gut F1 - Fortified	1.108	18.9	8.5	16.9	113%
BB - Gut F2 - Fortified	1.113	18.2	8.2	16.2	108%
FH - Muscle F1 - Control	1.048	0.3	0.1	0.2	
FH - Muscle F2 - Control	1.052	0.3	0.1	0.2	-
FH - Muscle F1 - Fortified	1.049	11.4	5.4	7.3	48%
FH - Muscle F2 - Fortified	1.085	11.9	5.5	7.3	49%
FH - Liver F1 - Control	0.493	ND	-	-	-
FH - Liver F2 - Control	0.499	ND	-	-	-
FH - Liver F1 - Fortified	0.629	5.6	4.5	12.3	82%
FH - Liver F2 - Fortified	0.524	3.5	3.4	9.3	62%
FH - Gut F1 - Control	1.047	ND	-	-	-
FH - Gut F2 - Control	1.013	ND	-	-	-
FH - Gut F1 - Fortified	1.078	7.7	3.6	9.1	61%
FH - Gut F2 - Fortified	1.004	8.5	4.2	10.7	72%

Recovery and suppression effects in exposure experiment samples and method detection limits

Due to the variability in analyte recovery observed using different sample matrices (see results section: Recovery and Liquid chromatography-tandem mass spectrometry suppression effects experiments above), it was decided that an internal standard should be incorporated into the sample extraction procedure. For this the microcystin variant desmethyl-Microcystin-LR (dmMC-LR) was used. A known amount of dmMC-LR was added to each sample prior to extraction, so the nodularin results could be compensated for analyte losses according to the concentration of microcystin measured in each sample. Aliquots of the dmMC-LR standard were also dried and analysed alongside the fish samples to ensure accurate determination of the level of microcystin added. Further control samples, for each tissue from each fish species, were also processed with actual samples to better assess the

levels of mass spectrometer suppression/enhancement and enable compensation of these effects in the results.

When the different tissue extracts from the exposure experiment were assessed for mass spectrometer suppression/enhancement effects, the average effects observed ranged from 92% suppression (8% of the expected result) to 40% enhancement (140% of the expected result). This was dependent mostly on the tissue type, but to some degree on fish species. The muscle samples showed the least mass spectrometer ionisation effects and the most severe effects were observed in the gut samples. The suppression effects observed for MC-LR were less severe than those for Nod-R and enhancement effects for MC-LR were observed in the majority of the sample matrices (Table 7).

Because of the severity of suppression/enhancement effects observed in some of the sample matrices, nodularin concentrations in the samples (combination of Nod-R, dmNod-R) were adjusted for these effects using the average value for the corresponding sample matrix (Table 7). Due to the analyte losses observed during preparation of the extracts, the nodularin concentrations were also adjusted for the observed recovery of internal standard (dmMC-LR) in each individual sample. Internal standard recoveries in food slurry samples averaged 59% and 55% for Black Bream and Southern Sand Flathead, respectively. In Black Bream muscle and liver tissue recoveries averaged 59% and 30% respectively, and in Southern Sand Flathead averaged 66% and 25% in muscle and liver tissues, respectively (Table 7, data for individual samples are detailed in Appendix A). Matrix specific detection limits are also detailed in Table 7. All results presented herein are adjusted based on the average LC-MS/MS suppression/enhancement effects and dmMC-LR recovery levels for each individual sample.

Table 7: Calculated detection limits in the assessed sample matrices, after incorporation of average mass spectrometer ionisation suppression and enhancement effects for nodularin (combination of nodularin-R and desmethyl-nodularin-R) and recovery levels of desmethyl-microcystin-LR.

Fish	Sample Matrix	Suppression / Enhancement Effects [a]		dmMC-LR Recovery [b]	Matrix Specific Detection Limit (µg/kg) [c]
		Nod-R/dmNod-R	dmMC-LR		
Black Bream	Food	−42%	+37%	59%	0.07
	Muscle	−12%	+21%	59%	0.05
	Liver	−55%	-13%	30%	0.19
	Gut	−92%	-90%	110%[d]	0.27
Southern Sand Flathead	Food	−36%	+40%	55%	0.07
	Muscle	−8%	+20%	66%	0.04
	Liver	−12%	+13%	25%	0.12
	Gut	−80%	-85%	67%[d]	0.19

[a] Average mass spectrometer suppression/enhancement effects observed for this sample matrix. [b] Average recovery of desmethyl-microcystin-LR observed for this sample matrix. [c] Calculated using the analytical detection limit (0.05 ng/mL) and assuming 1 g of sample was used. [d] Previously determined (see Recovery and LC-MS/MS suppression effects experiments Table 6).

Measured concentrations of nodularin in spiked slurry and fish doses

Expected nominal and measured concentrations of nodularin (combination of Nod-R and dmNod-R) in the *N. spumigena*-contaminated slurries are shown in Table 8. The LC-MS/MS analysis detected an average of 38,570 µg/kg nodularin in the high dose food slurry for Black Bream. In the contaminated slurry for Southern Sand Flathead, LC-MS/MS analysis detected an average of 6,720 and 38,905 µg/kg nodularin for the low and high dose food, respectively. These concentrations were on average ca. 4-times greater in the high doses than the expected concentrations and ca. 2-times for the low dose (Table 8). There was no nodularin detected in any of the slurry prepared as a control (Table 8).

Table 8: Liquid chromatography-tandem mass spectrometry analysis of nodularin (the combination of nodularin-R and desmethyl-nodularin-R) in slurry samples fed to fish.

Slurry Sample	Total Nodularin (ng/mL)[a]	dmMC-LR (ng/mL)[a]	dmMC-LR recovery	Measured Nodularin Content[b] (µg/kg)	Expected Nominal Nodularin Content (µg/kg)
FH 200 µg/kg	34,400	25.3	32%	41,180	10,000
FH 200 µg/kg	27,720	23.8	30%	36,630	
FH 50 µg/kg	10,060	63	80%	5,210	3,000
FH 50 µg/kg	11,590	39.3	50%	8,230	
BB 200 µg/kg	42,600	40.8	46%	36,850	10,000
BB 200 µg/kg	60,910	37.9	42%	40,290	
FH control	ND	54.5	69%	0	0
FH control	ND	54.7	69%	0	
BB control	ND	68.3	76%	0	
BB control	ND	63.5	71%	0	

[a] Concentrations were adjusted for mass spectrometer suppression/enhancement effects using the corresponding average value from Table 7. [b] adjusted based on the dmMC-LR recovery levels for each individual sample. ND = Not detected (<0.05 ng/mL). FH = Southern Sand Flathead, BB = Black Bream

Average fish weights, lengths and the doses received are shown in Table 9. There were significant differences in the weight and body length of control and nodularin exposed Black Bream (ANOVA: Weight, $F(1, 32) = 5.96$, $P = 0.02$; Length, $F(1, 32)= 6.92$, $P = 0.013$). However there were no differences between weight and length of Southern Sand Flathead in the different treatments (ANOVA: Weight, $F (2, 41) = 1.06$, $P = 0.36$; Length, $F(2, 41) = 0.81$, $P = 0.45$). As the measured nodularin concentrations in the *N. spumigena* containing slurry's were greater than the expected concentrations based on nominal dosing, greater than planned doses of nodularin were gavaged to the fish (see Table 9). Black Bream were dosed at an average of 870 µg nodularin/kg b.w., while Southern Sand Flathead received average doses of 106 or 776 µg nodularin/kg b.w. for the low and high dose treatments, respectively. While dosed levels were higher than expected across all treatments, there remained a significant difference in the nodularin doses delivered. There was ca. 6-times more nodularin in the high dose for flathead compared to the low dose (Table 9). No nodularin was detected in the control doses (Table 9).

Table 9: Mean (± S.E.) fish weights, total length and measured total nodularin (as combination of nodularin-R and desmethyl-nodularin-R) dose in Black Bream and Southern Sand Flathead.

	Southern Sand Flathead			Black Bream	
	Control	Low dose	High dose	Control	High dose
Weight (g)	114.3 ± 8.7[a]	98.8 ± 6.7[a]	102.0 ± 7.4[a]	1121.2 ± 58.4[a]	789.1 ± 100.3[b]
Length(cm)	23.3 ± 0.6[a]	32.3 ± 9.9[a]	22.4 ± 0.5[a]	39.5 ± 0.5[a]	33.2 ± 1.9[b]
Nodularin (µg/kg)	-	106.3 ± 5.9	775.6 ± 24.3	-	869.6 ± 58.9

Note: There were 13 and 21 fish for controls and high dose of Black Bream, respectively. For Southern Sand Flathead there were 12 controls, 16 low dose and 16 high dose fish. Different subscript letters denote a significant difference ($P<0.05$) in mean weight or length of either Black Bream or Southern Sand Flathead in different treatments.

Nodularin uptake and tissue distribution

Nodularin was detected in the liver, muscle and gut tissues of Black Bream and Southern Sand Flathead (Table 10). Control muscle tissues of Black Bream and Southern Sand Flathead contained no detectable levels of nodularin (see Appendix A). Control liver tissues from Black Bream contained no or little nodularin (0.5-0.7 µg/kg w.w.), while one Southern Sand Flathead fish contained 1.6 µg/kg w.w. nodularin in its liver (see Appendix A). No nodularin was detected in gut tissue of Black Bream, however one Southern Sand Flathead contained 0.3 µg/kg w.w. nodularin in the gut (see Appendix A).

For both species, the primary organ where nodularin accumulated was the liver, followed by the muscle (Figure 1), while some also remained in the gut. In both Black Bream and Southern Sand Flathead, dosed at a similar level, ca. 80% of toxin detected was in the livers. The main difference between the two species was the distributions of toxin in gut and muscle tissues (Figure 1). A greater percentage of toxin was detected in the muscle of the Southern Sand Flathead (4.5% compared to 2.3% in Black Bream), while in Black Bream greater amounts of nodularin remained in the gut (18.3% compared to 13.5% in Southern Sand Flathead). The level of nodularin exposure fish received resulted in differences in tissue toxin distribution. In Southern Sand Flathead exposed to 106 µg nodularin/kg b.w. 56% of toxin detected was in the liver, and 3.4% in the muscle, while 40% remained in the gut. In fish dosed at 776 µg nodularin/kg b.w. 81.5% of toxin detected was in the liver, and 4.5% in the muscle tissue, while 14% remained in the gut (Figure 1).

Highest concentrations of nodularin were measured in the livers of Southern Sand Flathead exposed to 776 µg/kg b.w., followed by Black Bream and then Southern Sand Flathead dosed at 106 µg/kg b.w. (Table 10). Concentrations accumulated into liver tissues of Black Bream were on average half that accumulated into Southern Sand Flathead at the same dose (Table 10). Concentrations in livers of Southern Sand Flathead dosed at the lower nodularin level (106 µg/kg b.w.) were on average 6-times lower than those in the higher dosed fish (Table 10).

Significantly lower levels of toxin were detected in muscle tissues of both fish species, compared to livers. Nodularin concentrations in muscle tissues of Black Bream averaged 0.07 µg/kg w.w., compared to 4.11µg/kg w.w. in the liver. In Southern Sand Flathead muscle nodularin concentrations averaged 0.11 and 0.6 µg/kg w.w., compared to 1.76 and 10.84 µg/kg w.w. for fish dosed at 106 and 776 µg/kg b.w. respectively (Table 10). Nodularin detected in the muscle tissue of Black Bream was 10-times less than that measured in Southern Sand Flathead given a similar dose. While muscle tissue concentrations in Southern Sand Flathead dosed at 106 µg/kg b.w. were 5-times lower than those in higher dosed fish (776 µg/kg b.w.; Table 10). As a percentage of dosed toxin, Black Bream accumulated an average 0.53%, while Southern Sand Flathead accumulated an average 1.41% and 1.91% for fish dosed at 776 and 106 µg/kg b.w., respectively (Table 10).

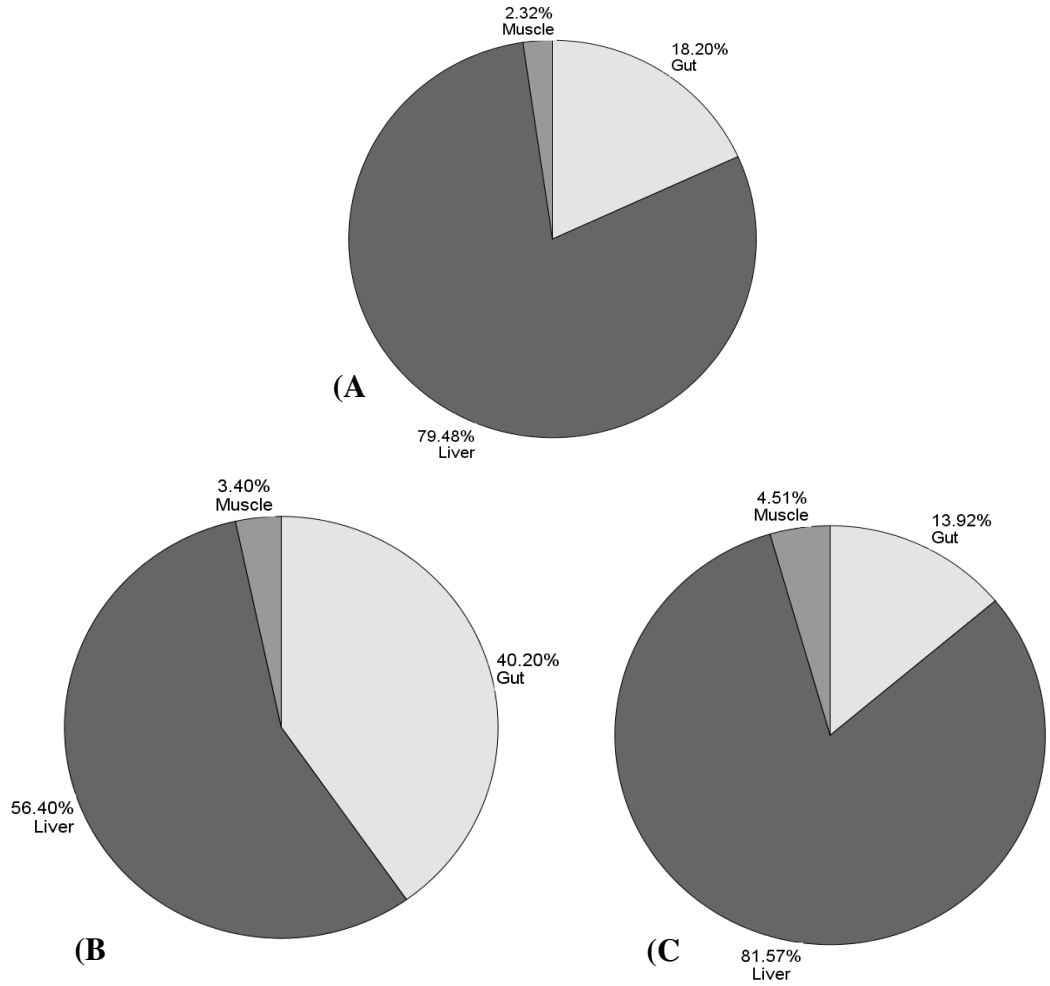

Figure 1: Relative distribution of nodularin (combination of nodularin-R and desmethyl-nodularin-R) in different tissues of fish: (A) Black Bream, (B) Southern Sand Flathead dosed at 106 µg/kg b.w. and (C) Southern Sand Flathead dosed at 776 µg/kg b.w..

Table 10: Concentrations of nodularin (as a combination of nodularin-R and desmethyl-nodularin-R) detected in muscle and liver tissues of Black Bream and Southern Sand Flathead following feeding with *Nodularia spumigena* contaminated slurry. * Mulvena et al (2012). N = 21 for Black Bream. N = 16 fish in each treatment for Southern Sand Flathead.

Species	Dose administered (µg/kg b.w.)	Tissue	Nodularin concentration (µg/kg w.w.)			
			Min.	Max.	Mean	Median
Black Bream	870	Liver	0.78	24.54	4.11	2.95
		Muscle	0	0.62	0.07	0
		Total nodularin	0.78	24.87	4.17	3.15
		Nod accumulated (% initially dosed)	0.1	3.17	0.53	0.36
Southern Sand Flathead	106	Liver	0.12	8.76	1.76	1.2
		Muscle	0	0.9	0.11	0
		Total nodularin	0.12	8.8	1.87	1.4
		Nod accumulated (% initially dosed)	0.13	7.45	1.91	1.2
Southern Sand Flathead	776	Liver	0.56	35.92	10.84	7.8
		Muscle	0	4.35	0.6	0
		Total nodularin	0.56	35.97	11.44	7.8
		Nod accumulated (% initially dosed)	0.08	4.5	1.41	0.98
*Health Alert Guideline for Fin-fish (whole fish)			24 µg/kg			

Nodularin accumulation and depuration

Distinct accumulation and depuration patterns were observed between species, tissues and dose (Figures 2 and 3). Nodularin was detected in both liver and muscle tissues of Black Bream and Southern Sand Flathead 24 hr post gavage and was still detected up to 20 days post gavage (Figures 2 and 3).

In Black Bream, nodularin concentrations steadily increased in livers to peak at 14 days post gavage, then declined, while for Southern Sand Flathead given a similar dose, nodularin in livers increased sharply at 24 hr, then declined slowly over the following 19 days (Figures 2 and 3). In muscle tissues the opposite occurred. In Black Bream, nodularin concentrations peaked 24 hr post gavage then declined slowly and were undetectable after 20 days. In Southern Sand Flathead, nodularin concentrations fluctuated over the first 2 days, sharply increased at 7 days, then declined to undetectable levels at 14 days (Figures 2 and 3).

In Southern Sand Flathead exposed to a low dose of nodularin (106 µg/kg b.w.) low levels of nodularin accumulated into both liver and muscle tissues over the first 7-14 days. In livers, nodularin concentrations peaked 14 days post gavage and in muscle at 20 days (Figure 3). Peaks in muscle tissues of Southern Sand Flathead occurred 6 days following peaks in livers for fish dosed at both low and high doses (Figure 3).

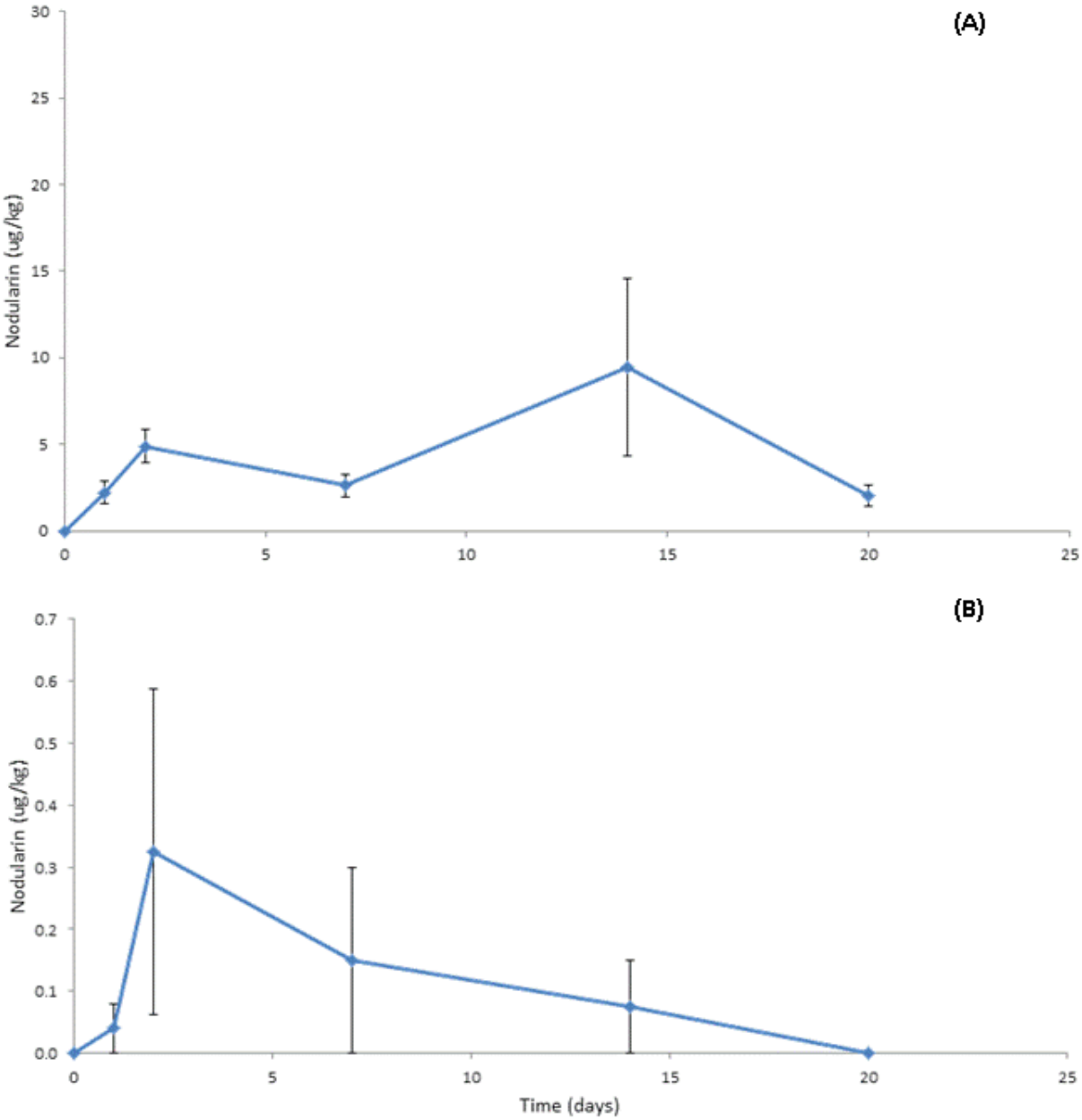

Figure 2: Accumulation and depuration of nodularin (as a combination of nodularin-R and desmethyl-nodularin-R) in Black Bream tissues following exposure to an average dose of 870 µg/kg b.w.. (A) Liver, (B) Muscle. Data are the mean (n = 4) and error bars = ± standard error.

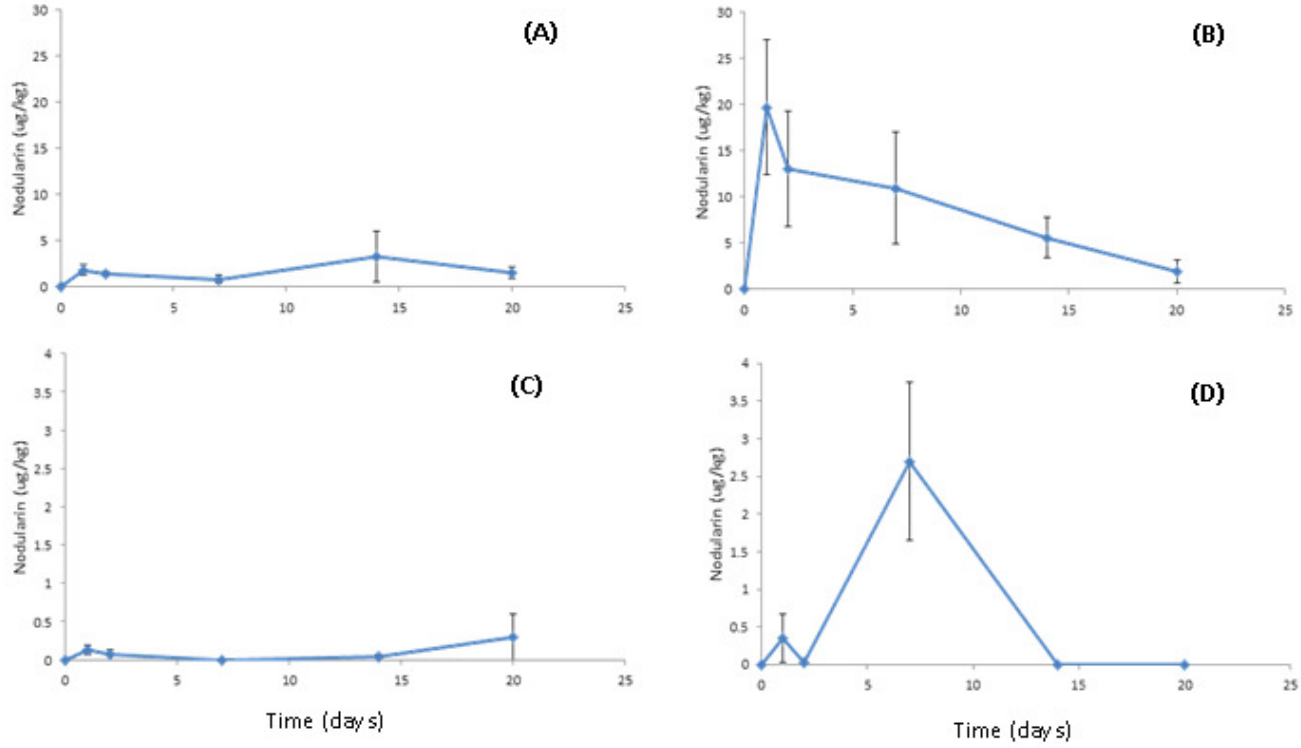

Figure 3: Accumulation and depuration of nodularin (as a combination of nodularin-R and desmethyl-nodularin-R) in Southern Sand Flathead tissues. (A) Liver of fish dosed at an average 106 µg/kg b.w. (B) Liver of fish dosed at an average 776 µg/kg b.w. (C) Muscle of fish dosed at an average 106 µg/kg b.w. (D) Muscle of fish dosed at an average 776 µg/kg b.w. Data are the mean (n = 3) and error bars = ± standard error.

Discussion

Uptake and tissue distribution

In aquatic environments, nodularin is mainly released into the water during toxic *N. spumigena* blooms as cells die and lyse (Myers, 2008). Thus seafood, such as fish are more likely to be exposed to nodularin through ingestion of toxic *N. spumigena* or contaminated food, and to a lesser extent through dissolved toxin. Fish may uptake and accumulate nodularin via two main routes: it can be absorbed via the gills and skin or through the gastrointestinal tract (Ibelings and Chorus 2007). Once absorbed, transport of nodularin to various organs and tissues can take place. Distribution patterns of nodularin in the organs of fish are an important basis for understanding nodularin intoxication mechanisms and for risk assessment.

In the current study, uptake of nodularin through the gastrointestinal tract in two fish species, the omnivorous Black Bream and carnivorous Southern Sand Flathead, was examined. In both species nodularin was absorbed through the gastrointestinal tract and transported into the liver and muscle tissues. Various fish species have been shown to accumulate nodularin under bloom conditions (Sipia et al 2006; Karjalainen et al 2008; Mazur-Marzec et al 2006; Sipia et al 2001a,b; Eaglesham et al 2002; Van buynder et al 2001; Department of Health Victoria 2014; Stewart et al 2012; Dolamore et al 2017). These studies have shown that the highest concentrations of nodularin accumulate in the liver and lower concentrations accumulate in the muscle. A limited number of laboratory studies utilising different routes of exposure (intraperitoneal injection and feeding), have also demonstrated uptake and translocation of nodularin into organs and tissues including the liver, gallbladder and muscle (Kankaanpaa et al 2002; Engstrom-Ost et al 2002; Platt 2005; Paakkonen et al 2008; Vourinen et al 2009; Persson et al 2009; Myers et al 2010). While nodularin is clearly transported into various organs and tissues in fish, following uptake, the primary organ of accumulation in most species is the liver (Ibelings and Chorus 2007; Sotton et al 2015). In Black Bream and Southern Sand Flathead ca. 80% of accumulated nodularin was detected in the livers, with 2-4.5% accumulated in muscle tissues. This is in line with observations from various fish species in both field and laboratory studies where reports of 80-100% accumulated toxin is detected in the liver and between 0.1%-18% in muscle tissues (Sipia et al 2001a; Kankaanpaa et al 2002; Sipia et al 2006; Cazenave et al 2005; Mazur-Marzec et al 2007).

Nodularin concentrations in liver and muscle

Concentrations of nodularin in fish livers were up to 58-times higher than those detected in muscle tissues. This result is in agreement with reports for various fish species exposed to nodularin in the wider literature whereby concentrations in livers have been reported to be orders of magnitude greater than in muscle tissues. For instance, nodularin content in livers of flounders caught in the Baltic Sea during toxic *N. spumigena* blooms have been reported in the range of 20 to 2,230 µg/kg d.w, while concentrations in muscle tissues were less than 200 µg/kg d.w, some 30-times lower (Sipia et al 2001a,b; Sipia et al 2002; Sipia et al 2006; Karlsson et al 2003; Kankaanpaa et al 2005; Marzur-Marzec et al 2006).

The concentrations of nodularin detected in tissues of fish, including sea trout, Black Bream and flounders, following oral exposure (237-44 µg/kg b.w) under laboratory conditions ranged 19-3194 µg/kg d.w. and 34-723 µg/kg d.w. in liver and muscle tissues respectively (determined by ELISA or HPLC; Platt 2005; Kankaanpaa et al 2002; Vourinen et al 2009). Direct comparison of these results with the nodularin concentrations detected in Black Bream and Southern Sand Flathead liver and muscle tissues in the current study is challenging without applying a conversion, as all the results expressed concentrations per kg dry weight (d.w.) while in the current study we expressed them per kg wet weight (w.w.). DHHS (2017) applied a conversion based on the wet weight of Black Bream and

dusky flathead being equivalent to one fifth of dry weight in order to make comparisons between studies investigating concentrations of mercury in tissues. Applying this conversion to the current study results and comparing with the dry weight concentrations in fish exposed under similar conditions detailed above indicates that Black Bream and Southern Sand Flathead accumulated nodularin to similar levels into their livers, however lower levels into their muscle tissue (Black Bream: 122.7 µg/kg and 3.1 µg/kg d.w. equivalent into liver and muscle respectively; Southern Sand Flathead: 179.6 µg/kg and 21.71 µg/kg d.w. equivalent in liver and muscle respectively).

During identical exposure conditions, the Southern Sand Flathead, a predatory carnivore, accumulated higher concentrations of nodularin into liver and muscle tissues compared to Black Bream, which is an opportunistic omnivore. Although early knowledge suggested there was no clear relationship between feeding type and toxin concentrations in fish tissues (Ibelings and Chorus, 2007), an increasing number of studies have reported differences in the levels of toxin accumulated into tissues among species of different trophic levels or feeding guilds (Kopp et al 2013; Xie et al 2005; Rezaitabar et al 2017; Ni et al 2017). For instance, Kopp et al (2013) reported highest concentrations of microcystins in livers of carnivorous fish, followed by herbivores and omnivores from ponds and reservoirs in the Czech Republic and Xie et al (2005) reported greatest concentrations of microcystins to be detected in tissues of carnivorous fish (*Culter ilishaeformis*) followed by the omnivorous fish (*Carassius auratus*) and lowest in phytoplanktivorous fish (*Hypophthalmichthys molitrix*) and herbivorous fish (*Parabramis pekinensis*). Rezaitabar et al (2017) reported carnivores to have highest concentrations in muscle tissues, while phytoplanktivores, had greater concentrations of MC-LR in livers. Conflicting results have been reported in the published literature. For instance, Jia et al (2014) found no difference in the concentrations of microcystins accumulated by phytoplanktivores and carnivores collected during a bloom in Lake Taihu, China. Other studies have reported highest concentrations of toxins in phytoplanktivores followed by omnivores and lowest in carnivores (Amrani et al 2014; Bukaveckas et al 2017; Ni et al 2017). The results from the current study and those reported from field studies during cyanobacterial blooms suggest that a consideration of species-specific differences in feeding habits, could be important to understanding the accumulation and persistence of cyanobacterial toxins in different fish species and should therefore be considered in assessment of risks to human health and in consideration of choosing an appropriate species as a sentinel in monitoring programs.

While various field studies together with results of the current study suggest that cyanotoxin concentrations may in part be a function of feeding group or trophic level, it is likely that a number of other factors also play a role in the concentrations of toxin accumulated, such as exposure history. In the current study, fish exposed to higher concentrations of nodularin accumulated greater amounts of toxin. Persson et al (2009) exposed flounders to increasing doses of nodularin via i.p. injection and while they did not find significant differences between treatments, fish dosed at 50 µg/kg b.w. accumulated 4-times higher toxin concentrations in their livers than fish exposed to 2 µg/kg b.w.. Similarly highest levels of toxin in fish tissues have been reported to coincide with highest levels of toxins measured in bloom material during field studies (Karjalainen et al 2008; Ni et al 2017).

Accumulation and elimination patterns

Time-dependant accumulation and depuration of nodularin was observed which differed depending on tissue, species and dose administered. In Black Bream and Southern Sand Flathead, fed similar dosages of nodularin, accumulation into muscle and liver tissues occurred shortly after exposure (within 24 h), however thereafter differed. In Black Bream nodularin continued to accumulate in the liver, however was removed from muscle tissue. In contrast in Southern Sand Flathead, nodularin continued to accumulate in muscle tissue, however was removed from the liver. In a study by Kankaanpaa et al (2002) on the accumulation of nodularin into sea trout following oral exposure (440 µg/kg b.w.), similar levels of nodularin were found in muscle and liver 24 hr post gavage. This was

followed by removal from muscle tissues (undetectable after 8 days) and continual accumulation into liver tissues. In a study of the accumulation of nodularin into muscle and livers of Black Bream following a single oral dose at 233 µg/kg b.w., nodularin was absorbed via the gastrointestinal tract and distributed into both muscle and liver tissues during the first 24 hr (Platt 2005). After which it was removed from muscle, but continued to accumulate into liver and renal tissues (Platt 2005), which is similar the patterns observed in Black Bream during the current study. These results suggest that Southern Sand Flathead transport and accumulate nodularin into livers faster than Black Bream; however this happens much slower into the muscles.

In fish administered different dosages of nodularin, different accumulation patterns were also observed. In contrast to the rapid accumulation, followed by early elimination of nodularin from liver tissues of Southern Sand Flathead dosed at 776 µg/kg b.w., fish dosed at 106 µg/kg b.w. gradually absorbed the toxin from the gastrointestinal tract into the liver, reaching a peak 14 days post gavage and then stabilising. Nodularin was transported to muscle tissues, in fish dosed at both levels, as early as 24 hr post gavage. In fish administered the high concentration, accumulation generally increased till 7 days post gavage and then elimination began, while in fish administered the lower dose, after 24 hrs there was basically no nodularin detected in muscle again until 20 days post gavage. For both the low and higher dosed fish the peak in nodularin accumulation into muscle tissues occurred six days post the peak in the livers.

Of the amount of nodularin administered in food only a small amount was absorbed by the fish. For instance Southern Sand Flathead absorbed an average of 1.9% and 1.4% (low and high doses respectively) of originally administered nodularin and Black Bream 0.53%, this being mostly accumulated in the livers. This is in agreement with reports in the wider literature (Kankaanpaa et al 2002; Bury et al 1998; Tencalla and Dietrec 1997). For instance in sea trout 0.05-0.53% of dosed nodularin (440 ± 50 µg/kg b.w.) was absorbed into the liver (Kankaanpaa et al 2002), while in rainbow trout dosed with microcystin (0.64 – 5,873 µg/kg b.w.) 0.17-1.5% was absorbed into fish livers (Bury et al 1998; Tencalla and Dietrec 1997).

Nodularin determination: Analytical techniques, quality control and quality assurance

Determining and particularly quantifying the presence of cyanotoxins in seafood requires appropriate analysis of the toxin concentrations in tissues. This is not always straightforward with differences in sample processing, e.g.: extraction procedures, use of sample clean-up, and determination methods (HPLC, LC-MS/MS, ELISA, PPIA), all impacting the final toxin quantification. High accuracy is required for reliable results, especially when concentrations are to be used in risk assessment and the provision of advice around seafood safety.

In the current study, fish tissue samples were prepared by extraction in methanol, removing lipids with hexane, 'cleaning-up' the samples with solid phase extraction cartridges and drying the 'cleaned-up' extracts in glass vials. Dried extracts were then resuspended and analysed using LC-MS/MS. A number of experiments and steps were included in the sample processing and analytical determination to assess the reliability and quality of the data obtained. These included spike recoveries to assess sample loss during processing and fortification of extracted samples to assess mass spectrometer suppression/enhancement effects (see methods section).

Mass spectrometer suppression effects were more severe for nodularin-R compared to those observed for MC-LR where only slight levels of enhancement were observed for some of the sample matrices. The level of suppression/enhancement was dependant mostly of tissue type, with smallest effects observed in muscle tissue and gut samples the greatest, but to some degree on fish species. Mass spectrometer suppression effects for nodularin were in the range of 8-12%, 12-55% and 80-92%

for muscle, liver and gut tissues respectively. Karlssonn et al (2005) observed mass spectrometer suppression for nodularin of 38% for fish liver samples. Similarly Karlssonn et al (2005) also reported greater suppression effects for nodularin compared to various microcystin variants. Sipia et al (2006) reported no clear mass spectrometer suppression/enhancement effects in liver and muscle samples of flounder and roach, reporting effects to range -10 to +40%. Previous levels of mass spectrometer enhancement effects (e.g.: +20% to +30%) have been detected using the current LC-MS/MS method in muscle samples from New Zealand shortfin eels (*Anguilla australis*; Dolamore et al 2017). Cadel-six et al (2014) reported low mass spectrometer effects for microcystin in intestines (+1%) and muscle (+15%) tissues of rainbow trout, however higher levels for livers (+23%).

Recovery experiments are important quality assurance and quality control, which allows assessment of losses of toxin during sample extraction and processing. Recovery of nodularin from tissues in the current study ranged 56-82% for liver tissues, 48-80% for muscle and 61-113% for gut. These are in the range of recoveries published in the wider literature for nodularin in liver (28-84%) and muscle (40-84.9%; Stewart et al 2012; Van buynder et al 2001; Karlsson et al 2003; Sipia et al 2007). While the nodularin recoveries were in the range of those reported in the wider literature, our study found that they varied significantly between species and tissues. Due to this variability together with high mass spectrometer suppression effects observed for nodularin we employed an internal standard, dmMC-LR, into every sample. This level of QA/QC has not been reported before, in fact very few studies report any sort of recovery assessments, let alone account for them in provision of their results. Recovery of dmMC-LR, the toxin used as an internal standard in the current study, ranged from 25-30% in livers, 59-66% in muscle. Unfortunately, dmMC-LR could not be measured in the gut samples as the sample matrix severely affected the chromatography for this compound. Recovery of dmMC-LR from fish tissues has been reported to range from 28-75% in liver and 35-76% in muscle in the wider literature (Ni et al 2017; Karjalainen et al 2008; Cadel-six et al 2014; Stewart et al 2012).

High variability in sample results were observed between individual fish. This is often reported in studies investigating cyanotoxin uptake in fish under field conditions (Karjalainen et al 2008; Kankaanpaa et al 2005; Sipia et al 2006; Jia et al 2014; Acou et al 2008), although has been observed in laboratory studies as well (Paakkonen et al 2008; Cazenave et al 2005). This could be due to differences in metabolism of different species (Jia et al 2014), feeding habits (discussed earlier), in studies where not reported and accounted for due to differences in recovery between tissues and species and it could be due to an inadequate number of samples. For the current study it is unlikely related to methods due to the QA/QC applied with internal standards in each sample or differences in feeding habits of fish as the fish were all administered a dose in the same manner. It is most likely that in the current study the variability is due to differences in metabolic rates of individual fish and low sample numbers.

The sample processing and analytical QA/QC conducted as part of this study indicates that to have high reliability data you need to ensure appropriate controls are added to your sample processing and analysis steps. Otherwise there may be an over- or under-estimation of the concentrations of toxins in tissues which could have significant implications for providing advisories regarding seafood safety. At minimum recovery experiments should be conducted on each tissue matrix being examined and run with each sample batch. The inclusion of an internal standard in every sample is the only way to fully evaluate any losses during processing and provide the highest level of reliability in data obtained. Ideally, this internal standard would be a 'heavy' version the analyte of interest (i.e., the same compound with isotopes incorporated to shift the mass of the compound by several Daltons to allow simultaneous, but specific, detection of both compounds by mass spectrometry). Unfortunately, a 'heavy' version of nodularin-R is not available, therefore, fortification of each sample with dmMC-LR which is structurally similar to nodularin-R was used. However, the use of dmMC-LR (or other structurally similar microcystin congeners) relies on that compound not being present in the samples, so may not be feasible for samples collected in the field. Further addition of a greater number of

replicates would potentially reduce variability in results. The results from the present study demonstrate why performing the analysis without any quality control measures is not recommended.

Food safety and hazard control measures for Gippsland Lakes

Risks from exposure to cyanotoxins in seafood are becoming increasingly recognised, with measures to control these risks being identified and increasingly implemented (Johnson et al 2010; Mulvenna et al 2012; NSW Food Authority 2017). In the Gippsland Lakes measures to control risks to humans from exposure to nodularin through consumption of fish are managed through restrictions of fish collection when certain alert levels are exceeded (Van buynder et al 2001; Mulvena et al 2012). Current health alert guidelines applied during blooms in the Gippsland lakes are those outlined in Mulvena et al (2012). Mulvena et al (2012) suggest a safe guideline for human consumption of microcystin and/or nodularin in fish of 24 µg toxin/kg (for a 2-16 year age group). This guideline is based on the assumption that nodularin is at least as hepatotoxic as microcystin for intraperitoneal exposure in experimental animals and due to its similar mode of action presents at least the same level of risk as microcystins to human health. *Nodularia spumigena* blooms in the Gippsland Lakes during 2011-2012 resulted in provision of advisories based on the detection of nodularin in whole Black Bream samples in exceedance of the 24 µg/kg guideline value (Department of Health 2014; Poon 2012).

In the current study, Black Bream and Southern Sand Flathead were exposed to nodularin doses (average range 106-869 µg/kg b.w.) in the range of concentrations which they may encounter in food items during *N. spumigena* blooms in the Gippsland Lakes (nodularin concentrations detected in prawns in range 56 to 22,430 µg/kg d.w and mussels 31 to 2,500 µg/kg d.w, Van Buynder et al 2002; Eaglesham et al 2002; Department of Health Victoria, 2014). Provided with a single oral dose, nodularin was detected in Southern Sand Flathead and Black Bream at levels exceeding the suggested health alert guideline of 24 µg/kg whole fish (Black Bream average maximum total nodularin (muscle and liver) measured 24.9 µg/kg w.w., Southern Sand Flathead 36 µg/kg w.w.), indicating potential for these species to accumulate nodularin to levels that may pose risk to human consumers. This is consistent with reports for concentrations of nodularin detected in tissues of Black Bream during recent blooms (2011-12) in the Gippsland Lakes, where nodularin concentrations in whole fish exceeded health alert guidelines (24 samples of 36 total; detected nodularin levels ranged 16-203 µg/kg w.w., Department of Health Victoria 2014). Exceedances in fish in the current study were due to the occurrence of nodularin levels in liver, where majority of the toxin was accumulated. Concentrations in muscle tissues reached maximums of 0.62 µg/kg w.w. and 4.35 µg/kg w.w. in Black Bream and Southern Sand Flathead, respectively, which are well below the health guideline. Again this data corresponds well with most recently collected field data on Black Bream during cyanobacterial blooms in the Gippsland Lakes, whereby concentrations of nodularin in gut and gilled fish never exceeded 16 µg/kg limit of detection (Poon 2012; NSW Food Authority 2017). Also reports of nodularin concentrations detected in muscle tissues of various fish species caught during blooms in the Gippsland Lakes whereby concentrations detected have ranged 0.7-7.5 µg/kg (Van buynder et al 2001; Poon 2012). In most cases, whole fish of Black Bream and Southern Sand Flathead or most other fin-fish species caught in the Gippsland Lakes would not be eaten and concentrations of toxin in the muscle would be the highest risk that needs to be considered. Control measures to limit exposure to whole fish in the Gippsland Lakes have included advising recreational fishers to gut and gill fish prior to cooking and consuming. Commercial fishers have also been able to sell fish as long as it is filleted or gutted and gilled (together with confirmation of no nodularin presence in muscle tissue) (Van Buynder et al 2001; Victorian Bays and Inlets Fisheries Association 2013). The current study results suggest that a gut and gill advisory could be a viable measure to limit the exposure to nodularin, however such an advisory should only be given based on analytical confirmation of toxin levels in tissues.

Current advisories in the Gippsland Lakes are provided based on measurement of nodularin in Black Bream. This species is relied upon as a sentinel, whereby it is used as an indicator species to provide advisories to not eat fish or gut and gill fish before consuming. The use of Black Bream as a sentinel was based on the commercial and recreational importance of the species together with detection of highest concentrations of nodularin in Black Bream compared to all other fish species during a bloom in the Gippsland lakes in 2002 (Eaglesham et al 2002), its potential to accumulate nodularin to a greater degree than other fin-fish due to its dietary preferences for shellfish and prawns which are known to accumulate toxins and potential to spend majority of time within areas experiencing blooms compared to other fish species (Anonymous 2011). Results from the current study suggest that the use of a single species may not afford protection early enough or for long enough time after blooms start to subside. Southern Sand Flathead accumulated significantly greater concentrations of nodularin into tissues than Black Bream, with the ability to uptake nearly 3-times more of available toxin, following exposure to similar doses. Furthermore, peaks in nodularin concentrations occurred more rapidly in Southern Sand Flathead compared to Black Bream. As there are a number of fish species of commercial and recreational importance in the Gippsland Lakes that represent varied feeding groups and trophic levels we would suggest that further investigation into uptake, accumulation and depuration of nodularin in a greater range of species be undertaken to ascertain which would be most appropriate as a sentinel. Larger, higher-frequency sample sets, spanning multiple years and investigating multiple trophic levels are recommended before having confidence in the choice of sentinel species.

Conclusions

This study demonstrates that nodularin accumulates primarily into the liver of Southern Sand Flathead and Black Bream and to a lesser extent the muscle tissues. These are important species both recreationally and commercially in the Gippsland Lakes. Our results suggest that, removal of the liver (and other intestinal organs) would greatly reduce the risk of adverse effects to human health from consumption of both species. However, nodularin does accumulate in muscle tissue and the data from this study indicate that the concentrations of nodularin in diet may have an impact on the levels detected in fish. Under conditions of prolonged exposure the accumulation potential into tissues could be greater than observed in the current study. There are reports in the wider literature of cyanotoxin concentrations in muscle tissue of fish reaching concentrations that exceed tolerable daily intake values set by WHO (Ibelings and Chorus 2007; Sipia et al 2006; Stewart et al 2012; Ni et al 2017) and therefore we suggest that monitoring of tissue concentrations and consideration of potential health risks be assessed for any instance when there is a bloom and need to provide advisories around seafood safety.

The study has indicated that nodularin uptake, tissue distribution, accumulation and elimination varies between species. Black Bream, the species currently used as a sentinel species to provide health advisories in the Gippsland Lakes, accumulated lower concentrations of nodularin into tissues than that of Southern Sand Flathead and differences in accumulation patterns were observed. These results identify that further research is needed on species of commercial and recreational importance in the Gippsland lakes to make sure the sentinel will provide not only an early indication of risk, but also indicate when it is safe to consume fish following the decline of a bloom.

Lastly, in order to obtain data that is of high reliability it is recommended that spike recoveries be conducted in all tissue matrices and for each species in any study. If possible the use of an internal standard is highly recommended as it significantly reduces the chances of over- or under-estimation in results.

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Appendices

Appendix A: Raw data of nodularin concentrations in fish tissues as determined using LC-MS/MS.

Table 1: Liquid chromatography-tandem mass spectrometry analysis of nodularin (the combination of nodularin-R and desmethyl-nodularin-R) in muscle samples from Black Bream (BB) fish.

#	Sample Label	Weight (g)	Total Nodularin (ng/mL)[a]	dmMC-LR (ng/mL)[a]	dmMC-LR Recovery	Nodularin Content (µg/kg)
35	BB_CONTROL_F1_24Hr M	1.021	ND	37.4	36%	-
36	BB_CONTROL_F2_24Hr M	1.031	ND	74.5	72%	-
37	BB_CONTROL_F3_24Hr M	1.065	ND	42.5	41%	-
38	BB_CONTROL_F4_24Hr M	1.027	0.2	66.0	64%	0.1
39	BB_CONTROL_F5_24Hr M	1.018	0.1	69.4	67%	0.1
40	BB_200_F1_24Hr M	1.006	ND	27.3	26%	-
41	BB_200_F2_24Hr M	1.049	ND	44.2	43%	-
42	BB_200_F3_24Hr M	1.090	0.4	71.4	69%	0.2
43	BB_200_F4_24Hr M	0.994	ND	23.5	23%	-
44	BB_200_F5_24Hr M	1.010	ND	50.1	48%	-
45	BB_200_F1_2D M	1.034	ND	46.9	52%	-
46	BB_200_F2_2D M	1.089	0.2	34.9	38%	0.2
47	BB_200_F3_2D M	1.070	ND	40.3	44%	-
48	BB_200_F4_2D M	1.022	1.6	62.4	69%	1.1
49	BB_200_F1_7D M	1.052	ND	66.4	73%	-
50	BB_200_F2_7D M	1.027	ND	40.9	45%	-
51	BB_200_F3_7D M	1.029	ND	78.1	86%	-
52	BB_200_F4_7D M	1.068	0.9	64.3	71%	0.6
53	BB_CONTROL_F1_14D M	1.032	ND	38.5	42%	-
54	BB_CONTROL_F2_14D M	1.007	ND	52.1	57%	-
55	BB_CONTROL_F3_14D M	1.052	ND	41.0	45%	-
56	BB_CONTROL_F4_20D M	1.037	ND	43.0	47%	-
57	BB_200_F1_14D M	1.021	0.3	40.6	45%	0.3
58	BB_200_F2_14D M	1.057	ND	68.7	76%	-
59	BB_200_F3_14D M	1.057	ND	86.6	95%	-
60	BB_200_F4_14D M	1.017	ND	63.1	70%	-
61	BB_CONTROL_F1_20D M	1.017	ND	52.4	58%	-
62	BB_CONTROL_F2_20D M	1.009	ND	72.4	80%	-
63	BB_CONTROL_F3_20D M	1.055	ND	61.7	68%	-
64	BB_CONTROL_F4_20D M	1.062	ND	44.1	49%	-
65	BB_200_F1_20D M	1.051	ND	61.5	68%	-
66	BB_200_F2_20D M	1.023	ND	56.0	62%	-
67	BB_200_F3_20D M	1.067	ND	80.2	88%	-
68	BB_200_F4_20D M	1.049	ND	82.7	91%	-

[a] Concentrations were adjusted for mass spectrometer suppression/enhancement effects using the corresponding average value from Table 1. ND = Not detected (<0.05 ng/mL).

Table 2: Liquid chromatography-tandem mass spectrometry analysis of nodularin (the combination of nodularin-R and desmethyl-nodularin-R) in muscle samples from flathead (FH) fish.

#	Sample Label	Weight (g)	Total Nodularin (ng/mL)[a]	dmMC-LR (ng/mL)[a]	dmMC-LR Recovery	Nodularin Content (µg/kg)
147	FH_CONTROL_F1_24Hr M	1.016	ND	66.5	57%	-
148	FH_CONTROL_F2_24Hr M	1.049	ND	72.4	62%	-
149	FH_CONTROL_F3_24Hr M	1.059	0.7	44.0	38%	0.9
150	FH_CONTROL_F4_24Hr M	1.073	0.5	72.3	62%	0.4
151	FH_CONTROL_F5_24Hr M	1.052	24.7	51.7	45%	26.3
152	FH_CONTROL_F6_24Hr M	1.023	2.4	111.4	96%	1.2
153	FH_50_F1_24Hr M	1.007	ND	35.3	30%	-
154	FH_50_F2_24Hr M	1.046	0.5	82.8	71%	0.3
155	FH_50_F3_24Hr M	1.010	0.07	51.6	44%	0.1
156	FH_50_F4_24Hr M	1.059	0.2	66.4	57%	0.1
157	FH_200_F1_24Hr M	1.051	ND	57.8	50%	-
158	FH_200_F2_24Hr M	1.066	ND	41.9	36%	-
159	FH_200_F3_24Hr M	1.030	0.05	57.8	50%	0.1
160	FH_200_F4_24Hr M	1.066	0.6	25.9	22%	1.3
161	FH_50_F1_2D M	1.003	0.2	54.3	47%	0.2
162	FH_50_F2_2D M	1.089	ND	58.3	50%	-
163	FH_50_F3_2D M	1.019	ND	66.8	58%	-
164	FH_200_F1_2D M	1.027	ND	66.2	57%	-
165	FH_200_F2_2D M	1.074	0.07	45.7	39%	0.1
166	FH_200_F3_2D M	1.089	ND	91.8	79%	-
167	FH_50_F1_7D M	1.066	ND	54.1	51%	-
168	FH_50_F2_7D M	1.029	ND	75.0	70%	-
169	FH_50_F3_7D M	1.023	ND	92.2	86%	-
170	FH_200_F1_7D M	1.052	7.6	89.0	83%	4.4
171	FH_200_F2_7D M	1.010	5.8	106.4	100%	2.9
172	FH_200_F3_7D M	1.009	1.3	89.1	83%	0.8
173	FH_50_F1_14D M	1.091	0.1	72.4	68%	0.1
174	FH_50_F2_14D M	1.008	0.05	73.1	68%	0.04
175	FH_50_F3_14D M	1.004	ND	86.4	81%	-
176	FH_200_F1_14D M	1.010	ND	77.1	72%	-
177	FH_200_F2_14D M	1.020	ND	65.4	61%	-
178	FH_200_F3_14D M	1.081	ND	90.2	84%	-
179	FH_CONTROL_F1_20D M	1.046	0.1	82.3	77%	0.1
180	FH_CONTROL_F2_20D M	1.049	1.5	99.2	93%	0.8
181	FH_CONTROL_F3_20D M	1.014	ND	52.3	49%	-
182	FH_CONTROL_F4_20D M	1.049	0.2	64.6	60%	0.2
183	FH_CONTROL_F5_20D M	1.046	ND	69.0	65%	-
184	FH_CONTROL_F6_20D M	1.008	ND	102.0	95%	-
185	FH_50_F1_20D M	1.019	1.5	92.7	87%	0.9
186	FH_50_F2_20D M	1.027	ND	91.9	86%	-
187	FH_50_F3_20D M	1.055	ND	81.1	76%	-
188	FH_200_F1_20D M	1.025	ND	83.9	79%	-
189	FH_200_F2_20D M	1.026	ND	100.9	94%	-
190	FH_200_F3_20D M	1.079	ND	74.1	69%	-

[a] Concentrations were adjusted for mass spectrometer suppression/enhancement effects using the corresponding average value from Table 1. ND = Not detected (<0.05 ng/mL).

Table 3: Liquid chromatography-tandem mass spectrometry analysis of nodularin (the combination of nodularin-R and desmethyl-nodularin-R) in liver samples from Black Bream (BB) fish.

#	Sample Label	Weight (g)	Total Nodularin (ng/mL)[a]	dmMC-LR (ng/mL)[a]	dmMC-LR Recovery	Nodularin Content (µg/kg)
1	BB_CONTROL_F1_24Hr	1.023	ND	16.0	13%	-
2	BB_CONTROL_F2_24Hr	1.067	ND	88.4	69%	-
3	BB_CONTROL_F3_24Hr	1.007	ND	40.8	32%	-
4	BB_CONTROL_F4_24Hr	1.021	ND	29.6	23%	-
5	BB_CONTROL_F5_24Hr	1.038	ND	37.7	30%	-
6	BB_200_F1_24Hr	1.013	0.9	40.5	32%	1.3
7	BB_200_F2_24Hr	1.022	0.8	10.6	8%	4.4
8	BB_200_F3_24Hr	1.038	0.4	8.5	7%	2.9
9	BB_200_F4_24Hr	1.001	0.4	19.4	15%	1.4
10	BB_200_F5_24Hr	1.037	1.0	63.1	49%	1.0
11	BB_200_F1_2D	1.051	1.3	12.3	11%	5.5
12	BB_200_F2_2D	1.073	1.1	19.4	17%	3.0
13	BB_200_F3_2D	1.004	3.2	28.5	25%	6.2
14	BB_200_F4_2D	1.009	0.8	2.5	2%	- [b]
15	BB_200_F1_7D	1.012	1.9	30.5	27%	3.4
16	BB_200_F2_7D	1.008	1.5	54.5	49%	1.5
17	BB_200_F3_7D	1.052	5.6	73.8	66%	4.0
18	BB_200_F4_7D	1.007	1.8	64.5	58%	1.6
19	BB_CONTROL_F1_14D	1.024	0.4	33.4	30%	0.7
20	BB_CONTROL_F2_14D	1.026	0.4	45.3	40%	0.5
21	BB_CONTROL_F3_14D	1.010	ND	40.2	36%	-
22	BB_CONTROL_F4_14D	1.026	ND	23.5	21%	-
23	BB_200_F1_14D	1.028	14.4	32.1	29%	24.5
24	BB_200_F2_14D	1.006	1.9	26.0	23%	4.1
25	BB_200_F3_14D	1.012	2.1	52.3	47%	2.2
26	BB_200_F4_14D	0.920	6.2	54.8	49%	6.9
27	BB_CONTROL_F1_20D	1.014	ND	9.9	9%	-
28	BB_CONTROL_F2_20D	1.006	ND	69.7	62%	-
29	BB_CONTROL_F3_20D	1.015	ND	21.3	19%	-
30	BB_CONTROL_F4_20D	1.033	ND	43.7	39%	-
31	BB_200_F1_20D	1.059	0.4	11.8	11%	1.6
32	BB_200_F2_20D	1.058	1.0	14.5	13%	3.7
33	BB_200_F3_20D	1.027	1.1	29.1	26%	2.1
34	BB_200_F4_20D	1.078	0.4	29.6	26%	0.8

[a] Concentrations were adjusted for mass spectrometer suppression/enhancement effects using the corresponding average value from Table 1. [b] Nodularin content was not calculated as dmMC-LR recovery was very low. ND = Not detected (<0.05 ng/mL).

Table 4: Liquid chromatography-tandem mass spectrometry analysis of nodularin (the combination of nodularin-R and desmethyl-nodularin-R) in liver samples from flathead (FH) fish.

#	Sample Label	Weight (g)	Total Nodularin (ng/mL)[a]	dmMC-LR (ng/mL)[a]	dmMC-LR Recovery	Nodularin Content (µg/kg)
103	FH_CONTROL_F1_24Hr	1.085	ND	13.9	11%	-
104	FH_CONTROL_F2_24Hr	1.031	ND	11.0	9%	-
105	FH_CONTROL_F3_24Hr	1.016	ND	40.4	32%	-
106	FH_CONTROL_F4_24Hr	0.869	ND	1.2	1%	-
107	FH_CONTROL_F5_24Hr	0.727	0.3	16.6	13%	1.6
108	FH_CONTROL_F6_24Hr	1.019	ND	64.9	51%	-
109	FH_50_F1_24Hr	0.944	0.6	11.4	9%	3.5
110	FH_50_F2_24Hr	1.003	0.3	18.1	14%	1.2
111	FH_50_F3_24Hr	0.429	0.2	35.1	27%	0.9
112	FH_50_F4_24Hr	0.796	0.1	5.8	5%	1.7
113	FH_200_F1_24Hr	0.603	0.7	11.6	9%	5.9
114	FH_200_F2_24Hr	0.355	1.1	22.1	17%	9.0
115	FH_200_F3_24Hr	0.537	2.3	7.7	6%	35.9
116	FH_200_F4_24Hr	0.656	2.8	9.8	8%	28.1
117	FH_50_F1_2D	1.011	0.07	4.5	4%	1.0
118	FH_50_F2_2D	0.618	0.3	14.0	11%	1.9
119	FH_50_F3_2D	0.836	0.3	16.4	13%	1.2
120	FH_200_F1_2D	0.399	1.3	8.8	7%	23.5
121	FH_200_F2_2D	1.017	1.7	7.5	6%	13.9
122	FH_200_F3_2D	1.061	0.5	15.9	12%	1.9
123	FH_50_F1_7D	1.085	0.1	45.2	38%	0.1
124	FH_50_F2_7D	1.024	0.6	49.7	42%	0.6
125	FH_50_F3_7D	0.407	0.6	51.8	44%	1.7
126	FH_200_F1_7D	0.453	2.0	11.5	10%	22.1
127	FH_200_F2_7D	1.016	0.8	38.9	33%	1.2
128	FH_200_F3_7D	0.704	0.6	5.6	5%	9.5
129	FH_50_F1_14D	1.066	0.2	73.6	63%	0.1
130	FH_50_F2_14D	0.609	0.7	7.3	6%	8.8
131	FH_50_F3_14D	0.819	0.3	23.4	20%	1.0
132	FH_200_F1_14D	0.566	1.3	17.1	15%	8.0
133	FH_200_F2_14D	0.492	1.5	24.0	20%	7.6
134	FH_200_F3_14D	1.034	1.3	60.4	51%	1.2
135	FH_CONTROL_F1_20D	1.033	ND	8.0	7%	-
136	FH_CONTROL_F2_20D	0.508	ND	35.9	31%	-
137	FH_CONTROL_F3_20D	0.655	ND	71.5	61%	-
138	FH_CONTROL_F4_20D	0.577	ND	20.3	17%	-
139	FH_CONTROL_F5_20D	0.408	ND	15.4	13%	-
140	FH_CONTROL_F6_20D	0.878	ND	86.9	74%	-
141	FH_50_F1_20D	1.043	0.5	55.2	47%	0.5
142	FH_50_F2_20D	0.356	0.4	44.1	37%	1.4
143	FH_50_F3_20D	0.644	1.5	54.0	46%	2.6
144	FH_200_F1_20D	1.035	0.5	38.8	33%	0.7
145	FH_200_F2_20D	1.043	0.8	81.9	70%	0.6
146	FH_200_F3_20D	0.636	2.1	44.3	38%	4.4

[a] Concentrations were adjusted for mass spectrometer suppression/enhancement effects using the corresponding average value from Table 1. ND = Not detected (<0.05 ng/mL).

Table 5: Liquid chromatography-tandem mass spectrometry analysis of nodularin (the combination of nodularin-R and desmethyl-nodularin-R) in gut samples from Black Bream (BB) fish.

#	Sample Label	Weight (g)	Total Nodularin (ng/mL)[a]	Nodularin Content (µg/kg)
69	BB_CONTROL_F1_24Hr M	1.017	ND	-
70	BB_CONTROL_F2_24Hr M	1.006	ND	-
71	BB_CONTROL_F3_24Hr M	1.008	ND	-
72	BB_CONTROL_F4_24Hr M	1.055	ND	-
73	BB_CONTROL_F5_24Hr M	1.033	ND	-
74	BB_200_F1_24Hr M	1.015	ND	-
75	BB_200_F2_24Hr M	1.059	9.0	3.9
76	BB_200_F3_24Hr M	1.047	ND	-
77	BB_200_F4_24Hr M	1.026	21.8	9.7
78	BB_200_F5_24Hr M	1.039	7.5	3.3
79	BB_200_F1_2D M	1.039	ND	-
80	BB_200_F2_2D M	1.050	ND	-
81	BB_200_F3_2D M	1.047	ND	-
82	BB_200_F4_2D M	1.014	ND	-
83	BB_200_F1_7D M	1.019	ND	-
84	BB_200_F2_7D M	1.012	ND	-
85	BB_200_F3_7D M	1.093	ND	-
86	BB_200_F4_7D M	1.010	4.2	1.9
87	BB_CONTROL_F1_14D M	1.055	ND	-
88	BB_CONTROL_F2_14D M	1.072	ND	-
89	BB_CONTROL_F3_14D M	1.036	ND	-
90	BB_CONTROL_F4_20D M	1.052	ND	-
91	BB_200_F1_14D M	1.010	ND	-
92	BB_200_F2_14D M	1.069	ND	-
93	BB_200_F3_14D M	1.075	ND	-
94	BB_200_F4_14D M	1.071	ND	-
95	BB_CONTROL_F1_20D M	1.083	ND	-
96	BB_CONTROL_F2_20D M	1.067	ND	-
97	BB_CONTROL_F3_20D M	1.019	ND	-
98	BB_CONTROL_F4_20D M	1.016	ND	-
99	BB_200_F1_20D M	1.021	ND	-
100	BB_200_F2_20D M	1.057	ND	-
101	BB_200_F3_20D M	1.064	ND	-
102	BB_200_F4_20D M	1.027	ND	-

[a] Concentrations were adjusted for mass spectrometer suppression/enhancement effects using the corresponding average value from Table 1. ND = Not detected (<0.05 ng/mL).

Table 6: Liquid chromatography-tandem mass spectrometry analysis of nodularin (the combination of nodularin-R and desmethyl-nodularin-R) in gut samples from flathead (FH) fish.

#	Sample Label	Weight (g)	Total Nodularin (ng/mL)[a]	Nodularin Content (µg/kg)
191	FH_CONTROL_F1_24Hr	1.049	ND	-
192	FH_CONTROL_F2_24Hr	1.010	ND	-
193	FH_CONTROL_F3_24Hr	1.087	ND	-
194	FH_CONTROL_F4_24Hr	1.049	ND	-
195	FH_CONTROL_F5_24Hr	1.026	ND	-
196	FH_CONTROL_F6_24Hr	1.001	ND	-
197	FH_50_F1_24Hr	1.018	7.7	5.6
198	FH_50_F2_24Hr	1.043	11.6	8.3
199	FH_50_F3_24Hr	1.033	ND	-
200	FH_50_F4_24Hr	1.055	4.9	3.4
201	FH_200_F1_24Hr	1.005	ND	-
202	FH_200_F2_24Hr	1.022	ND	-
203	FH_200_F3_24Hr	1.004	ND	-
204	FH_200_F4_24Hr	1.059	ND	-
205	FH_50_F1_2D	1.014	1.8	1.3
206	FH_50_F2_2D	1.000	ND	-
207	FH_50_F3_2D	1.007	ND	-
208	FH_200_F1_2D	1.020	9.7	7.1
209	FH_200_F2_2D	1.028	18.0	13.1
210	FH_200_F3_2D	1.051	1.9	1.3
211	FH_50_F1_7D	1.002	ND	-
212	FH_50_F2_7D	1.001	ND	-
213	FH_50_F3_7D	1.064	2.2	1.5
214	FH_200_F1_7D	1.066	3.1	2.1
215	FH_200_F2_7D	1.029	ND	-
216	FH_200_F3_7D	1.022	3.6	2.6
217	FH_50_F1_14D	1.048	ND	-
218	FH_50_F2_14D	1.022	ND	-
219	FH_50_F3_14D	1.010	ND	-
220	FH_200_F1_14D	1.096	3.0	2.1
221	FH_200_F2_14D	1.015	1.8	1.3
222	FH_200_F3_14D	1.022	ND	-
223	FH_CONTROL_F1_20D	1.038	ND	-
224	FH_CONTROL_F2_20D	1.076	ND	-
225	FH_CONTROL_F3_20D	1.047	ND	-
226	FH_CONTROL_F4_20D	1.005	ND	-
227	FH_CONTROL_F5_20D	1.022	ND	-
228	FH_CONTROL_F6_20D	1.018	0.4	0.3
229	FH_50_F1_20D	1.083	ND	-
230	FH_50_F2_20D	1.055	ND	-
231	FH_50_F3_20D	1.041	ND	-
232	FH_200_F1_20D	1.027	ND	-
233	FH_200_F2_20D	1.047	ND	-
234	FH_200_F3_20D	1.018	ND	-

[a] Concentrations were adjusted for mass spectrometer suppression/enhancement effects using the corresponding average value from Table 1. ND = Not detected (<0.05 ng/mL).

Appendix C: Objective 2 report: A review of protocols for managing the risks to seafood safety from cyanobacterial toxins: A Gippsland Lakes Perspective.

A review of protocols for managing the risks to seafood safety from cyanobacterial toxins: A Gippsland Lakes Perspective.

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Glossary

APHA means American Public Health Association.

Aquaculture means the controlled production of molluscan shellfish in natural and artificial systems.

AS means Australian Standard.

ASP means Amnesic shellfish poisoning

ASQAAC means Australian Shellfish Quality Assurance Advisory Committee

Australian Shellfish Quality Assurance Program (ASQAP) means an agreed, co-operative State-Federal-Industry program to underpin the safety of commercial shellfish produced in Australia. accordance with these guidelines are safe, wholesome and properly labelled.

BGA Blue-Green Algae

CMA Catchment Management Authority

Cyanobacteria are a division of microorganisms that are related to bacteria but are capable of photosynthesis. They are prokaryotic and represent the earliest known form of life on the earth

Cyanotoxins are toxins produced by cyanobacteria (also known as blue-green algae).

DEWLP Department of Environment, Water, Land and Planning

Depuration means the process that uses a controlled aquatic environment to reduce the level of certain pathogenic organisms that may be present in live shellfish.

DHHS Department of Health and Human Services

DSP means Diarrhetic shellfish poisoning

EPA Environment Protection Authority

EU European Union

FRDC Fisheries Research Development Corporation

Growing area means the body of water (i.e. bay, harbour, gulf, cove, lagoon, inlet, estuary or river) in which commercial species of bivalve molluscs grow naturally or are grown by means of aquaculture.

HAB Harmful Algal Bloom

Harvest area means an area that has been designated by the SSCA or another competent State authority for the purpose of growing and/or harvesting commercial shellstock and may include wildstock or aquacultured shellstock.

Harvester means a person who takes shellfish by any means for commercial purposes from a growing area.

Hepatotoxin means cyanobacterial toxins that cause liver injury.

LWM Local Water Manager (for BGA management)

Marine Biotoxins are toxic compounds that are produced by certain kinds of microscopic algae that are naturally present in marine waters

NATA National Association of Testing Authorities

NHMRC National Health and Medical Research Council

NSP is Neurotoxic shellfish poisoning

NSSP National Shellfish Sanitation Program in the United States of America

PIRSA Primary Industries and Regions South Australia

PSP is Paralytic shellfish poisoning

QSWAMP Queensland Shellfish Water Assurance Monitoring Program

RACC Regional Algal Co-ordination Committee

Seafood is any edible fish, prawns and shellfish collected from fresh, estuarine and marine waters for human consumption.

Shellfish means all edible species of molluscan bivalves such as oysters, clams, scallops pipis and mussels, either shucked or in the shell, fresh or frozen, whole or in part or processed.

State Shellfish Control Authority (SSCA) means the State government agency or agencies having the legal authority to classify shellfish growing areas, control the relaying, harvesting, depuration and handling of shellstock and to seize shellstock that is contaminated or has been harvested from prohibited or closed shellfish harvesting areas.

SPATT means Solid Phase Adsorption Toxin Tracking

TSP Toxic shellfish poisoning

TSQAP Tasmanian Shellfish Quality Assurance Program

USFDA United States Food and Drug Administration

VSQAP Victorian Shellfish Quality Assurance Program

WASQAP Western Australian Shellfish Quality Assurance Program

WHO World Health Organisation

Wildstock means molluscan shellfish that grow in natural conditions without the application of cultural practices and are commercially harvested as a wild resource.

Background to this review

In the past two decades, there has been an apparent increase in the frequency, intensity and geographical distribution of toxic algal blooms in Australian coastal, estuarine and freshwater systems. The formation of these toxic blooms may be aided by increased nutrients, principally nitrogen and phosphorus, entering these systems from anthropogenic sources and warmer water temperatures (Myers, 2008). Cyanobacteria, or more commonly known as blue-green algae, are the cause of the majority of freshwater harmful algal blooms reported in Australia and worldwide however also form blooms in estuarine and marine waters (Lopez et al., 2008). Mortality of fish (wild and cultured) and contamination of seafood (fish, prawns, bivalve molluscs) have been associated with blooms of various cyanobacteria species including *Anabaena circinalis, Microcystis aeruginosa* and *Nodularia spumigena* (Hallegraeff 1992; Jia et al. 2014; Ibelings and Chorus 2007; Berry et al. 2011; Ettoumi et al. 2011). There is growing concern from the public, government agencies, aquaculturists and the commercial fishing sector regarding safety of seafood harvested from waters containing cyanobacterial blooms and regarding health risks associated with consumption of seafood contaminated with cyanotoxins. In Australia the occurrence of cyanobacterial blooms has resulted in substantial economic losses in fish and shellfish sales. For example, commercial fishers reported large reductions in fish catch in 2001 due to a *Lyngbya* bloom in Moreton Bay, Queensland (GeoScience Australia, 2013). In New South Wales commercial fishers have had to divert their catches to bait and adopt a gut and gill policy for fish prior to sale due to blooms of toxic cyanobacteria (NSW Food Authority, 2012). These actions have been required to reduce the likelihood of exposure to toxins in the fish during consumption, however they are time consuming and costly to industry. Additionally government agencies have incurred increased economic burdens due to increases in toxic algal outbreaks with increased monitoring and analysis costs and provision of advice regarding seafood and recreational safety (GeoScience Australia, 2013).

The Gippsland Lakes, one of Australia's largest lake systems, situated in south-eastern Victoria, support a range of recreational and commercial activities, including fishing. The Lakes are home to Victoria's largest fishing port and commercial fishing within the lakes amounts for approximately 200 tonnes of seafood annually and $1.1 million per year (DEDJTR 2016). They are also home to a significant recreational fishery, which on an annual basis may equal or exceed the commercial sector catch for some species (DEDJTR 2016). In the last decade the lakes have experienced an increase in the frequency and intensity of cyanobacterial blooms, with twelve major blooms of since 1985 (Day et al. 2011). In 1999 and more recently 2011-12 and 2012-13 toxic blooms of *N. spumigena* have led to lengthy restrictions on the harvesting and sale of shellfish, prawns and un-filleted fin-fish due to the presence of nodularin toxin in seafood tissues.

Significant economic losses have been incurred by the fishing and tourism industry in the region during the restricted periods and to government agencies. Average annual costs of algal blooms in the Gippsland Lakes have been estimated as at least $9 million (AUD dollars) (Ladson and Tillard 2011). Due to the closures the commercial and recreational fishing sectors have expressed concerns about the monitoring process for toxins in seafood during blooms and how it is conducted.

The current protocol "managing risks to seafood safety from blue-green algal blooms in the Gippsland Lakes" (Anonymous, 2011) was designed to provide a response capability for ensuring seafood safety during toxic blooms in the Lakes. It involves a multi-agency approach between various government organizations, including Department of Health, DEWLP, PrimeSafe, Fisheries Victoria, EPA Victoria, Parks Victoria and local councils. In short, the protocol is based on weekly seafood sampling of mussels, prawns and fish at various sites once the bloom has been identified as toxin producing. If levels of toxins in the seafood reach health alert levels set by a scientific advisory committee on algal toxins in seafood (Mulvena et al., 2012), the Department of Health advise the general public through media releases and signage and PrimeSafe (Victoria's statutory authority responsible for regulation of commercial seafood safety) regarding the collection and sale of seafood from the lakes. PrimeSafe then advise the commercial fisherman that they need to cease commercial harvest and sale of prawns and

whether they need to gut and gill finfish prior to sale until levels of toxin in the tissues have declined to below the health alert levels (Anonymous, 2011).

While the protocol has been implemented during blooms in the Lakes since 2011 a number of issues have been identified as requiring further research to provide confidence in design and execution. More specifically, issues identified include:

1. Weekly sampling of seafood is intensive and costly
2. Advisory periods have been lengthy due to issues in sampling and analysis
3. Provision of advisories is given based on nodularin concentrations in sentinel species, with little scientific understanding of uptake and elimination rates for toxins in all species harvested.
4. The gut and gill advisory is time consuming and costly for commercial operators
5. There is no secured funding for event response during toxic blooms
6. There is a lack of certified laboratories in Australia for the routine analysis of cyanobacterial toxins.

In order to provide recommendations to government and industry around practices recognised nationally and internationally to monitor and provide advice around seafood safety during harmful algal blooms a review of the current Gippsland Lakes protocol and comparison to programs for seafood safety applied worldwide is needed. This review is part of a larger project developed through consultation with Seafood Industry Victoria (SIV) and fishing operators in the Gippsland Lakes to develop management recommendations to assist in advisories around seafood safety during toxic bloom events in Gippsland Lakes. The review aims to provide an understanding of the nationally and internationally recognised practices for seafood monitoring programs and identify management and monitoring strategies that may be incorporated into the Gippsland Lakes protocol to improve the programs design and implementation.

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Introduction

Microscopic organisms, known more commonly as phytoplankton or microalgae, form the basis of the food web in marine, estuarine and freshwater environments. Under favourable conditions, particularly in systems over-enriched with nutrients with elevated water temperatures, sufficient light intensity and low water flow, these microalgae can rapidly increase in concentration forming what is known as an "algal bloom" (Myers 2008; Drobac et al. 2013). Algal blooms can have considerable negative impacts of the environment and on public health. They create unsightly, highly turbid waters, often with floating scum layers that deter recreational use and foul shorelines. Of greater concern are blooms of species that produce toxins which can have detrimental effects on the health of both humans and animals; for example, livestock and wildlife can be exposed when they encounter blooms at their source of drinking or cooling water while humans may be exposed as a result of drinking contaminated water, through recreational activities such as swimming in affected water or via consumption of seafood which has accumulated the toxins (Chu, 2011; Berry et al. 2011; Nagoda and Busse 2013). These blooms are commonly referred to as "Harmful Algal Blooms" or HABs and negative impacts from HABS not only affect the environment and human health, but have flow on effects to local economies of affected areas through economic losses to commercial fishing industry, goods and service providers to industry, recreational and indigenous fishers and tourism operators (Hallegraef 1992' Ettoumi et al. 2011; Chu 2011). In the past two decades, there has been an increase in the frequency, intensity and geographical distribution of HABs not only in Australia but globally, being a widespread phenomenon in coastal, estuarine and freshwater systems (Dillenberg and Dehnel, 1960; Hallegraeff, 1992; Sellner, 1997; Codd et al.., 1999; Falconer, 1999; Haider et al.., 2003; Burch 2008; Hudnell 2010).

Cyanobacteria, also known as blue green algae, are photosynthetic bacteria that are found naturally in freshwater and estuarine environments and less commonly in marine waters (Worcester and Taberski 2012; Hudnell 2010). They are the cause of the majority of freshwater HABs worldwide, with approximately 2000 species of cyanobacteria, of which over 40 have been identified as being toxin producers (Apeldoorn et al. 2007; Lopaz et al. 2008; Higman et al. 2014). Individual species may produce one or more toxins and individual toxins may have a number of different analogues, with bloom toxicity varying depending on environmental parameters such as salinity, nutrients and light (Apledoorn et al. 2007; Myers 2008; Higman et al. 2014). Surveys in different parts of the world have found that between 45% and 90% of cyanobacterial blooms are toxic (Global Water Research Coalition / Water Quality Research Australia, 2009). Toxins are largely retained within the cyanobacterial cells during bloom development and are released on cell death (Myers 2008). Cyanobacterial toxins can be classified into three main types – hepatotoxins, neurotoxins and dermatoxins (WHO 1999; Haider et al.., 2003; Apeldoorn et al. 2007; Drobac et al. 2013). The common toxins, causative agents and illnesses caused are shown in Table 1. The hepatotoxins are unique to the cyanobacteria and include microcystins, nodularin and cylindrospermopsin. They are commonly produced by the *Microcystis*, *Nodularia* and *Cylindrospermopsis* species. Hepatotoxins produce symptoms including nausea, vomiting and acute liver failure. In general these symptoms will appear rapidly, but may occur as late as several days following exposure to high amounts (Table 1). Neurotoxins include anatoxin a, anatoxins a(s) and saxitoxins and are commonly produced by the cyanobacteria species *Anabaena* and *Oscillatoria*. Consumption of large amounts of these toxins can result in muscle cramps, twitching, paralysis and cardia or respiratory failure. Symptoms appear within a few hours of exposure, but may take up to 36 hours to manifest themselves (Table 1). Dermatoxins, include lipopolysaccharides, which are produced by all cyanobacteria and Lyngbyatoxin-a and Aplysiatoxins which are most commonly produced by *Lyngbya* species. Dermatoxins are contact irritants and can result in severe dermatitis and conjunctivitis, and in severe cases irritation of airways leading to breathing difficulties.

Table 1. Cyanotoxins and their associated health effects (WHO, 1999; Chu 2011; Ettoumi et al. 2011).

Toxin Type	Toxins	Species	Symptoms
Hepatotoxins	Microcystin	*Microcystis, Planktothrix, Nostoc, Anabaena, Anabaenopsis*	gastroenteritis, nausea, vomiting and muscle weakness, liver failure
	Nodularin	*Nodularia*	
	Cylindrospermopsin	*Cylindrospermopsis, Aphanizomenon, Raphidiopsis*	
Neurotoxins	Anatoxin a	*Anabaena, Planktothrix, Aphanizomenon*	death by paralysis of peripheral skeletal muscles, then respiratory muscles, leading to respiratory arrest
	Anatoxin a (s)	*Anabaena*	
	Saxitoxins	*Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Planktothrix,* possibly *Microcystis aeruginosa.*	
Dermatoxins	lipopolysaccharides	All cyanobacteria	contact irritants, severe dermatitis and conjunctivitis, stomach cramps, nausea, fever, headaches, irritation of airways and breathing difficulties.
	Lyngbyatoxin-a	*Lyngbya*	
	Aplysiatoxins	*Lyngbya, Schizothrix, Planktothrix*	

There is growing evidence to suggest that cyanotoxins accumulate in shellfish. They can potentially cause sickness following consumption of these shellfish and other contaminated food products (Drobac et al. 2013; Higman et al. 2014). Numerous researchers have documented the occurrence and accumulation of cyanotoxins in various aquatic organisms including zooplankton, bivalves, gastropods, crustaceans, fish and water birds (see reviews and papers by Jia et al. 2014; Stewart et al. 2012; Berrye tal 2011; Etoumi et al. 2011; Ibelings and Chorus 2007; Ferao-Filho et al. 2011; Myers et al. 2010; Drobac et al. 2013). Cyanotoxins may be removed from fish and shellfish through depuration once exposure to toxic cells or toxins is removed. The rates of depuration however are species dependant (Myers et al. 2010; Jui et al. 2014). Once harvested cyanotoxins remain in fish tissues and cooking contaminated seafood does not render it harmless (VanBuynder et al. 2001; Myers et al. 2010; CDPH, 2010). Most often seafood contaminated with cyanotoxins looks and tastes the same as uncontaminated product. In the absence of adequate seafood safety controls for seafood harvesting and processing by both commercial and recreational fishers, the risk to human health is significant.

Ibelings and Chorus (2007) reviewed the consequences for public health from the accumulation of cyanobacterial toxins in "freshwater" seafood collected from coastal and freshwater environments. By far the bulk of information on cyanotoxins relates to the hepatotoxin microcystin with more limited information available on cylindrospermopsin, anatoxins and nodularin (Butler et al. 2012; Nagoda and Busse 2013; Ibelings and Chorus 2007; Drobac et al. 2013). In light of the findings of their review, Ibelings and Chorus concluded that exposure of humans to cyanotoxins through food may be underestimated and that concentrations reported in fish, mussels and shellfish from around the world have the potential to impact human health. The highest concentrations of cyanotoxins are typically found in the liver and gut of fish, or the hemolymph and hepatopancreas in shellfish (see reviews and papers by Jia et al. 2014; Stewart et al. 2012; Berry et al. 2011; Etoumi et al. 2011; Ibelings and Chorus 2007; Ferao-Filho et al. 2011; Myers et al. 2010; Drobac et al. 2013; Rita et al. 2014). For fish, these tissues are not typically eaten and their removal significantly lowers cyanotoxin exposure in humans (Myers et al. 2010; Ibelings and Chorus 2007; Drobac et al. 2013). However, elevated concentrations of cyanotoxins have been measured in edible portions of fish (muscle) and shellfish (muscle or whole) and there are various communities around the world that eat whole fish (Jia et al. 2014; Stewart et al. 2012 Berry et al. 2011; Etoumi et al. 2011; Ibelings and Chorus 2007; Ferao-Filho et al. 2011; Myers et al. 2010; Drobac et al. 2013; Rita et al. 2014). Ibelings and Chorus (2007) recommended that control measures should be implemented in areas where cyanobacterial blooms are a common occurrence and coincide with commercial seafood operations, and monitoring and surveillance of seafood quality should include cyanotoxin testing. For this purpose, guideline values or alert levels are also required (Ibelings and Chorus 2007).

Seafood is an important food source, not only in Australia, but globally. It is also a significant provider of employment and a direct and indirect source of revenue, with around 56 million people being directly employed in fisheries and aquaculture, and some 200 million employed along the value chain from harvesting to distribution worldwide. In 2012, fish production amounted for 157 million tonnes, of which marine fisheries and aquaculture accounted for 100 million tonnes, and inland fisheries and aquaculture making up the balance (United Nations, 2014). The safety of seafood products as a food source is of great importance from both a public health and economic viewpoint. Internationally and in Australia the impacts of marine HABs on public health and the economy are well recognised (Todd, 2001; US DHHS, 2007). Many countries, including Australia, manage the problems associated with marine biotoxins through implementation of regulatory monitoring programs based on a combination of phytoplankton monitoring and testing of seafood (mostly shellfish) flesh for toxins (Todd, 2001; US DHHS 2007). These programs are well planned, have relevant Federal and State legislative backing and State authorities have the necessary approval to initiate action if required. The programs are generally funded by industry or a combination of government and industry and have well documented protocols for managing a toxic bloom event (Todd, 2001; US DHHS, 2007). The objective of the biotoxin monitoring programs are to ensure that toxins levels in seafood are below levels that pose a threat to public health while providing confidence in seafood providers.

While risks of exposure to algal toxins through the consumption of seafood are well known from the marine environment (Todd, 2001; Higman et al. 2014), significantly less is known about freshwater and estuarine cyanotoxins. In the United States, between 1983 and 1992 seafood ranked third on the list of products which caused foodborne disease (Lipp and Rose, 1997 as seen in Ibelings and Chorus, 2007), a portion of which was attributed to the consumption of shellfish that had accumulated biotoxins. Aquaculture, in particular, has an inherent risk of developing cyanobacterial blooms based on methods of production, environment and organic inputs (Botana, 2008). In particular inland aquaculture of fish species such as carp, catfish, tilapia, milkfish and shrimp or prawns have been considered to have a medium to high risk for the development and occurrence of cyanobacterial blooms (Botana, 2008). In the past, health risks for consumers from cultured seafood products were traditionally considered negligible, because the liver, viscera or hepatopancreas were considered the main tissues for toxin accumulation and are predominantly not consumed (Rita et al. 2014). Recent studies, however, have shown concentrations of cyanotoxins in the edible portions of wild and farmed fish, prawns and mussels at levels that could exceed the provisional tolerable daily intake (TDI) proposed by the World

Health Organisation (WHO) if consumed (see reviews and papers by Kann, 2008; Jia et al. 2014; Stewart et al. 2012; Berry etal 2011; Etoumi et al. 2011; Ibelings and Chorus 2007; Ferao-Filho et al. 2011; Myers et al. 2010;Drobac et al. 2013; rita et al. 2014).

It is clear that the health risks to human consumers needs to be assessed and managed for cyanotoxins from freshwater and estuarine environments in a similar way to that undertaken for marine biotoxins. There are far fewer regulations in regards to cyanotoxins in seafood and subsequent monitoring compared to marine biotoxins. Worldwide, the assessment of risk to human health from cyanotoxin exposure and development of regulations has been concentrated on exposure through drinking water and recreation (i.e: bathing, swimming) with far fewer countries developing guidelines and monitoring of cyanotoxins in seafood (Ibelings et al. 2014; Burch, 2008).

Aims and objectives:

This review aims to provide an overview of regulation, management and monitoring programs for cyanotoxins in seafood in Australia and worldwide. In an attempt to compile information about monitoring for cyanotoxins in seafood, an internet search coupled with inquires with government agencies regarding monitoring protocols to provide seafood safety during cyanobacterial blooms was undertaken. While no programs were purposely excluded from this review, we did not go so far as to contact everyone. The review is divided into two sections. Section one provides a review of cyanotoxin regulation, management and monitoring in seafood in Australia and overseas. Section two provides details of internationally recognised practices for biotoxin monitoring and a comparison of the Gippsland Lakes protocol for providing seafood safety during toxic blue green algal blooms with that of biotoxin programs used in Australia and internationally.

Section 1: Regulations and monitoring of cyanotoxins in seafood

Health Alert Guidelines

The risk involved in eating fish and shellfish containing cyanotoxins has received increased attention in recent years. Ibelings and Chorus (2007) provide a comprehensive review of this issue and Ibelings et al. (2014) provide an update of the current approaches to cyanotoxin risk assessment and management around the Globe, including regulations for food. Worldwide there are far fewer regulations for exposure to cyanotoxins via food as there are for exposure via drinking water and recreation (Ibelings et al. 2014). By far the bulk of literature on cyanotoxins in seafood pertains to microcystins, with limited information on other cyanotoxins such as nodularin, cylindrospermopsin, saxitoxins and the anatoxins (Butler et al. 2012; Berry et al. 2011; Rita et al. 2014). The major factors contributing to cyanotoxin exposure through fish and shellfish consumption are the concentrations of the toxins in seafood and the consumption patterns (Todd, 2001; Drobac et al. 2013). While internationally there are no formal guidelines for cyanotoxins in seafood (with the exception of saxitoxin in some countries) an increasing number of countries are developing health alerts which document acceptable/unacceptable levels of cyanotoxin in edible portions of seafood that may be applied to provide seafood safety during toxic cyanobacterial blooms (Table 2). The approaches taken to develop alert values and their adoption in regulation and providing advisories varies worldwide (Chorus, 2012; Ibelings et al. 2014), with the main focus on microcystin toxins as they are widely regarded as the most significant source of risk to human health from cyanobacteria around the World (Butler et al. 2012; Berry et al. 2011; Rita et al. 2014).

Regulations in Australia

Currently in Australia, there are no regulatory guidelines that advise on safe levels of cyanotoxins in seafood at a national level (with the exception of saxitoxins in bivalve shellfish), however in Victoria 'health guideline' levels for cyanotoxins have been derived (Mulvenna et al. 2012). In order to provide a scientific basis for decisions on risks to public health from seafood consumption during toxic cyanobacterial blooms, the Victorian Department of Health convened an independent scientific panel to complete a risk assessment regarding seafood safety in the Gippsland Lakes, Victoria's only large inland water body with commercial and recreational fishing where re-occurring toxic cyanobacterial blooms occur (Mulvenna et al. 2012). Health guideline values were derived through this assessment (based on seafood consumption by 2-16 year age group as they are considered more susceptible to the effects of toxins) for fish, prawns, mussels and molluscs and the cyanotoxins microcystin, nodularin, saxitoxin and cylindrospermopsin, all of which have been reported in Australian fresh and/or estuarine environments as well as worldwide (Mulvenna et al. 2012). The Victorian health alert levels are shown in Table 3 and may be applied to commercially and recreationally harvested fish, prawns and molluscs. Following the development and implementation of these guidelines for cyanobacterial blooms in the Gippsland Lakes, the New South Wales State Algal Advisory group (a group of government agencies that respond to algal issues in NSW) adopted the Victorian health guideline levels as an interim measure to provide advice around seafood safety during cyanobacterial blooms in NSW (NSW Food Authority, 2012; Chorus, 2012).

Table 2: Guidance values and other regulations or recommendations for managing cyanotoxins in seafood (adapted from Ibelings et al. 2014).

Country	Management framework	Toxin	Guideline or trigger values	Reference
Australia (Victoria and NSW)	Guidelines applied in regulation of commercial and recreational harvest during toxic blooms.	Microcystins and Nodularin	24-51 µg/kg whole organism	Mulvenna et al. 2012
		Cylindrospermopsin	18-39 µg/kg whole organism	
		Saxitoxins	800 µg/kg whole organism	
USA (California)	Guidelines applied on a voluntary basis by local, regional, State and tribal entities	Microcystins	10 µg/kg wet weight	Butler et al., 2012
		Cylindrospermopsin	70 µg/kg wet weight	
		Anatoxin-a	5000 µg/kg wet weight	
USA (Ohio)	Guideline to do not eat fish.	Microcystins	28µg/kg fish fillet	Illinois EPA 2012
USA (Illinois, Kansas)	Guidelines to only eat fish if gutted and gilled. Do not eat shellfish.	Microcystins	20µg/L in water	Illinois EPA 2012
Denmark	European regulations on food hygiene and control of products of animal origin	No specific guideline values for cyanotoxins. No harvesting of shellfish if algal toxins of any type detected in water or flesh.		Christoffersen and Warming 2012
France	French Agency for Food Safety opinion.	Microcystins	Exposure to toxins in water and fish: 5.6 µg/kg (adult) 1.4µg/kg (child) Exposure through fish consumption only: 28µg/kg (adult) 7µg/kg (child)	Arnich 2012
		Saxitoxins	800 µg/kg	

Table 3: Health Guideline values for cyanobacterial toxins in seafood for Victoria (From Mulvenna et al. 2012).

Toxin	Health guideline value (µg/kg whole organism)		
	Fish	**Prawns**	**Mussels or Molluscs**
Microcystin-LR^ or equivalent toxins, i.e. Nodularin	24	32	51
Cylindrospermopsin and deoxy-cylindrospermopsin	18	24	39
Saxitoxins	800	800	800

^ due to lack of suitable standards and adequate comparative toxicity data, the recommendation of the Australian Drinking Water Guidelines is to treat all microcystins as having equivalent toxicity to microcystin-LR. The guideline value therefore represents the sum value for all detectable microcystins and nodularin.

International regulations

Internationally the development of guidelines for safe levels of cyanotoxins in seafood is limited. In the United States, California and Ohio provide guideline values for safe levels of cyanotoxins in fish and shellfish (Table 2). The Californian guidelines apply to the consumption of sport fish and shellfish and are not applied to the consumption of commercially harvested fish and shellfish (Nagoda and Busse 2013; Butler et al. 2012). They are not mandatory guidelines rather were developed for applicaiton on a voluntary basis by local, regional, State or tribal entities throughout California (Butler et al., 2012; Butler et al. 2012; Hudnell et al. 2012). The State of Ohio developed criteria for the consumption of microcystin-contaminated fish using tolerable daily intake (TDI) protocols based on lifetime exposure used in the Uniform Great Lakes Sport Fish Consumption Advisory (Illinois EPA 2012). They reported that microcystin concentrations of over 28 µg/kg fish fillet should not be eaten (Table 2). A memorandum from Illinois EPA regarding consumption of fish during toxic cyanobacterial blooms recommended that the World Health Organization's (WHO) guidance of 20µg/L microcystin in recreational waters could be applied as a water microcystin concentration at which to recommend "no consumption of fish" from cyanobacterial affected waters (Illinois EPA 2012). This recommendation was based on estimates of bioconcentration factors for microcystin in fish fillets and the 28µg/kg microcystin concentration in fish fillets derived in Ohio as a "do not eat" level (Illinois EPA 2012). In the State of Illinois the EPA will advise against eating fish unless the entrails are discarded, if microcystin is detected at 20ug/L, through media statements such as: "Due to the uncertainty about levels of toxins that can accumulate in fillets it may be best to wait a week or two after algal blooms are over before eating fish from waters where a bloom is occurring" (Illinois EPA, 2014).

Denmark have no specific regulatory limits for cyanotoxins in seafood however harvesting of bivalve molluscs (including mussels, cockles, clams, scallop and oysters) may only occur if no algal toxins, of all known types, are detected in water and shellfish and standards for food hygiene are met (Table 2) (Danish Veterinary and Food Authority, 2007; Christoffersen and Warming 2012). While France has no regulatory levels for cyanotoxins in seafood, in 2008 the French Agency for Food Safety (AFSSA) issued an opinion specifically regarding the contamination of seafood with microcystin toxins (Arnich 2012). They acknowledged that based on the TDI for chronic exposure set by WHO (0.04 µg/kg bw/day) and a daily consumption of 86 g fish and an intake of microcystin contaminated water, the maximum safe concentration of microcystin in edible parts of seafood for adults would be 5.6µg/kg and for children 1.4µg/kg. If data are available to show the absence of microcystins in drinking water, the maximum safe concentration of microcystin in edible parts of seafood would be 28 and 7 µg/kg of , respectively (Table 2) (Arnich, 2012). In 2009, AFSSA recommended to "do not eat fish" during a toxic bloom producing saxitoxins based on Australian, Brazilian and New Zealand guideline values for safe levels in seafood tissues (Arnich 2012).

Management and Monitoring programs:

Monitoring and Management practises in Australia for cyanotoxins

Currently in Australia there are no nationally recognised practices for the management of cyanotoxins in seafood. National frameworks for managing cyanobacterial blooms and their associated toxins in Australia are focused on providing States and Territories overarching guidelines to manage risks from exposure through drinking water and recreational contact. Examples are The Australian Drinking Water Guidelines (2004), The Guidelines for Managing Risks in Recreational Water (NHMRC 2008) and The Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC and ARMCANZ, 2000). Identification of risks posed by cyanobacterial toxins in seafood must therefore be identified at the State level and included in regional or local area cyanobacterial co-ordination and contingency plans.

Australian States and Territories undertake routine monitoring at designated sites known to have re-occurring cyanobacterial blooms or that pose a risk to recreational users as determined by methods described in Australian Guidelines for Managing Risks in Recreational Water (NHMRC 2008). Victoria is the only State that has developed and applies guideline values for cyanotoxins in seafood to direct commercial and recreational fishing activities for protection of public health from consumption of cyanotoxin-contaminated seafood (Stewart et al. 2012; Mulvenna and Orr 2012). The Victorian Department of Health assumed a leadership role in providing seafood safety in Victoria, through development of the protocol "Protocol for managing the risks to seafood safety from blue-green algal toxins in the Gippsland Lakes". This protocol describes the procedures for monitoring and providing public health advice regarding consumption of seafood and advisories to commercial and recreational harvesters of seafood during toxic blooms. In short, management actions are based on measured toxin levels in seafood; however collection of seafood samples is initiated based on detection of toxic phytoplankton at alert levels during routine phytoplankton monitoring. The program is currently co-ordinated and funded by government agencies with assistance from industry in collection of seafood samples (Anonymous, 2011).

Despite the occurrence of toxic cyanobacterial species, significant cyanobacterial blooms and recognition of the ability of seafood to accumulate cyanobacterial toxins in other Australian States and territories, currently there are no documented protocols for providing seafood safety in regards to cyanobacterial toxins or monitoring of toxins in seafood in the event of a bloom anywhere except Victoria. NSW has experienced several extensive potentially toxic cyanobacterial blooms in fresh and estuarine waters since 2009 (NSW Food Authority, 2012). These blooms have resulted in a range of seafood safety interventions including: advising recreational fishers to gut, gill and wash any fish caught in bloom area prior to cooking and to not collect yabbies and freshwater mussels (NSW Food Authority, 2012); Industry voluntarily diverting commercial harvest seafood to bait and adopting a gut and gill policy for fish prior to sale (NSW Food Authority, 2012); and Government mandating closure of areas to recreational and commercial harvest of seafood during an algal bloom (NSW Food Authority, 2012; NSW Government, 2012).

The decision to provide advisories and closures in NSW described above were generally made based on concentrations of toxic cells in water collected through regional or local area monitoring programs for algal blooms in recreational waters and not measures of toxins in seafood tissues (*Pers. Comm.* Alison Lewis, Hunter Regional Coordinating Committee representative). In 2012 NSW Food Authority undertook a review of the risk assessment for their Seafood Safety Scheme (NSW Food Authority, 2012). This review identified that there is a role for the NSW Food Authority in the whole-of-government approach to the management of cyanobacterial blooms. It determined that overall the risk to seafood consumers from cyanobacterial toxins in NSW is low, however, there are risk management activities ranging from issuing advisory information through to issuing recreational and commercial fishing closures that may be warranted during cyanobacterial blooms. The report stated that: "The Victorian health alert guidelines will provide valuable assistance during blooms" (NSW Food authority 2012).

South Australia, Western Australia, Tasmania, Northern Territory and the Australian Capital Territory do not monitor for cyanotoxins in fish, however they conduct routine phytoplankton monitoring as part of programs to provide protection for recreational users of regional or local water bodies (Mulvenna, 2012; Mulvenna and Orr, 2012). These programs are based on the Guidelines for Managing Risks in Recreational Water (NHMRC 2008) three tier framework, which is based on alert levels corresponding to Surveillance, Alert and Action Mode (NHMRC 2008). Advisories to the public are provided during "alert" and "action" levels of this framework via on site signs, information via the Internet and telephone hotlines about the increased risks for skin irritation and gastro-intestinal illness from contact in bloom affected areas (NHMRC, 2008). At the "Action" level the public is typically advised to refrain from recreation involving water contact (particularly with the risk of oral uptake), and authorities may temporarily close water bodies for primary contact recreation such as swimming (NHMRC, 2008). Advisories are provided as press releases or in information leaflets informing the public about the risks of consuming contaminated seafood, for example "any fish harvested from Blue-green algal affected water should have gills and guts removed prior to cooking. People should not eat whole fish, or shellfish or crustaceans collected from the bloom affected water body. The type of algae affecting the water body produces toxins that can concentrate in shellfish and crustaceans and also accumulate in the liver and internal organs of fish. Ingesting BGA toxins can lead to serious illness" (SA Department of Health, 2008; NSW Government, 2012a). These advisories are based on cyanotoxin levels in water or cell counts/biovolume measures. While Western Australia does not have an official shellfish monitoring program in freshwaters the Western Australia Health policy recommends people not to consume shellfish collected recreationally (Koutsoukos, 2010). They provide an Environmental Health Guide about wild shellfish collection which provides advice around the risks of eating recreationally gathered shellfish and fish from algal affected waters (Koutsoukos, 2010; Department of Health, 2008).

International practices for monitoring and management of cyanotoxins

Internationally, like Australia, there are no guidelines or regulations for the implementation of monitoring programs for cyanotoxins in seafood as there are with marine biotoxins in shellfish. Denmark appears to be the only country that routinely monitors cyanobacterial toxins in seafood. Cyanotoxins are considered together with marine biotoxins in routine seafood monitoring, where commercial harvesting of mussels is subject to closure by regulatory authorities in the event that any toxins of any type are detected through their monitoring program (Danish Veterinary and Food Authority, 2007; Christoffersen and Warming 2012). The monitoring program has been in place since 1991, is funded by industry, and involves weekly sampling of both phytoplankton and shellfish year round. Closures based on cyanobacteria are for the species *Nodularia spumigena* and may be initiated by phytoplankton reaching a triggered response level (Danish Veterinary and Food Authority, 2007).

In a report by the Scottish Government in 2012 assessing the risks to public health from cyanobacteria in inland and inshore waters it was stated: "Another potential source of intoxication for both animals and humans is via bioaccumulation of cyanotoxins in the food chain. The principal concern here would be the accumulation of toxins in shellfish including freshwater and brackish water mussel and fish" (Scottish Government, 2012). The report suggests that local action plans and proactive risk assessments should consider the need for advice to avoid eating freshwater shellfish. Further the report stated: "fish should not be consumed if fish mortalities or abnormal behaviour ware observed in water bodies containing mass populations of cyanobacteria. In the event of cyanobacterial scum being present or cyanobacterial cell numbers exceeding 20,000 per ml, toxin analysis of fish intended for consumption should be carried out. If toxins are detected expert advice would need to be sought on whether concentrations are sufficient to justify restrictions on the consumption of fish". The report also advised that the liver and gut of fish caught in waters affected by cyanobacteria should not be fed to pets (Scottish Government 2012). While the UK do not actively monitor for cyanotoxins in seafood, a recent report into the development of a monitoring programme for marine biotoxins in the UK, identified that there was potential for cyanotoxins to contaminate shellfish and that the risk should be assessed (Higman et al. 2014. In particular the report identified that a review of shellfish harvesting

areas should be made to assess potential for influx of cyanotoxins into harvesting areas. This would allow determination of areas of high risk which could be incorporated into any future monitoring programs (Higman et al. 2014). The report acknowledged that there was strong evidence for occurrence of cyanobacterial blooms in the UK and there is evidence from other regions of the world to suggest that risk exists and without monitoring programs the risk is potentially significant (Higman et al. 2014).

In the United States there is no routine monitoring of seafood for cyanotoxins in any of the 48 States (Hudnell et al. 2012; Jabusch, 2013). In 2012-13 surveys of cyanotoxins in lakes, reservoirs and coastal wetlands across California resulted in questions regarding the risks of eating fish and shellfish (Nagoda and Busse, 2013). California health advisory levels for cyanotoxins in fish were developed in 2012; however they are applied on a voluntary basis, and in most instances, as with all other US States, monitoring for cyanobacterial toxins in seafood is not directly undertaken (Nagoda and Busse, 2013; Butler et al., 2012; Jabusch, 2013). Advisories around seafood safety are based on cell concentrations of toxic species and/or cyanotoxin levels in water and are provided as part of advisories for recreational use of water bodies (Nagoda and Busse, 2013; Jabusch, 2013; Hudnell et al. 2012). Media releases and signs erected at the sites of toxic blooms will contain information relating to seafood safety generally in the form of a Statements such as: "*at minimum organs be removed and discarded prior to cooking fish fillets and that caution be taken with shellfish*" or in some instances "*shellfish should not be consumed*" (Oregon Health Authority; 2011; Kasich et al. 2014; Illinois EPA, 2014; Hardy, 2008).

Internationally there is growing awareness of the emerging risk from cyanobacterial toxins and potential risks from consumption of toxin contaminated seafood. There is however a lack of data on cyanotoxins in lakes, on which judgments about health risks can be made. Monitoring is considered to be expensive and/or there are no commercial laboratories that have the ability to analyse for these toxins. Providing an advisory based on phytoplankton and or/cyanotoxins monitoring to "not eat" or "eat when gutted and appropriately filleted" is therefore the approach adopted in many countries.

Summary of cyanotoxins regulation, monitoring and management in seafood:

Around the world governments recognise the risks cyanotoxins pose to human health in regards to exposure through drinking water and reactional waters (Ibelings et al. 2014; WHO 1999) and increasingly they are starting to acknowledge potential risk of exposure through seafood consumption (Ibelings et al. 2014; Nagoda and Busse, 2013; Chorus 2012; Higman et al. 2014). Overall regulations and monitoring for cyanotoxins in seafood lags far behind those developed for marine biotoxins in shellfish. Well-developed frameworks, including the EU directive, US National Sanitary Survey Program and Australian Shellfish Quality Assurance Program, providing appropriate legislative backing are available for guiding the development of monitoring programs to provide seafood safety and manage risks from marine biotoxin outbreaks. To date, internationally Denmark are the only country to routinely monitor and manage risks posed by cyanotoxins in shellfish (Christoffersen and Warming, 2012; Ibelings et al. 2014). Nationally, Victoria have taken a lead in managing risks posed by cyanotoxins in seafood through the development of health alert levels in seafood and providing a contingency plan to monitor cyanotoxins in seafood during toxic blooms in a lake frequented by cyanobacterial blooms and being a significant commercial and recreational fishery (Mulvenna and Orr 2012; Stewart et al. 2012). In most countries and Australian States and Territories the risks posed by cyanotoxins in seafood are considered to be low or are not understood and routine or event based monitoring to provide advice around safety of consumption during toxic bloom events is not conducted.

Worldwide research indicates that seafood may accumulate cyanobacterial toxins to levels that pose threats to humans through consumption (see reviews and papers by Kan, 208; Jia et al. 2014; Stewart et al. 2012; Berry etal 2011; Etoumi et al. 2011; Ibelings and Chorus 2007; Ferao-Filho et al. 2011; Myers et al. 2010;Drobac et al. 2013; Rita et al. 2014) and various researchers have documented the need for risk assessments and monitoring protocols, similar to that in marine systems, where cyanobacterial

blooms occur frequently and seafood is harvested (whether for commercial or recreational purposes) if public health safety is to be maintained (Van Buynder et al. 2001; Kan, 2008; Stewart et al. 2012; Rita et al. 2014; Berry et al. 2011; Jui et al. 2014). In order to provide seafood safety in relation to cyanotoxins there is a need to understand risks posed to consumers and in water bodies where risk is identified, strategies are needed to deal with this risk. The potential for public health effects from marine biotoxins is well recognised by public health and food regulatory agencies in Australia and throughout the world, with all Australian States and most overseas countries having regulatory requirements stating the minimum requirements for marine biotoxin monitoring programs, the need for contingency management plans that outline how biotoxin events will be managed and the maximum permissible toxin levels allowed in shellfish for human consumption. For freshwater and estuarine cyanotoxins the potential for public health effects is recognised by health authorities in areas where blooms regularly occur, however regulations for permissible levels allowed in seafood and for standardised monitoring programs in both Australia and overseas are far behind those for marine biotoxins. Cyanotoxins can be accumulated in seafood and pose risks similar to those of marine toxins. The risk assessment based approach currently applied in marine biotoxin management could easily be applied to assess risks for seafood produced or harvested from freshwater systems to determine if a cyanotoxin monitoring program is required.

Section2: Review of current monitoring program for providing seafood safety in the Gippsland Lakes and comparison with marine biotoxin management programs in Australia

Marine biotoxin monitoring

The potential public health effects from marine biotoxins are well recognised by public health and food authorities in Australia and worldwide. Most countries have national guidelines which outline to the procedures needed to meet obligations to protect consumers and comply with requirements laid down in national and international legislation.

These guidelines specifically relate to biotoxins and reflect current best practice and the legal requirements. They outline the procedures for:-

1. Collection and delivery of shellfish and phytoplankton samples
2. Analysis of shellfish samples
3. Assigning a status to a production area
4. Communication of results
5. Additional management procedures

Two well established international marine biotoxin monitoring and management guidelines that will be referred to in this review include: The United States of America Food and Drug Administration (USFDA) National Shellfish Sanitation (NSSP) Model Ordinance and the European Union 91/492/EEC directive (US FDA, 2013; Office of Official Publications of European Communities, 2003).

In Australia the Australian Shellfish Quality Assurance Programs (ASQAPs) Manual of Operations (ASQAP, 2009) provides guidance on development of marine biotoxin monitoring programs to meet Federal and State legislation governing the control of shellfish growing areas, and harvesting, processing and distribution in relation to biotoxins. In relation to marine biotoxin monitoring, Section 28 Biotoxin Management of the Manual of Operations states that:

"Section 28 Marine Biotoxin Control

28.1 *The SSCA must develop and implement a marine biotoxin management plan for all commercial shellfish harvesting areas.*

28.2 *The biotoxin management plan must define for every growing area:*

(a) The responsibilities of all parties involved in the management plan

(b) Hydrographic details describing predominant currents and circulatory patterns

(c) Species of shellfish cultured/harvested

(d) Sample sites

(e) Sampling frequencies

(f) Sampling methods

(g) Methods of analysis for water and shellfish samples

(h) Laboratories used for sample analysis

(i) Alert level/s for toxic/potentially toxic algal species

(j) Potentially toxic algal species list

(k) Actions to be taken by SSCA when either alert levels are exceeded or toxins are found in shellfish below closure levels

(l) Closure procedures including closure criteria, notification of closures to marine farmers and relevant authorities, public announcements, management during closures, product recall

(m) Opening procedures including opening criteria, notification of opening to marine farmers and relevant authorities, public announcements, procedures for opening inactive or seasonal growing areas

(n) Case definitions of toxic syndromes

28.3 *A guidance document for developing, implementing and reviewing a marine biotoxin management plan is attached in Appendix V.*

28.4 *Representative samples of shellfish and/or water must be collected for biotoxin and/or algal analysis during all harvest periods unless the SSCA has adequate data to demonstrate there is no biotoxin risk.*

28.5 *The SSCA must review the position of sampling stations and the frequency of sampling in each growing area based on an analysis of all data, incorporating historical data where possible.*

28.6 *A harvesting area, or portion(s) thereof, must be closed for the harvesting of shellfish when the SSCA determines that:*

(a) toxins in shellfish are found to be above the levels prescribed in Standard 1.4.1 of the Australia New Zealand Food Standards Code, shown below; or

Toxin Group	*Maximum Limit*
Paralytic Shellfish Poison (Saxitoxin equivalent)	*0.8 mg/kg*
Amnesic Shellfish Poison (Domoic Acid equivalent)	*20 mg/kg*
Diarrhetic Shellfish Poison (Okadaic Acid equivalent)	*0.2 mg/kg*
Neurotoxic Shellfish Poison	*200 MU/kg*

(b) algal cell counts for known toxic species go above those prescribed for closure in the growing areas management plan in the absence of toxicity data; or

(c) cases of illness consistent with the case definitions for any toxin syndrome have resulted from the consumption of shellfish from a particular area; or

(d) the SSCA determines a closure is necessary for any other reason.

28.7 *When sufficient data exists to establish that certain shellfish species or shellfish products do not accumulate marine biotoxins, then the biotoxin management plan may exempt these species from biotoxin closures.*

28.8 *The SSCA may exempt specific shellfish species from a marine biotoxin closure when adequate data exists to demonstrate that the species complies with Standard 1.4.1 of the Food Standards Code. In this case regular testing must be conducted on the species in the open status.*

28.9 *A marine biotoxin closure must remain in effect until the SSCA has data to show that the toxin content of the shellfish in the harvesting area complies with Section 1.4.1 of the Australia New Zealand Food Standards Code.*

28.10 *The determination to return a harvesting area to the open status must consider whether toxin levels in the shellfish and/or algal cells counts from adjacent areas are declining.*

28.11 *Where toxicity tests are taken for the purpose of opening a growing area the laboratory must use AOAC and/or APHA approved methods.*

28.12 *All toxicity testing methods that are not AOAC or APHA approved will be regarded as screening methods only.*

28.13 *A growing area must be closed when screening methods indicate toxins above or potentially above the maximum levels given in Standard 1.4.1 of the Australia New Zealand Food Standards Code pending results from an approved method.*

28.14 *The justification for returning a harvesting area to the open status must be adequately documented.*

28.15 *The SSCA must maintain a copy of all of the following records:*

(a) all information, including monitoring data, relating to the levels of biotoxins in the shellfish harvesting areas

(b) copies of notices placing harvesting areas in the closed status

(c) evaluation reports

(d) copies of notices returning harvesting areas to the open status

(e) copies of any public announcements made.".

The marine biotoxin management protocols for each State detail regular monitoring programs and also document/outline the administrative procedures, regulations and resources necessary for:

- Initiating an emergency shellfish sampling and analysis program
- Closing harvesting areas and embargo shellfish
- Preventing harvesting of contaminated shellfish
- Providing product recall
- Disseminating information of the occurrence of blooms.

State marine biotoxin management plans are designed around both routine and event based monitoring (ASQAP, 2009). While the methods and implementation vary by State generally they use a combination of phytoplankton and flesh monitoring in order to provide protection for consumers against consumption of toxin contaminated shellfish (Anonymous, 2015; DPIF, 2005; NSW Food Authority, 2014; DHHS, 2014; Biosecurity SA, 2014; DOH, 2015; Ecowise Environmental, 2009). All State programs are developed primarily for commercial harvest shellfish and are funded in most part by industry. The programs are based around marine shellfish including mussels, scallops, pipis, clams, and oysters and are for management of marine toxins including ASP PSP, DSP, NSP (Anonymous, 2015; DPIF, 2005; NSW Food Authority, 2014; DHHS, 2014; Biosecurity SA, 2014; DOH, 2015; Ecowise Environmental, 2009).

While there are currently no routine programs for recreationally harvested shellfish an outbreak of shellfish poisoning resulting from recreational shellfish gathering has the potential to cause considerable economic damage to commercial industry (Todd, 2001). In most States industry works with Government agencies, providing access to results of phytoplankton and biotoxin monitoring so as advice to recreational gathers can be provided, albeit from commercial growing areas only.

Gippsland Lakes seafood monitoring protocol

Nodularia blooms are a recurring problem in the Gippsland Lakes. Since 1986, there have been eight major blooms. Before 1986, however, blooms were rare, with only a handful of blooms reported in almost 200 years of European settlement (Holland et al. 2013). The Gippsland Lakes are a significant commercial and recreational fishery for Victoria (GLMAC, 2013). Key Species commercial significance in the lakes include Black Bream, eastern king prawns, school prawns, yellow eye mullet, tailor, river garfish, estuary perch, Australian anchovy, dusky flathead, luderick, Australian salmon, silver trevally, leatherjackets and sea mullet (DSEWPE, 2010). While key species of recreational significance include dusky flathead and Black Bream, as well as snapper, whiting, squid, mussels and prawns (DSEWPW, 2010).

Toxins were first assessed in seafood from the Gippsland Lakes in 1999 and 2001(Eaglesham et al. 2002; Van Buynder et al. 2001), thereafter a working group including the Department of Health and then Department of Primary Industries developed triggers and alert level responses to apply during toxic algal blooms to provide seafood safety. These alert levels were presented in a paper "Nodularin uptake by seafood in a cyanobacterial bloom" by Van Buynder et al. 2001. From 2001, Victoria were the first Australian State to provide guidance on risks to seafood safety from cyanotoxins, including in Regional Contingency plans for the Gippsland Lakes a section on "Alert levels and management actions for waters used for harvest of seafood for human consumption" (DPI, 2001).

Currently, the management of public health with respect to cyanotoxins in the Gippsland Lakes is set out in the "Protocol for managing the risks to seafood safety from blue-green algal toxins in the Gippsland Lakes "(Anonymous, 2011). The protocol forms a part of the regional co-ordination plan for blue green alga in East Gippsland. The protocol is an event based monitoring protocol that provides a

means to ensure the protection of the public against the adverse health effects of cyanotoxins from recreational and commercial harvesting of seafood with the Lakes. It defines the roles and responsibilities of agencies in the event of a toxic bloom which poses a risk to seafood safety, the actions to take place and what procedures are to be followed to effectively manage seafood safety and to ensure agency preparedness to manage the risk. The protocol is designed for management of seafood safety from blue-green algal toxins produced within the Gippsland Lakes and applies to four different toxins: microcystins, nodularin, saxitoxins and cylindospermopsin. Seafood in this protocol being defined as fish, mussels, molluscs and prawns.

Challenges to the current event based seafood monitoring protocol for the Gippsland Lakes identified by government and industry include:

- Lack of funding for seafood monitoring and toxin analysis
- Lack of communication regarding processes involved in sampling and analysis
- Lack of knowledge around seafood uptake of toxins to provide guidance on sampling protocols
- No laboratory in Victoria to undertake toxin analysis.

The review:

An evaluation of the Gippsland Lakes "Protocol for managing risks to seafood safety from blue green algal toxins in the Gippsland Lakes" in comparison to Australian State marine biotoxin management plan documents was undertaken. Information was collected from internet searches, database searches and contact with State/territory biotoxin program managers. Sources of information used to conduct the review included: State Shellfish Biotoxin Management Plans and/or monitoring program outlines; Response protocols and operational procedures for Harmful Algal blooms (recreational); Relevant State legislation and follow-up contact with Shellfish Quality Assurance Program managers via telephone and/or email for clarification and further details.

In order to provide comparison between practices in marine biotoxin programs and that of the Gippsland Lakes information was summarised in common format to highlight key elements. The subheadings for comparison were as follows:

- Program administration
- Phytoplankton and biotoxin monitoring
- Sampling procedures
- Closure and opening protocols
- Notification procedures
- Triggers for review

At the end of the comparison strengths and weaknesses of the Gippsland Lakes protocol are assessed and comments provided to assist in the process of improvements.

Program administration:

Marine biotoxin management programs for commercially harvested (wild and aquaculture) shellfish in Australian States are administered by both government agencies and authorities or are contracted out by the commercial shellfish industry (Todd, 2001). Industry fully supports the biotoxin management programs and has active involvement in their operation. Recreational programs are administered by government; however they often rely on commercial programs for data collection and analysis (Todd, 2001; Hay et al. 2000). Each States marine biotoxin management plan clearly documents the roles and responsibilities of agencies and industry. Tasmanian, South and Western Australia programs are managed by the State Shellfish Quality Assurance Program (SQAP) manager (Biosecurity SA, 2010; DHHS, 2014; DOH, 2015), while New South Wales and Queensland programs are overseen by the

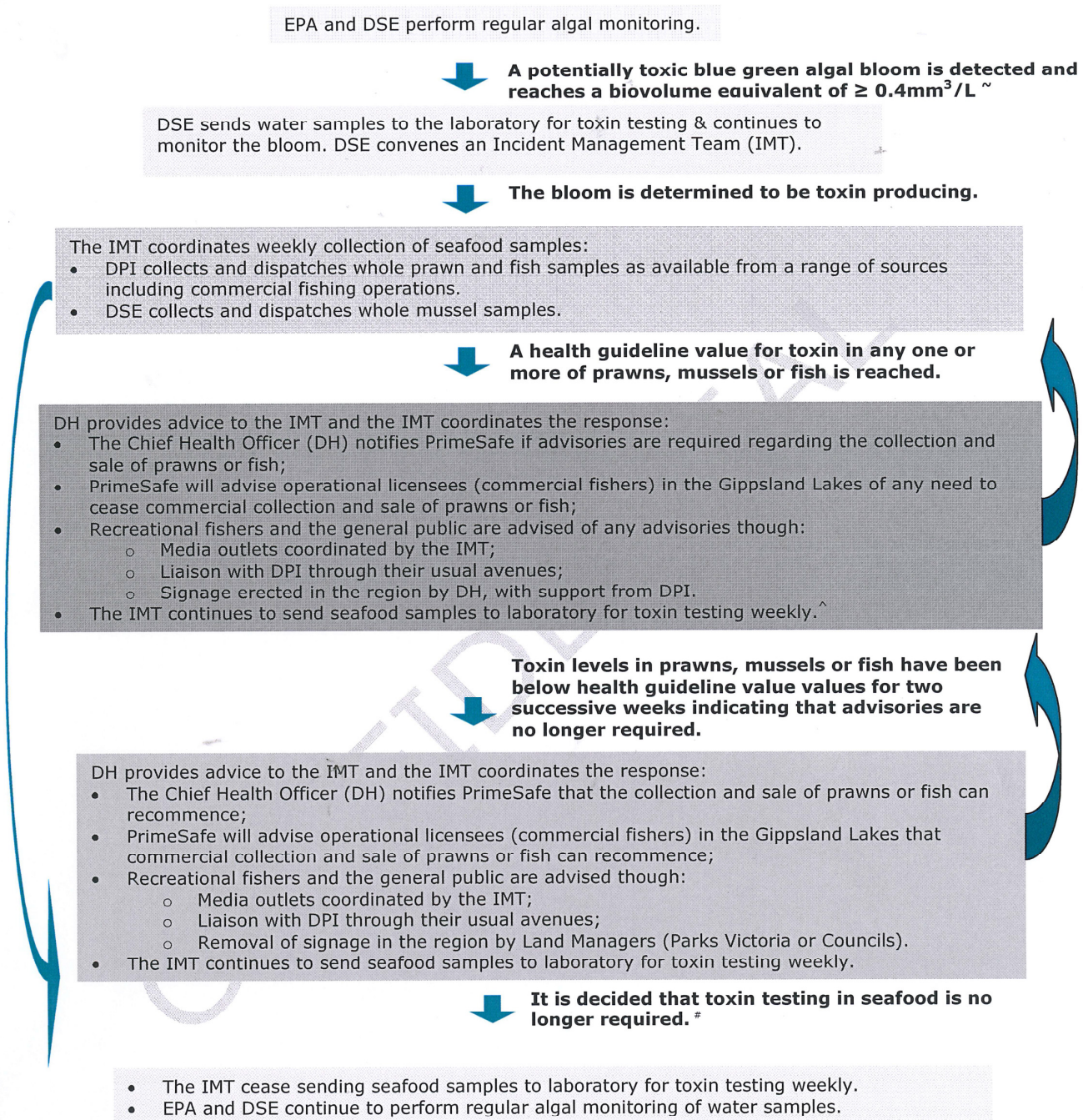
State SQAP manager and a local area manager is responsible for program management (NSW Food authority, 2014; DPIF, 2005). In Victoria, the SQAP was transitioned over to an industry managed program, whereby industry has tendered out the program and a harvest area manager is responsible for program administration (Anonymous, 2015; Ecowise environmental, 2009). These program managers are responsible for:

- Conducting or co-ordinating phytoplankton and shellfish monitoring
- Investigation of toxic events
- Closure and opening harvest areas (usually based on advice from legislating authorities)
- Early warning and official reporting of toxic events
- Communication between relevant statutory authorities, government agencies and industry

Funding for programs in Australia is fluid and is supported by government and industry either directly or through levies and licence fees (DPIF, 2005; NSW Food Authority, 2014; DHHS, 2014; Ecowise Environmental, 2009). For States and Territory's with recreational programs government has the responsibility of the costs (Hay, 2000; Anonymous, 2015; DPIF, 2005; NSW Food Authority, 2014; DHHS, 2014; Biosecurity SA, 2014; DOH, 2015; Ecowise Environmental, 2009).

Internationally management programs have often been initiated and planned by government agencies, with few countries having programs initiated by the private sector or a combination of government and private organisations (Todd, 2001). Currently in New Zealand, Ireland, Chile and Denmark programs are administered and run by industry for commercial harvest (Rhodes et al. 2003; Danish Food and Veterinary Authority, 2007; Food Safety Authority Ireland, 2014). The New Zealand government run a recreational program separately (Hay et al. 2000). Internationally there are a mix of models in place to fund monitoring in commercial growing areas, however where reactional programs exist they are government funded (Todd, 2001; Danish Food and Veterinary Authority, 2007; Food Safety Authority Ireland, 2014). For instance in New Zealand industry pays for all the monitoring undertaken in commercial areas on a user pays system, whereas the Ministry of Health monitors the non-commercial areas. The ministry of health has a data sharing agreement with the commercial industry to use their data to assist in dealing with marine biotoxin problems in nearby recreational areas (Todd, 2001; Hay et al. 2000; Rhodes et al. 2013).

Department of Environment, Water, Land and Planning (DEWLP) are the administering agency for algal blooms in the Gippsland Lakes (Mulvenna and Orr 2012; Warwick et al. 2009). DEWLP are the State-wide co-ordinators for Blue Green algae in Victoria and currently rely on a system of 10 regional co-ordinators (convening agencies) for regional emergency planning and preparedness for regional blooms. (). Local water managers are responsible for management of blooms within local water storages and water ways (DEPI, 2014, Mulvenna and Orr 2012; Warwick et al. 2009; DEPI, 2014). As regional coordinators for East Gippsland region DEWLP is the administering agency for blue green algal blooms in the Gippsland Lakes (DEPI, 2014). The main role of DEWLP includes: the development, co-ordination and implementation of algal bloom contingency strategies; co-ordination of regional media relations and public information programs related to blue-green algae; the development, co-ordination and implementation of regional algal monitoring programs; co-ordination and implementation of training programs for algal sampling and identification of when warnings should be issued and which agency should issue those warnings and advisories for drinking and recreational uses (Warwick et al. 2009; DEPI, 2014). A part of the DEWLP Blue Green Algal co-ordination plan for East Gippsland is the "Protocol for managing risks of seafood safety from blue-green algal toxins in the Gippsland Lakes" (Anonymous, 2011). This is the key document setting out the roles and responsibilities of agencies in toxic blue-green alga blooms in the Gippsland Lakes. Major factors discussed include guidelines for cyanotoxins in seafood; seafood monitoring; seafood sampling strategy; incident notification; lifting advisories and a flow chart of the response process (Figure 1).



~ This level equates to the Alert mode/Amber level in the *Guidelines for Managing Risks in Recreational Water* (National Health and Medical Research Council 2008). At this biovolume algal/water samples are able to be reliably tested for toxins.

^ If commercial fishing has been prohibited for prawns or fish and bait fishers are unable to provide samples then it may be necessary to pay fishers to collect the samples.

This will be decided by the IMT, based on the growth status of the bloom and other environmental conditions.

Figure 1: The monitoring and management process to provide advisories around seafood safety during toxic cyanobacterial blooms in the Gippsland Lakes Protocol (From Anonymous, 2011).

There is no lead agency listed as being responsible for administering this protocol rather in the event that DEWLPs routine phytoplankton monitoring shows increases in toxic phytoplankton species to recreational amber alert levels (Anonymous, 2011) DEWLP will convene an Incident Management Team (IMT). This IMT manage the response to cyanobacterial blooms and is made up of representatives from government agencies and authorities. There is no reference to industry's role and responsibility in the program. The IMT co-ordinates all aspects of response to blooms including seafood sampling, media response and communications based on advice from the Victorian Department of Health (DH). Procedures for incident response and notification are provided in the protocol, however details regarding responsible agency for reporting and management of documents relating to bloom event and decisions made throughout the event are not documented. There is provision for an incident controller; however who would take this role is not detailed, rather in order to provide notifications a number of agencies are involved. For instance for all aspects the Chief Health officer (CHO) from Victorian Department of Health must provide advice to the IMT to co-ordinate any response. The CHO must notify PrimeSafe of any need to provide advice to commercial operators to cease harvesting. PrimeSafe must then notify commercial operators to cease harvesting. There are no details of who would provide surveillance during the event of an advisory in the Lakes.

Funding for the sampling and analysis of seafood during a toxic bloom event is provided by Government, specifically the Department of Health. DEWLP are responsible for costs related to routine phytoplankton monitoring, which is run under a separate program. Industry currently contributes through collection of samples. During an event, personnel provided to assist in the co-ordination of the bloom would be funded by the government agency with which they are employed.

Program Legislation:

Legislation provides government agencies the means to protect public health from toxins in seafood products. It provides a means to control harvesting by commercial and recreational fishers and take action if needed to protect human health. For marine biotoxin programs legislation for administering safety of commercially cultured and wild harvest shellfish consists of both National and State legislation (Table 4). Nationally there are two standards, which are referred to in Australian State and Territory legislation, which apply when providing seafood safety from biotoxins;

The Australia New Zealand Food Standards Code - Standard 4.2.1 - Primary Production and Processing Standard for Seafood (Australia Only) - F2012C00775. This standard outlines the regulations for seafood products harvested and sold for human consumption in Australia. Specifically Division 2 – Seafood safety requirements states that:

> *3 General seafood safety management*
>
> *A seafood business must systematically examine all of its primary production and processing operations to identify potential seafood safety hazards and implement controls that are commensurate with the food safety risk.*
>
> *5. Inputs and harvesting areas*
>
> *(1) A seafood business must take all reasonable measures to ensure inputs do not adversely affect the safety or suitability of the seafood.*
>
> *(2) A seafood business must not harvest seafood in an area if it is known, or ought reasonably be known at the time, that the seafood, if harvested in the area, may not be safe or suitable when sold for human consumption.*

For bivalve molluscs there are specific regulations relating to marine biotoxins which are outlined in Division 3 – Harvesting and other requirements for bivalve molluscs, it states that:

(1) A seafood business that engages in the primary production or processing of, or manufacturing activities concerning, bivalve molluscs must implement a documented food safety management system that effectively controls the hazards.

Editorial note:

'Hazard' is defined in Standard 3.1.1 as a biological, chemical or physical agent in, or condition of, food that has the potential to cause an adverse health effect in humans.

Under subclause 1(2) of this Standard, the requirement for a food safety management system in subclause 16(1) does not apply to retail sale activities concerning bivalve molluscs.

(2) A seafood business is taken to comply with subclause (1) if it implements –

(a) a food safety program set out in Standard 3.2.1; or

(b) a food safety management system set out in the Commonwealth Export

Control (Processed Food) Orders; or

(c) the Codex Alimentarius Hazard Analysis and Critical Control Point System

(HACCP) for food safety management set out in Annex C to CAC/RCP 1-1969, revision 4 (2003); or

(d) any other Hazard Analysis and Critical Control Point (HACCP) based food safety management system recognised by the Authority.

(3) For the purposes of subclause (1), a seafood business must comply with –

(a) the conditions of the ASQAP Manual specified in the Schedule to this Standard; or

(b) conditions recognised by the Authority.

Australia New Zealand Food Standards Code - Standard 1.4.1 - Contaminants and Natural Toxicants - F2015C00052. This standard outlines the maximum residue limits for four of the marine biotoxins (DSP, ASP, NSP, PSP). These levels apply to only to bivalve molluscs.

To export seafood from Australia there are also two acts that must be complied with. These are the Export Control Act 1982 and the Export control act (Fish and Fishery Products) Orders 2005 (Table 4).

At the State level a number of acts and regulations are involved in the provision of seafood safety (see table 4). The details of these and how they are administered differs across the Australian States. Public health is generally dealt with under Health Acts, while controls on harvesting of shellfish are given under Fisheries, Food and Seafood Safety Acts (Anonymous, 2015; DPIF, 2005; NSW Food Authority, 2014; DHHS, 2014; Biosecurity SA, 2014; DOH, 2015; Ecowise Environmental, 2009). It is a requirement of ASQAP (2009) for all State marine biotoxin management documents to provide detail of the State legislation to support the protocols and ensure authorities can take appropriate action in event of a biotoxin event. This legislation provides appropriate authorities with power to enact restrictions on harvesting of seafood for both commercial and recreational fishers in each State.

Details of legislation that allows for provision of seafood safety in the Gippsland Lakes program are not explicitly specified although there are references to some regulations in the roles and responsibilities section (Anonymous, 2011). PrimeSafe is listed as the statutory authority operating under the Seafood Safety Act 2003 to regulate the safety of seafood across Victoria. They issue operational licences to Victorian seafood harvesting and processing businesses and ensure they comply with the Australia and New Zealand Food Standards Code and have authority to restrict harvesting by commercial industry in the Lakes (Anonymous, 2011). PrimeSafe acts on the advice of the Chief Health Officer of Department of Health about the potential public health effects of cyanobacteria which impact on seafood safety.

DEWLP are responsible for the management of commercial and recreational fishing activities under the Fisheries Act 1995 and the Fisheries regulations 1998 (Anonymous, 2011). Department of Health in Victoria has responsibilities for water contamination under the Public Health and Wellbeing Act 2008 (commenced operation 2010), Food Act 1984, the Safe Drinking Water Act 2003 and the Safe Drinking Water Regulations 2005 (Warwick et al. 2009). In the Emergency Management Manual Victoria, The Department of Health is nominated as the Control Agency for food/drinking water contamination (Warwick et al. 2009). However, with the exception of the Seafood Safety Act (2003), Fisheries Act 1995 and Fisheries Regulations 1998 the other Acts mentioned above are not detailed in the Gippsland Lakes protocol.

Table 4: National and State legislation, codes and guidelines used to provide seafood safety in Australian marine biotoxin protocols and Gippsland Lakes protocol (From Anonymous, 2015; DPIF, 2005; NSW Food Authority, 2014; DHHS, 2014; Biosecurity SA, 2014; DOH, 2015; Ecowise Environmental, 2009).

Level	State						
	Victoria	NSW	QLD	South Australia	Tasmania	WA	Gippsland Lakes
National	• Australian and New Zealand Food Safety Code and Standard 4.2.1 - Primary Production and Processing Standard for Seafood (Australia Only) • Australia New Zealand Food Standards Code - Standard 1.4.1 - Contaminants and Natural Toxicants. • Export Control Act 1983 • Export Control (Fish and Fisheries Products) Orders 2005 • Australian Quality Assurance Program Operations Manual						
State	• Health Act 1958 • Fisheries Act 1995 • Food Act 1984 • Seafood Safety Act 2003 • Environment Protection Act 1970	• NSW Fisheries Management Act 1994 • NSW Food Act 2003 • NSW Food Regulation 2010 • NSW Public Health Act 1991	• Food Act 1981 • Fisheries Act 1994 • Food Production (Safety) Act 2000	• Section 43 of the Fisheries Act • Primary (Produce)(Food Safety Scheme) Act 2004 • (Primary Produce)(Food Safety Scheme)(Seafood) Regulations 2006	• Primary Produce Safety (Seafood) Regulations 2014 • Primary Produce Safety Act 2011 (PPSA) • Public Health Act 1997 • Food Act 2003 • Living Marine Resources Management Act 1995	Not provided in the Marine Biotoxin Monitoring and Management Plan 2015 provided.	• Seafood Safety Act 2003 • Fisheries Act 1995 • Fisheries Regulations 1998

Phytoplankton and biotoxin monitoring:

State marine biotoxin monitoring programs all employ a combination of phytoplankton monitoring and shellfish testing. In Victoria, South Australia, Western Australia and New South Wales routine phytoplankton monitoring is conducted to provide an early warning of the presence and abundance of potentially toxic species and development of blooms in shellfish harvesting areas (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015). Tasmania is the only State that does not use phytoplankton monitoring routinely to trigger shellfish sampling, rather uses this data as a means to identify causative species in the event of shellfish contamination (DHHS, 2014). Full details of the procedures, including sites, sampling frequency, collection and processing procedures and laboratories for sample analysis are provided in all State

protocols to provide consistency in methods undertaken and efficiency in procedures from sampling to receiving results.

Shellfish samples for biotoxin analysis are collected both routinely and when measured phytoplankton levels indicate that a toxic species is increasing in abundance in or adjacent to the harvesting area or if the concentration of cells for a particular phytoplankton species have reached trigger values. For Victoria, Western Australia, South Australia, New South Wales and Queensland shellfish are collected when phytoplankton levels reach appropriate trigger levels (Anonymous, 2015; EcoWise Environmental, 2009; DOH, 2015; Biosecurity SA, 2014; NSW Food Authority, 2014; DPIF, 2005), while in the Tasmanian biotoxin monitoring program shellfish sampling is conducted routinely as the early warning method of biotoxin contamination (DHHS, 2014). Shellfish are sampled either monthly or weekly, depending on the risk factors for the area, (determined prior to a site being able to be licensed for harvest) and analysed for biotoxins (Anonymous, 2015; EcoWise Environmental, 2009; DOH, 2015; Biosecurity SA, 2014; NSW Food Authority, 2014; DPIF, 2005; DHHS, 2014).

In all programs, all species harvested in a growing area are sampled and analysed for toxin and closures are based on all species harvested. There is provision in the South Australian program for the use of the sentinel bivalve, the mussel, if evidence is available to show that they accumulate biotoxins more rapidly and to higher concentrations than other species (Biosecurity SA, 2014). Similarly in the South Australian program closures may be species specific if evidence is available to show that a particular species does not accumulate biotoxins (Biosecurity SA, 2014). Routine biotoxin analysis is undertaken in Western Australia quarterly and in New South Wales and South Australia monthly irrespective of phytoplankton levels and a full biotoxin screen is conducted (DOH, 2015; NSW Food Authority, 2014; Biosecurity SA, 2014). In the Western Australian program shellfish are collected with phytoplankton samples and are stored chilled at 5^{o}C or frozen (DOH, 2015). The samples are stored for 6 weeks on a rotational basis, which means that samples are available for toxin testing if phytoplankton monitoring indicates presence of a potentially toxic at a cell concentration exceeding a prescribed “threshold level” (DOH, 2015). Threshold or trigger levels for potentially toxic species and regulatory levels of toxins in shellfish are detailed in each States protocols.

No routine monitoring for phytoplankton is conducted as part of the Gippsland Lakes protocol; however phytoplankton levels are used as a trigger to collect seafood samples (Anonymous, 2011). The DEWLP conduct routine phytoplankton monitoring in the Gippsland Lakes as part of the East Gippsland Regional Co-ordination Plan for Blue Green Algae (Anonymous, 2011; FAAM, 2014). Data from this program is relied on to provide the early warning indicators of the presence and abundance of potentially toxic species and of the development of algal blooms (Anonymous, 2011). Phytoplankton monitoring is conducted weekly at six sites across the lakes throughout the year (FAAM, 2014). The program follows a tiered risk based management approach (DEPI, 2014). At a level of ‘moderate’ which is where a potentially toxic blue green algal bloom is detected and cells reach a biovolume equivalent of 0.4mm^3/L water samples will be collected and tested for toxicity and monitoring of cell numbers is increased (Anonymous, 2011; DEPI, 2014). An incident management team is convened and if the bloom is determined to be toxin-producing seafood samples are collected (Anonymous, 2011).

In the Gippsland Lakes protocol it states that “seafood testing is used as the early warning of blue-green algal toxins in seafood that may pose a risk to human health” and analysis of whole seafood samples begins as soon as toxins are detected in algal and/or water samples (Anonymous, 2011). Sentinel commercially relevant species of fish, prawns and mussels are collected to assess toxins on all occasions. Advisories are provided for all species commercially and recreationally harvested based on toxin levels in these sentinel species. Health guideline values for providing advisories are detailed in the protocol. There are no routine analyses undertaken for cyanotoxins in seafood in the Gippsland Lakes (Anonymous, 2011).

Sampling procedures:

Sampling procedures are a key component of a monitoring program as they provide information needed for producing advisories around public health and harvesting control. The key parts include sample collection, handling and turnaround time, analysis and reporting. These steps are discussed below and compared for the Gippsland Lakes protocol and marine biotoxin protocols.

Sampling sites

The ASQAP operations manual (2009) stipulates a number of criteria for the establishment of sampling sites for phytoplankton and biotoxin sample collection. These criteria, detailed below, are used in all State marine biotoxin monitoring protocols to establish sites.

In the establishment of sampling sites for routine toxic phytoplankton and marine biotoxin sample collection general factors that need to be considered include (ASQAP, 2009; Canadian Food Inspection Agency, 2014):

- the history of phytoplankton and marine biotoxin activity in the area;
- coverage of all major commercial and recreational harvesting areas;
- the need to sample seasonal fisheries immediately prior to and during their open season;
- accessibility of sample sites in all weather conditions;
- environmental factors likely to influence sampling (currents, retention zones) and impacts of inputs (rivers, drains, groundwater)

Routine sites may need to change according to ongoing results or as monitoring programs are modified and adapted. In the event of a bloom additional sites may be included to determine the extent of the bloom; however these would not need to be monitored regularly only when a bloom occurs in the harvesting area (ASQAP 2009).

Specific criteria for the selection of phytoplankton sampling sites include (ASQAP, 2009):

- sites are representative of the water filtered by the shellfish being monitored;
- consideration should be given to the tidal stage to ensure that samples collected represent the water the shellfish are about to filter rather than the water they have already filtered;
- the water samples should be collected so the entire depth of harvest area are sampled;
- Samples should be collected using bottles and hoses for quantitative analysis in preference to nets (qualitative).
- Sites should be located in areas where past experience has shown toxins are likely to appear first.

Specific criteria for sites for seafood collection include:

- Shellfish should be collected from sites representative of current harvesting
- Shellfish should be collected from areas that represent exposure to the bloom

While for all plans the sampling sites are not necessarily listed in the States official marine biotoxin management plan, as part of contingency and local area biotoxin plans developed in each harvesting area (a requirement of the State management plans) the sites must be detailed for both routine and event monitoring. During events these sites may change to represent areas of the bloom (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015).

In the Gippsland Lakes protocol there is a lack of information regarding sites for sampling during a bloom event and/or how sites will be selected. No details are provided for routine phytoplankton monitoring or reference to other documents regarding phytoplankton procedures and therefore sites

used for monitoring. Information regarding sites for seafood sample collection during blooms are brief and lack detail. Minutes from a teleconference held in December 2011, supplied by Seafood Industry Victoria, provide more detailed information regarding sample sites during toxic bloom events in the Gippsland Lakes (Anonymous, 2011). In short, the Gippsland Lakes protocol details the following information regarding sampling sites:

- Samples are to be collected from the area of the bloom where possible.
- Mussels will be sourced from hard substrates both on the shore and in the lakes. Exact site location will depend on the location and size of the bloom.

The teleconference minutes provided the following information regarding sampling sites (Anonymous, 2011a):

- Four sites with the Gippsland Lakes should be sampled during a bloom for fish and prawns and four sites along the Kalimna rock wall (approx. 50m apart) for mussels.

Sampling frequency

Phytoplankton

Australian and international protocols for phytoplankton sampling suggest that it should be undertaken frequently and regularly, and the frequency should remain constant throughout the year, as potentially harmful species can occur at any time (ASQAP, 2009; US FDA, 2013; Office of Official Publications of European Communities, 2003). Internationally, weekly phytoplankton sampling is the norm (Todd, 2011; Danish Food and Veterinary Authority, 2007; Rhodes et al. 2013), while in Australian States the frequency of phytoplankton monitoring is generally fortnightly for marine programs (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015). In some States phytoplankton monitoring is conducted year round, such as Victoria (Anonymous, 2015), while in other States monitoring is conducted during the period of highest risk, outside of which frequency it's reduced to monthly (ie: South Australia undertake fortnightly from Oct-April and monthly May-Sept) (Biosecurity SA, 2014). Queensland oyster industry monitors phytoplankton monthly during harvest season and in Tasmania monthly year round (DPIF, 2005). In the event of identification of a potentially toxic species and/or high abundance of a potentially toxic species during routine phytoplankton monitoring, each States protocol requires the frequency of phytoplankton monitoring be increased to weekly at minimum (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015).

Details of phytoplankton monitoring are not provided in the Gippsland Lakes protocol, nor is there any reference to protocols describing phytoplankton monitoring protocols (Anonymous, 2011). As part of their role as the regional co-coordinators for the Gippsland region, including the Gippsland Lakes, DEWLP conduct routine phytoplankton monitoring to provide advice around recreational safety in the Lakes (FAAM, 2014). Phytoplankton monitoring is conducted at six sites within the Lakes on a weekly basis throughout the year (FAAM, 2014).

Seafood

Shellfish sampling occurs as both routine and as a triggered response in marine biotoxin protocols for Australian States and internationally (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015; Danish Food and Veterinary Authority, 2007; Murray, 2009). In Victoria, South Australia, Queensland, New South Wales and Western Australia shellfish are collected as a response to increases in abundance of phytoplankton species or identification of potentially toxic species (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015; DPIF, 2005). In this instance shellfish

samples are collected at a minimum of weekly intervals once phytoplankton reaches defined trigger levels.

Routine sampling of shellfish occurs in some Australian States and is common practice in European and United States programs ((Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015; DHHS, 2014; Rhodes et al. 2013; Danish Food and Veterinary Authority, 2007; Murray, 2009). The frequency of seafood collected varies and is usually based on a risk assessment for the harvest area and species being harvested. Internationally, New Zealand, Ireland and Canada undertake routine sampling at intervals from weekly to monthly, while Denmark conduct mussel sampling weekly year round (Danish Food and Veterinary Authority, 2007; Food Safety Authority Ireland, 2014; Todd, 2001; Canadian Food Inspection Agency, 2014). In New South Wales and South Australia shellfish are collected monthly, however for South Australia this is only during the peak risk period (October-April) (NSW Food Authority, 2014; Biosecurity SA, 2014). In Western Australia shellfish are collected in conjunction with the phytoplankton sample and frozen. In the event phytoplankton indicates potentially toxic species at or approaching trigger levels the shellfish samples are assessed for biotoxins (DOH, 2015). Tasmania is the only Australian States that use shellfish biotoxin monitoring routinely instead of phytoplankton monitoring (DHHS, 2014). They have classified harvesting areas as low to high risk and sampling frequency is based on this classification. A Low risk area is defined as areas having no history of potentially toxic or toxic algal cysts being present in numbers of concern, medium risk – areas may have had toxic cysts identified in sediments, or toxic cell in the water column (DHHS< 2014). These areas may have been affected by blooms in the past as a consequence of being seeded from surrounding areas. Blooms have been infrequent (once every 5-10yrs) and some closures have occurred. High risk areas have experienced frequent biotoxin closures. Usually these are areas where *G. catenatum* blooms are initiated and where there is a history of high toxin levels in the bivalve shellfish during algal blooms (DHHS, 2014). In low risk areas shellfish are sampled monthly for biotoxins, while in medium and high risk areas they are sampled weekly year round (DHHS, 2014).

In Victoria and South Australia the protocols stipulate that in the event of a closure where the restriction on harvesting is likely to be prolonged, sampling of shellfish may be conducted less frequently and performed when there is a decline in phytoplankton levels (Biosecurity SA, 2014; Anonymous, 2015; EcoWise Environmental, 2009). This is mainly to save resources and costs. Phytoplankton should be continually monitored and in order to re-open harvesting areas shellfish samples must be collected and analysed for biotoxins (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015).

The sampling for seafood (prawns, mussels and fish) in the Gippsland Lakes is based on a triggered response (Anonymous, 2011). Seafood is sampled on a weekly basis as soon as the DEWLPs phytoplankton monitoring program detects toxin in algal and/or water samples. The protocol does provide a means to increase the sampling frequency of seafood, stating the: “In the event that either the rate of seafood toxin accumulation or of toxic algal growth increases rapidly then testing frequency may be increased” (Anonymous, 2011). Definitions for what constitutes a “rapid increase in algal growth” or “rates of toxin accumulation” that would trigger increased sampling frequency are not detailed in the protocol.

Species sampled

Phytoplankton

Phytoplankton species that are enumerated vary from country to country. Most European countries enumerate the whole phytoplankton community and/or conduct full phytoplankton counts (Higman et al. 2014; Chu, 2011). In Australian programs full enumeration of species is undertaken (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015). Each States management plans provide a list of the species that must be able to be identified together with species known to be present in Australian waters and proven to produce toxins either in Australia

or internationally; potential toxin producing species (ie toxicity untested/unclear) known to be present in Australian coastal waters; and other potential toxin producing species world-wide that may be present in Australian waters (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015). While full enumeration is time consuming it provides a much greater likelihood of identifying new harmful species should they become present in the water column.

The Gippsland Lakes management protocol does not provide any details regarding phytoplankton monitoring and therefore details about the species of cyanobacteria of interest or potential risk to producing toxins. DEWLP however undertake routine monitoring of phytoplankton in the Gippsland Lakes and this sampling includes full enumeration of species in the samples (FAAM, 2014).

Shellfish /seafood

Seafood samples should include and represent those being harvested. If this is not possible, or for routine toxin monitoring seafood samples should be of those that are most likely to reveal the early presence of biotoxin and which are most likely to show the highest toxin levels (ASQAP, 2009; US FDA, 2013).

In international biotoxin monitoring programs all species to be harvested must be sampled during a toxic bloom event and in order to open an area for harvest (US FDA, 2013; Office of Official Publications of European Communities, 2003). During routine sampling sentinel species, most often mussels may be used as early warning indicators of the occurrence of marine biotoxins in a harvesting area. For example Puget Sound, in the US, has a sentinel mussel monitoring program. This program was established by the Washington State Department of Health as an early warning indicator for marine biotoxins. Mussels register PSP toxins more quickly than other shellfish and are consequently used as "sentinels" to determine whether PSP toxins are increasing in a given area (Puget Sound Institute, 2015). Under this monitoring program, mussels are placed in cages and set in strategic growing areas throughout Puget Sound. Mussel samples are then collected either biweekly or monthly and tested for PSP levels (Puget Sound Institute, 2015). Rising PSP levels in these mussels trigger more targeted and frequent sampling regimes in other shellfish species in the affected areas (Puget Sound Institute, 2015). New York State Department of Conservations marine biotoxin monitoring program also use sentinel mussels at set monitoring locations (New York State, 2015). In the US some States have used passive samplers, artificial substrates that accumulate toxins, known as SPATT (Solid Phase Adsorption Toxin Tracking) to monitor toxins in water from shellfish growing areas as an early warning system to occurrence of toxins in shellfish (Nagoda and Busse, 2013). SPATT bags are sampling devices constructed of resins that adsorb specific toxins, which are deployed in a water body for a fixed amount of time (Kudela, 2011). SPATT provide an integrated sample and supplement grab samples, which are subject to variability due to spatial and temporal heterogeneity in toxin expression in water bodies (Nagoda and Busse 2013).

In Australia, marine biotoxin monitoring programs sample all species that are harvested and analyse toxins in each species if sampling of shellfish is in response to phytoplankton counts. All species to be harvested must be sampled and show toxin levels below guideline levels for an area that was closed to harvest to be re-opened (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015). In monitoring programs that employ the sampling of shellfish routinely bivalve molluscs may be used as a sentinel, for instance in South Australia this is specified in their biotoxin management protocol (Biosecurity SA, 2014).

In the Gippsland Lakes protocol the species sampled to determine advisories are popularly fished species including Black Bream (*Acanthopagrus butcheri*), blue mussels (*Mytilus edulis*), eastern king prawns and school prawns (*Melicertus plebejus* and *Metapenaeus macleayi*, respectively) (Anonymous, 2011). Prawns and mussels are targeted as they are the only crustaceans and molluscs collected in significant numbers for human consumption while Black Bream is the most extensively targeted finfish

in the Lakes by both commercial and recreational fishers (Anonymous, 2011a). Previous data on accumulation of nodularin toxin in fish during algal blooms in the Gippsland Lakes in 2002 showed Black Bream to accumulate the highest concentrations of toxin, although the sample numbers (1 fish each) and species assessed (3) were limited (Eaglesham et al 2002). Further basis for the finfish choice are that these fish would accumulate a higher degree of toxin due to their diet and residence time in the lakes compared to other species (Anonymous, 2011a).

Sample collection and handling

The collection and handling of samples is a critical step in monitoring programs, as samples collected provide the critical information regarding the presence and occurrence of potentially toxic species and /or toxins and are used as the basis for decisions around monitoring and seafood harvesting. International and national programs (including the US NSSP, EU Directive and ASQAP) all provide guidance on the sample collection and handling requirements needed to provide satisfactory samples for phytoplankton and shellfish biotoxin analysis to support management decisions. State biotoxin plans all provide detailed procedural guidelines for sample collection methods of phytoplankton and shellfish in Appendices. All personnel collecting samples must be trained by an appropriate training authority in collection of both phytoplankton and shellfish and these processes are audited regularly (ASQAP, 2009; US FDA, 2013). Procedures for collection, on site processing, handling and transport are documented in each State biotoxin plan. Handling times, the time between collection of samples and when they are sent to the laboratory for analysis, is specified at 24hrs in most marine biotoxin plans, so as to reduce any degradation of samples(Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015). All samples must arrive at a laboratory with appropriate sample details, such as date, time of collection, species, sampler, site, site code on a laboratory request form; in many of the State protocols a template for these are supplied (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015). Typical methods applied in marine biotoxin plans for phytoplankton and shellfish sample collection and handling are provided below.

Phytoplankton samples

Phytoplankton samples are typically collected at shellfish harvesting sites using a combination of qualitative and quantitative methods. The approaches to phytoplankton sample collection most often employed in marine biotoxin monitoring are:

Plankton net tows: This is the simplest sample collection technique and is used for qualitative analysis of species. Samples are concentrated into a net of pre-determined mesh size, which means some small harmful algal species may be missed. Additionally if there is a high biomass of a particular species, the concentrating effect of the net tow may make it difficult to identify low abundance species (Higman et al. 2014).

Integrated water Hose/ Lund tube sampler: This is the most common method of sample collection both nationally and internationally in biotoxins programs. An integrated water sample is taken by dropping a hose to a set depth and then closing off the top of the hose and retrieving the bottom of the hose with a rope. The sample is then mixed in a bucket and subsampled for analysis of average phytoplankton concentration over the depth sampled (Higman et al. 2014).

Samples are collected to provide sufficient volume to allow the testing to be carried out with sample to spare. In general, 500ml to 1L is collected. Samples are fixed on site, using Lugol's iodine fixative, and then sent to a laboratory for analysis. During transport samples should be maintained to reduce major changes in temperature, however not placed on ice or refrigerated (ASQAP 2009). In State marine biotoxin programs industry are responsible for organising collection of phytoplankton samples and their transport to the appropriate laboratory for analysis. Industry either undertakes sampling themselves or contracts the work out to an appropriately qualified contractor (ie: Australian Laboratory Services (ALS)).

Seafood samples

Samples of shellfish need to be sufficient enough to allow for testing of all groups of toxins if necessary and be representative of a random sample from each site. The minimum number of shellfish ASQAP recommend be collected at any site is 12 (ASQAP, 2009; US FDA, 2013), while State programs recommend between 15-40 shellfish, depending on species, be collected from each sampling site. This will allow for 100g of shellfish flesh for each toxin to be analysed for. ASQAP (2009) require that all species harvested be sampled if sampling is in response to phytoplankton counts. During routine analysis shellfish samples may be those species that show accumulation of biotoxins before other species and to higher concentrations. For shellfish, samples are collected randomly along lines, from baskets or the sea bed to be representative of those being harvested. Samples are often shucked or whole, depending on the species and protocol. Samples are placed into appropriately labelled bags (mesh or plastic) and then placed into clean dry eskies and chilled. Samples need to be maintained at specified temperatures for the particular species being harvested (for instance mussels 4-8^{o}C) (ASQAP, 2009). Samples should not be placed directly onto ice; rather it should be separated via newspaper or cloth from samples. It is preferred in most programs to provide fresh samples rather than frozen. However in the event that there may be delay in transport samples may be frozen. As with phytoplankton samples, shellfish samples are the responsibility of industry to collect, prepare and transport, in most marine protocols (both Australian and international), however a government agency may oversee this sampling.

Gippsland Lakes protocol

There are no details for phytoplankton sampling provided in the Gippsland Lakes protocol. Phytoplankton sampling is conducted by DEWLP, which is used to inform the implementation of Gippsland Lakes protocol and involves the use of qualitative vertical net tows of the whole water column and quantitative bottle samples (0-1m depths) for species counts and relative abundance estimates (FAAM, 2014). Quantitative bottle samples are preserved with Lugol's iodine fixative (FAAM, 2014).

There are no clear instructions on the collection, handling and dispatch of seafood samples in the Gippsland Lakes protocol. Samples are to be sourced to represent areas of the bloom (Anonymous, 2011). Sample size requirements are not specified in the protocol; however were provided in the teleconference minutes supplied additional to the protocol. In the minutes it is proposed to collect three samples of bream, approximately 100g of shucked mussels (15-20 mussels) and a minimum of 100g of prawns from each site (Anonymous, 2011a). Whole seafood samples (Fish, prawns and mussels) are to be frozen "as soon as practicable" after collection. Samples should be transported with ice packs in an esky to ensure they remain frozen upon reaching the laboratory and labelling details are provided (Anonymous, 2011a). Sample collection and dispatch during a bloom is the responsibility of DEWLP (Anonymous, 2011a). DEWLP staff must work with commercial fisherman and other sources to collect appropriate samples for analysis as available (Anonymous, 2011). Collection and dispatch of mussel samples is to take place when phytoplankton samples are taken. In the event of a harvesting closure commercial fishing operators may be engaged to collect the required samples for analysis (Anonymous, 2011).

Environmental information

All State programs for marine biotoxins recommend obtaining environmental parameters at the same time as phytoplankton and/or biotoxin sampling. Data collected includes physical data (salinity, water temperature, secchi depth), meteorological data (river runoff, rainfall, wind speed and direction, irradiance) and nutrient concentrations (nitrogen and phosphorus) (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014). This information helps to understand factors leading to bloom events and together with phytoplankton data helps to improve the understanding of toxic bloom events.

There is no information provided in the Gippsland lakes protocol in relation to measuring environmental data at the time of sampling or during the bloom.

Sample analysis and reporting

Accurate assessment of phytoplankton and toxins is important as these results are the basis for management decisions. All analysis should be undertaken by appropriately accredited laboratories and the methods used should be approved by national and international regulations and standards. ASQAP (2009) and international programs European Union 91/492/EEC directive and US NSSP all require that marine biotoxin plans state the methods of analysis for phytoplankton and biotoxins in shellfish, that a list of laboratories used for algal and biotoxin analysis be included in the plans, actions to be taken by laboratories if alert levels are exceeded or toxins are found in shellfish and timeframes for reporting of results (ASQAP, 2009; US FDA, 2013; Office of Official Publications of European Communities, 2003). All State marine biotoxin management plans provide a list of the laboratories, their contact details, addresses, means of transport, tests conducted, methods of analysis, accreditation credentials and turnaround times (time between submission of samples to a laboratory and reporting of the results). While there are a number of laboratories in Australia that are accredited for phytoplankton identification and enumeration, laboratories with the ability to analyse biotoxins in shellfish are more limited. In 2012, following a tender process issued by the Australian (ASCRC) on behalf of the "Australian Marine Bio-toxin Partnership" a preferred provider for assessment of biotoxins in shellfish was determined. The preferred provider, Advanced Analytical Ltd, developed and validated required tests for various shellfish matrices and received NATA accreditation for most of these methods. They are now the preferred laboratory for routine and event-based analytical work relating to biotoxins in shellfish for the Australian shellfish sector (Abalone Council Australia, 2012).

ASQAP (2009) specify that management plans should detail the methods of processing phytoplankton samples, including concentration procedures for phytoplankton samples as well as details for enumeration (Sedgewick rafter or Lund cell count) in order to provide an understanding of the errors associated with results. Further a list of the species that the laboratory must be able to identify should be provided. The South Australian program is the only one that includes detailed information regarding phytoplankton processing. All State programs provide details of the species that must be identified and reported quantitatively and the levels that trigger response. For shellfish samples, ASQAP (2009) states that "the methods to be used for toxin analyses should be specified" in the plans. State marine biotoxin management plans all document for each group of toxins the approved methods of analysis, toxin congeners included in each group and the detection and reporting limits.

Results need to be communicated in a timely manner to relevant parties (ASQAP, 2009). For phytoplankton results the sample turn-around time should be within 24hrs of receipt to the laboratory. For shellfish samples the turn-around time varies depending on the toxin being analysed, but generally is between 2-3 days of sample receipt (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014). Often laboratories will provide SQAP managers with a phone response upon initial analysis of samples if a toxic species is identified or toxins detected in shellfish samples and this will be followed by confirmatory documentation when the laboratory undertakes confirmatory testing (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014).

The Gippsland Lakes protocol does not provide any details for laboratories that may perform analysis of cyanotoxins in seafood samples. The teleconference minutes however provide details for Queensland Health and Forensic and Scientific Services laboratory that have the ability to assess cyanobacterial toxins (Anonymous, 2011a). There are no details regarding tests conducted, methods of analysis, detection limits, accreditation requirements and turnaround times. The analysis of samples has been noted as an issue for the Gippsland Lakes protocol. The work undertaken by the "Australian Marine Bio-toxin Partnership" to set-up a preferred provider for assessment of biotoxins in shellfish should benefit the Gippsland Lakes protocol in providing capability for cyanotoxins.

Phytoplankton monitoring conducted by DEWLP includes analysis of quantitative bottle samples through assessment of cell count estimates with a haemocytometer and Sedgewick Rafter chamber (acceptable error ±20%) (FAAM, 2014). Relative abundance estimates are made based on a system of Present (P), Common (C), Very Common (VC), or Dominant (D). For the most significant and toxic species in the sample the biovolume is determined and reported (FAAM, 2014).

Communication and notification

An effective monitoring and response plan needs to have open communication networks between all parties involved and all parties need to be clear on their roles. ASQAP and the US NSSP recommend that procedures to disseminate information to relevant stakeholders during toxic events need to be established to ensure there is clear flow of communication between all parties and timeframes around providing notifications for closures and re-opening events and to the public be defined (ASQAP, 2009; US FDA, 2013). Details that need to be included in communication and notification sections of biotoxin plans include: who is to be notified, when and how they are to be notified; contact details of all relevant stakeholders to be notified (ie: industry/growers/harvesters, government authorities, regulators, fisheries officials, health officials); criteria for determining when a public announcement is to be made and who is responsible for making these announcements) (ASQPA, 2009; US FDA, 2013).

Marine biotoxin management plans in Australia all contain details of the communication networks and who is responsible for providing notifications to relevant stakeholders when a toxic event occurs. Generally there is a central co-ordinator, such as the SQAP manager, for providing communication and notifications in marine programs around Australia (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014). They will receive results from the laboratories and are responsible for communicating these to appropriate stakeholders. In the event of a toxic species being identified at levels warranting a closure or biotoxins being detected in shellfish at or exceeding guideline values the program manager will contact all relevant parties and notify them of a closure to harvesting. Similarly they will notify all relevant parties when a closed harvest area is able to be re-opened (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014). These notifications are provided immediately upon receiving results from the laboratory, by the program manager, via sms or phone call. This is followed up with a confirmatory email or fax, generally within 24hrs (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014). Contact details for all relevant stakeholders are provided in appendices of each States plan. In some States there may be local area managers, which are contacted by the program manager to communication notifications to relevant parties, such as individual growers and harvesters (NSW Food Authority, 2014). The central co-ordinator will maintain regular communication during closure events and/or in the event of toxic species being detected in a harvesting area but not at levels to enforce a closure with relevant stakeholders and/or sectors in regards to levels of algal and/or toxins in shellfish. Some States have websites that contain up-to-date information regarding harvest area closures, e.g.: Tasmania and NSW, which is available for public viewing (NSW Food Authority, 2014; DHHS, 2014). Public announcements and health warnings are the responsibility of the health authorities in each state. They will co-ordinate and advise what is to be detailed in media releases and on signage to be erected at sites. Most State biotoxin management plans provide example press releases and signage in appendices.

Communication and notifications in the Gippsland Lakes protocol is the responsibility of various agencies, and is co-ordinated through the IMT (Anonymous, 2011). The Department of health are the lead agency for public health and provide all advice during a toxic bloom event to the IMT or relevant authorities and they then act (Anonymous, 2011). For example the Chief health officer must notify PrimeSafe of a need to cease commercial seafood harvest and then PrimeSafe will issue a notice to operational licensees to cease commercial collection of seafood. The Department of Health will advise the IMT to co-ordinate notice to recreational fishers through normal media outlets, although these are not defined (Anonymous, 2011). Recreational fishers will also be informed through liaison with DEWLP in their usual avenues, again methods not defined. Details for providing signage at sites

warning against recreational harvesting of seafood are provided in the protocol. Pre-prepared signs are held by the department of health and a list of locations for them to be placed is contained within the DEWLP Blue green algal regional co-ordination plan for the East Gippsland region (Anonymous, 2011). The Department of health is responsible for erecting these signs with support for DEWLP. Communication with the public is to be co-ordinated through a communications plan that will be prepared by the IMT as part of the incident action plan. This will outline the responsibility, format, content and distribution of media releases, fact sheets and information lines during blue-green algal blooms (Anonymous, 2011). The incident action plan is not detailed in the Gippsland Lakes protocol nor is it detailed where you can obtain a copy. The IMT will be the spokesperson for regional blooms in the Lakes, while the department of health is responsible for key messages regarding seafood safety and will provide communications where necessary for these (Anonymous, 2011). There are no details provided in the protocol of the methods to provide notifications, for instance sms, phone, email, fax or around the timeframes around providing notifications once laboratory results have been received.

Closure and re-opening criteria:

Criteria for closing and opening harvest areas should integrate public health, conservation and economic considerations. Principle items of concern are (ASQAP, 2009; US FDA, 2013):

- what are the criteria that need to be met to initiate action;
- number of samples required to initiate action;
- size of area to be closed; and
- type of harvesting restrictions to be invoked (all species or specific species).

Closure criteria:

Marine biotoxin management plans for Australian States base their criteria on those outlined in the ASQAP operations manual and while wording may differ slightly, however shellfish growing areas are closed for harvesting based on 5 main criteria (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014):

- Marine biotoxins are detected in shellfish at or exceeding regulatory limits as specified in section x or appendix x.
- Cases of human illness consistent with case definitions for NSP, PSP< DSP or ASP have been confirmed or suspected from consumption of shellfish.
- A toxic species of micro-algae is detected in growing areas above action levels (this may be used to close an area pending toxin results)
- If the biotoxin program manager determines a need for closure for any other reasons (eg: toxins present in neighbouring areas, potentially toxic species not recorded before is present).
- Industry instigated closure.

In the Victorian protocol a closure may also be instigated if a toxic phytoplankton species is detected below the regulatory limits but is rising rapidly and is likely to exceed trigger values in the next sample (Anonymous, 2015; EcoWise Environmental, 2009). In NSW and Tasmania if phytoplankton or shellfish samples (whichever is used as routine) are not submitted for analysis in accordance with the timetabled sampling frequency a harvest area may be closed pending submission and analysis of the required samples (NSW Food Authority, 2014; DHHS, 2014).

In the event that the criteria for closure are met, harvesting of shellfish is immediately terminated. All marine biotoxin management plans have detailed instructions as to closure procedures. In short the process generally involves the program manager notifying appropriate industry and government agencies immediately via phone or SMS and within 24hrs following up with confirmatory email or fax notice that harvesting is suspended (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014). Details of all parties to be notified are supplied in appendices in

the relevant State management and/or contingency plans. The program manager will then instigate contingency management plans that involve increased monitoring of both phytoplankton and shellfish.

The area of closure is most often that which extends to the nearest sample site below regulatory closure levels or at the discretion of the program manager (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014). In all plans the species to be covered by the closures is generally all species harvested in the affected area. In the South Australian plan there is provision for species specific closures if it is known that a particular species does not accumulate toxins (Biosecurity SA, 2014). Similarly internationally closures are generally based on all species harvested unless there is good scientific basis to allow a particular species to still be harvested as it does not accumulate toxins. An example of this is in the Irish biotoxin management plan where scallops may be harvested if the abductor muscle is removed as this is known to be where toxin is accumulated and when removed the remaining flesh is generally below levels of concern (Food Safety Authority Ireland, 2014).

There are no criteria provided in the Gippsland Lakes protocol for closure of harvesting, similarly there are no clear mechanisms for providing closures documented. Under "incident notification" it is documented that "upon advice from the chief health officer (DH) PrimeSafe will advise operational licensees of need to cease commercial collection and sale of prawns and fish from the Lakes" (Anonymous, 2011). There are no details provided for mechanisms for implementing the closure or timeframes around the implementation of a closure following detection of toxins in seafood at or above guideline values. A flow diagram at the end of the protocol document indicates that for a closure to take place Department of Health will notify PrimeSafe that advisories are needed regarding collection and sale of prawns and fish. PrimeSafe will then advise operational licensees in the Gippsland Lakes of any need to cease commercial collection and sale of prawns or fish when toxins are at level that exceeds alert level. Recreational fishers will be notified via media outlets and signage erected in the region (Anonymous, 2011).

There are no criteria provided based on cell concentrations of toxic species exceeding prescribed abundance levels, pending results of toxin testing in seafood, or based on reporting of human illness fitting case definitions for toxin exposure through seafood consumption. There is no criterion provided around industry instigated closures or closures decided upon by the IMT based on other reasons (eg: toxic species identified that has not been observed before).

PrimeSafe is the Statutory Authority operating under the Seafood Safety Act 2003 in Victoria and therefore has the power to regulate the safety of commercial seafood in the Gippsland Lakes. PrimeSafe acts on the advice of the Chief Health Officer of Department of Health about the potential public health effects of cyanobacteria which impact seafood safety (Anonymous, 2011).

In the Emergency Management Manual Victoria, Department of Health is nominated as the Control Agency for food/drinking water contamination and has authority to prohibit activities or impose restrictions in relation to food under the Public Health and Wellbeing Act 2008 and Food Act 1984. Department of Primary Industry has responsibilities for fisheries as defined in the Fisheries Act 1995 and Fisheries Regulations 1998 (Warwick et al. 2009).

Opening protocols:

In marine biotoxin protocols a shellfish growing area previously closed for harvesting may only be re-opened when sufficient evidence is provided to program manager to show shellfish harvesting can safely proceed. Specific criteria applied in making decisions around re-opening an area in State programs generally include one or more of the following (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014):

1. Biotoxin levels in shellfish are below regulatory limits or are negative;
2. Levels of toxic phytoplankton are below regulatory limits and showing a downward trend;

3. No cases of human illness have been notified to health authorities and consistence with case definitions for NSP, DSP, ASP or PSP have resulted since the date of collection of the first clear sample in an area or adjacent area.

All marine protocols have further criteria around point 1 that levels of biotoxins in shellfish must be below regulatory limits for consecutive samples (generally 2-3 samples) which are sampled at set time intervals apart (varies depending on toxin, typically 2-14 days) (Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014) . In NSW and SA protocols when a harvesting area is re-opened increased monitoring of seafood for biotoxins must be conducted for at least 2-4 weeks post opening and at weekly intervals (NSW Food Authority, 2014; Biosecurity SA, 2014).

Procedures for re-opening are documented in detail in all State programs and generally follow that of mechanisms applied for providing a closure. The program manager provides all notifications to industry and government agencies via SMS or phone initially, followed up by confirmation fax or email that harvesting may re-commence. Appropriate agencies or the program manager remove signage and provide media releases. The program manager prepares all documentation relating to the event and the factors leading to the decisions made and stores them as directed in the protocol l(Anonymous, 2015; EcoWise Environmental, 2009; Biosecurity SA, 2014; NSW Food Authority, 2014).

Seafood advisories, in the Gippsland Lakes protocol must remain in effect until toxins levels in samples have reached below relevant health guideline values for two successive weeks (Anonymous, 2011). The lifting of advisories is co-ordinated through the Incident Management team, however details of this process are not provided in the protocol. A flow diagram attached in the protocol indicates that the Department of Health provides advice to the IMT and the IMT then co-ordinates the response, in particular the chief health officer notifies PrimeSafe that collection and sale of prawns and fish can recommence; PrimeSafe then advises operational licensees in the Gippsland Lakes that commercial harvesting can recommence; recreational fishers and the general public are advised through media outlets and DEWLP liaison and removal of signage in the region. This flow diagram is not referred to in the protocol (Anonymous, 2011).

No criteria based on the absence or reduction in abundance of the causative toxic algal species to cell concentrations below prescribed abundance and/or criteria based on absence of seafood poisoning consistent with case definitions for PSP or hepatotoxic poisoning since the date of the first clear sample are provided. The flow diagram included with the protocol indicates that after re-opening decisions to cease seafood testing will be made by the IMT and based on the growth status of the bloom and other environmental conditions (Anonymous, 2011).

Triggers for review:

The biotoxin management protocols supplied by State program managers were mostly updated in 2014 or 2015 (Anonymous, 2015; Biosecurity SA, 2014; NSW Food Authority, 2014; DOH, 2015), with the exception of that from QLD which was from 2005 (DPIF, 2005) and the Victorian management plan which was from 2009 (EcoWise Environmental, 2009), however the operations manual supplied with this management plan was updated in January 2015 (Anonymous, 2015). Some plans state that they will be reviewed on an annual basis or as required to reflect changes in scientific and technical knowledge and the requirement of the authority, while other provide no stated plans concerning review of their plans. The Gippsland Lakes protocol states that reviews will be undertaken annually as part of the DEWLP review of the BGA regional co-ordination plan for East Gippsland Region (Anonymous, 2011). The protocol supplied and compared in this report was from September 2011.

Recommendations for the Gippsland Lakes Protocol

Key strengths of the Gippsland Lakes Protocol

- The Victorian government has taken a lead nationally and internationally in managing risks posed by cyanotoxins in seafood through the development of health alert levels in seafood and providing a contingency plan to monitor cyanotoxins in seafood during toxic blooms in the Gippsland Lakes. The protocol is the first of its kind in Australia and provides a response capability when toxic cyanobacterial blooms are reported by routine phytoplankton monitoring conducted by DEWLP in the Gippsland Lakes.
- Existing legislative powers allow PrimeSafe to restrict harvesting of seafood from the Gippsland Lakes if cyanotoxins are detected in seafood when health alert levels are reached. There are also legislative powers to restrict and provide advisories around recreational harvesting of seafood during a toxic bloom event.
- The program is based on routine phytoplankton monitoring, where all species of phytoplankton are assessed and advisories to restrict and resume harvesting of seafood are based on concentrations of cyanotoxins in seafood tissues.

Key Weaknesses of the Gippsland Lakes protocol

Funding for event based monitoring

The Victorian Department of health has the responsibility for providing advisories around seafood safety in the Gippsland Lakes and has paid costs associated with seafood sample processing and analysis during toxic blooms. Sample collection and transport costs have been the responsibly of DEWLP and or industry. However, there is no allocation of funding to implement emergency sampling protocols to prevent the harvesting of contaminated seafood during the development of toxic blooms in government budgets. Funding for event response in the Gippsland Lakes has been an ongoing issue over the years, with government agencies all trying to relinquish this responsibility. Routine Phytoplankton monitoring at 6 sites within the Gippsland Lakes is currently conduced and funded by DEWLP as part of their responsibility as regional area managers for the Gippsland Lakes and to provide assurance in recreational safety of the Lakes. Industry has supported the view that funding "should not necessarily be the sole responsibility of industry" as there are a number of other beneficiaries of a monitoring program, and general public health considerations related to the recreational use of the lakes. Cyanotoxin monitoring is necessary during toxic blooms to avoid possible food poisoning outbreaks and to protect the viability of the Gippsland Lakes commercial and recreational fisheries. There is a need to finalise the roles and responsibilities of industry and government agencies, and to determine the appropriate level of funding required to be contributed by which parties to an event based monitoring program in the lakes in order to make sure funding is available prior to any cyanotoxin event. As action must often be taken at very short notice, contingency funding should be made available at the start of each financial year.

Key elements and considerable detail concerning cyanotoxin monitoring and management are lacking in the current Plan.

The plan is missing a considerable amount of detail throughout. To improve the protocol consideration could be given to suggested components for marine biotoxin monitoring and management plans outlined in the ASQAP (2009) operations manual. Greater detail is needed concerning the seafood resources managed under the protocol, roles and responsibilities of industry in the protocol, sampling sites, sample collection and transport methods, phytoplankton triggers used to initiate seafood sampling, closure and opening criteria (and guidelines for their application), methods of communication and notification, laboratories for phytoplankton and toxin analysis, sample turnaround times, etc. Ideally, the Plan should be a stand-alone document that contains all necessary information to enable appropriate cyanotoxin contingency arrangements to proceed smoothly.

Details of commercial and recreational seafood resources the Gippsland Lakes and those managed under the protocol are missing.

The species commercially and recreationally harvested in the Gippsland Lakes and species and harvest areas managed under the protocol need to be defined.

Roles and responsibilities of government and industry need to be well defined

This has been an issue in the Gippsland Lakes protocols identified as far back as 1996 (Norman, 1996). In order to provide a co-ordinated and efficient response, roles and responsibilities of all stakeholders need to be defined and documented. The role of industry in the current protocol is not defined. Further who will be the lead agency of the IMT is not well defined. It has been identified in the past debriefs that there is a need for a single contact person to provide quality and timely information to all stakeholders. A lead needs to be defined so as in the event of a toxic bloom, time is not wasted in co-ordination and implementation of response and a communication protocol is maintained more efficiently if co-ordinated by a single person or agency.

Communication and notification section is unclear

Clear and open communication networks need to be established and written into the plan. Clear definitions of roles and responsibilities for not only government agencies but industry stakeholders involved in the program need to be defined. Notification protocols are provided but no actual contact details are listed for any individual or agency and details of the methods of communication/notification are not provided (e.g: sms, phone, fax, email). Details of proformas for laboratory requests and notification templates for advisories and public warning signs should be provided together with timeframes for providing appropriate notifications.

Laboratories- there is no detail provided regarding laboratories suitability qualified to analyse samples for phytoplankton and cyanotoxins.

A list of the appropriately accredited (should be NATA) laboratories, their methods of analysis, phytoplankton identification and enumeration and toxins analysis capabilities and detection limits and sample turnaround times needs to be provided in the protocol. Contact details and any special sample requirements should also be detailed. As of 2012, Advanced Analytical became the preferred provider for biotoxin analysis for the shellfish industry. They also have the capability to analyses cyanotoxins in seafood matrices. They have an office in Melbourne where sample can be delivered for transport to their Sydney Laboratory. Samples can also be directly couriered to the Sydney Lab.

Sample collection and handling details are lacking

Sample collection and handling details need to be better defined to ensure consistency in sampling methods and provide quality assurance and quality control in their collection, processing, analysis and reporting. The current methods documented are very ad hoc and would lead to inefficiencies in sample collection and analysis. The protocol needs documented procedures in place as to who will collect samples, when will samples be collected, how, where and what samples will be collected. Details of how the sample s are to be handled and transported once collected so as to preserve them for analysis need to be described.

Methods for phytoplankton monitoring are not provided in the Biotoxin Management Plan.

Seafood monitoring is not used as an early warning system in the Gippsland Lakes for providing seafood safety, phytoplankton monitoring triggers the sampling of seafood. Details of the phytoplankton monitoring program used to provide early warning of the onset of a toxic cyanobacterial bloom in the lakes and to initiate seafood monitoring need to be provided. Phytoplankton trigger levels used to initiate seafood sampling need to be defined within the protocol.

Use of sentinel species for providing advisories lacks scientific basis

The use of Black Bream as a sentinel species lacks objective scientific information to ensure it will be representative of all species harvested in the Gippsland Lakes. Given the use of indicator species to provide information regarding the toxin risk of other nearby species, it is imperative that the indicator species is relatively sensitive and accumulates toxins more efficiently than other species during algal bloom events (McLeod, 2014). A potential problem with indicator species is that uptake and elimination rates of toxins may vary between species, and spatial variation in occurrence of toxin producing algal blooms can occur over very small geographical scales leading to differences in exposure between species. In view of these potential issues, it is necessary to evaluate the appropriateness of particular species as indicators through assessment of a range of species toxin uptake and depuration rates (McLeod, 2014).

Regulatory sampling can greatly benefit if toxin uptake and depuration kinetics are known for each species and each toxin, however without this information risk of differences in species accumulation rates leading to incorrect advisories are high. Studies of accumulation of nodularin in fish have shown varied responses in accumulation of cyanotoxins in edible fish tissues (Ibelings and Chorus 2007; Berry et al. 2011; Jia et al. 2014; Stewart et al. 2012). Stewart et al. (2012) reported the highest concentrations of nodularin in aquatic/marine biota by natural exposures (41 - 48 mg kg^{-1} dry weight in livers, compared with 2.2 mg kg^{-1} dw in Baltic flounder liver) in Mullet collected from a Queensland Lake during a toxic *Nodularia spumigena* bloom. Scientific research is needed to support a basis for choosing a sentinel species. It is recommended that research in the use of sentinel species or samplers be undertaken to strengthen the scientific basis for policy decisions regarding which indicator species are appropriate to use in the event based monitoring program. Research recommendations include:

1. International programs for marine biotoxins have employed the use of passive samplers (SPATT bags) and/or bags of mussels deployed at set sampling sites as sentinels for occurrence of toxins in shellfish. The use of these types of sentinels could be investigated as sampling tools during times advisories are in place. Research into the use of these in place of weekly seafood sampling, once advisories are in place, could reduce costs associated with sample collection and handling while toxins are remaining at levels exceeding health guidelines. When these samplers indicate dropping toxin levels, seafood samples could then be collected and analysed for toxins in order to lift advisories.
2. It is recommended that when toxin-producing algal blooms occur in the lakes that monitoring of a range of harvested species is undertaken throughout and following the bloom events. This approach should strengthen the evidence base for the choice of indicator species and support a science-based decision on the appropriate indicator species to use. If such an approach is taken, it is recommended that a statistically based sampling programme be designed which involves sampling multiple species at the same time from each of several different monitoring sites.

The criteria for providing advisories to commercial and recreational fishers are not readily apparent and guidelines for their application are mixed with incident notification procedures.

There is no section in the protocol outlining the criteria for providing an advisory to restrict harvesting of seafood during a toxic bloom event and the criteria used for providing advisories are not readily apparent. A section needs to be included in the protocol defining the criteria used to provide advisories and the mechanisms for providing advisories. Advisories to restrict harvesting of commercial and recreational seafood in the Gippsland Lakes are based on a single criterion in the current plan: “A health guideline value for toxin in any one or more of prawns, mussels or fish is reached”. At this point the Department of Health will issue advice to PrimeSafe of need to inform commercial licensees of need to cease harvesting. Considerations for providing advisories based on criteria around cell concentrations of a toxic algal species exceeding action levels, and the cell concentration levels that could initiate a closure pending the results of toxin testing of shellfish meat should be included in the protocol. Further closure criteria should be added based on the reporting of human illness fitting case definitions for hepatotoxin or neurotoxin exposure. Case definitions for hepatotoxin and neurotoxin

exposure should be included in the protocols as well. Procedures for mechanisms to provide advisories should also be clearly documented and timeframes around providing advisories outlined.

Complementary re-opening criteria matching the closure criteria are required.

The current section on lifting advisories requires some amendment. It needs to be stated that the lifting of advisories can only occur when certain toxin criteria are satisfied. For all toxins, concentrations should be below the relevant regulatory limit in two consecutive samples collected over a minimum period time, e.g: 14 days. Additional criteria should be provided based on the absence or reduction in abundance of the causative toxic algal species to cell concentrations below prescribed threshold or 'action' levels, and criteria based on the absence of any shellfish poisoning reported since the date of the first "clearance" sample. Guidelines concerning the application of the criteria should also be documented.

Annual reviews of the Protocol are required.

The Gippsland Lakes protocol provided for this review was dated from September 2011. The protocol is supposed to be reviewed annually as part of the DEWLP review of the blue green algal response plan for the East Gippsland Region, however it appears this has not occurred. Annual reviews are needed to re-assess the phytoplankton and biotoxin monitoring strategy, to evaluate the efficacy of management procedures and inter-agency communications during the most recent toxic bloom events and incorporate any important variations made to standard operating procedures. Any changes should be documented as an addendum to the Management Plan and formally inserted into the Plan during annual reviews.

Conclusions

Marine biotoxin monitoring programs are well established in Australia and internationally. While cyanotoxins may present the same risks as marine biotoxins, currently little is undertaken in the way of monitoring and management of cyanotoxin risks from seafood consumption. The basic requirements for marine biotoxin monitoring programs include (adapted from Todd 2001):

1. A planned program that is adaptable without altering the main aims of the program. The program will have scope to cover recreationally and commercially harvested seafood (wild caught), will detail the stakeholders roles and responsibilities, the type and frequency of monitoring together with sites. A contingency plan for increasing sampling as necessary will be provided. Notification procedures for results will be documented, as are procedures for closure and re-opening of areas. Documented procedures for the recall and detention of contaminated shellfish and/or for public warnings in the case of recalls or area closures will be provided. There are also surveillance procedures for closed areas to ensure harvesting product does not occur.
2. The program has clear and relevant legislative backing available (on both a state and national level), and this legislation is concise and ensures authorities can take the appropriate action.
3. Appropriate and sufficient funding will be available to carry out the monitoring program and contingency funding will be available for use in the case of a toxin event.
4. Internationally the best practice involves a combination of phytoplankton monitoring and flesh testing. This is determined on a case by case basis, and monitors for known and potential risks in an area.
5. Phytoplankton monitoring plays an important role as an early warning, however internationally closures are based on flesh testing with a few exceptions that use phytoplankton.

Marine biotoxin programs provide a quality assurance for the seafood industry, which in turn creates consumer confidence in seafood products. They also provide economic benefits through access to market opportunities both domestically and internationally. Routine toxin monitoring is essential in areas used for recreational and commercial seafood harvest in order to provide assurance in safety of

seafood products. Without monitoring and management programs in place for providing seafood safety during toxic cyanobacterial blooms, in areas where seafood is commercially or recreationally harvested for human consumption consumers of this seafood are at risk from adverse public health effects from cyanotoxin poisoning and industry is potentially at risk from the adverse effects of a cyanotoxin event through loss in consumer confidence in seafood safety and therefore loss in the market of their products.

In Australia and internationally there is currently a lack of adequate data to be able to quantify the risk associated with cyanotoxins and seafood, although the scientific database is growing. All Australian States and Territories, with exception of Victoria, do not have management, monitoring and/or contingency plans in place to deal with risks around cyanotoxins in seafood. Similarly, internationally Denmark is the only country to routinely monitor and provide advice around seafood safety in relation to cyanotoxins. There have been advisories put in place in Australia and internationally around consumption of seafood for recreational gathers and access to commercially harvested seafood products has been suspended due to the occurrence of cyanotoxins in seafood products during toxic bloom events.

Clearly, there is strong need to evaluate risks posed by cyanotoxins in seafood and develop monitoring and management systems that will enable local, state and federal agencies and industry to work together in developing early warning systems and providing accurate forecasts on bloom occurrence, development and transport to make it possible to develop realistic mitigation strategies that minimise the risks to human health and reduce economic impacts. Given that there have been relatively few, if any, cases of cyanotoxins in seafood causing human illness; it is easy for countries to become complacent about the actual risks of cyanotoxin contamination in seafood. However as the potential for human health risks have been shown through the occurrence of cyanotoxins in freshwater, estuarine and marine seafood at levels posing risk to human health governments and industry need to be pro-active and educated about the issues of cyanotoxins in seafood, and prepared for events rather than being reactionary.

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Appendix D: Co-investigators, support staff and Intellectual Property related to project

Researchers:

Dr Jackie Myers (CAPIM, The University of Melbourne)

Dr Vincent Pettigrove (CAPIM, The University of Melbourne)

Co-investigators:

Dr Kathryn Hassell (CAPIM, The University of Melbourne)

Dr Sara Long (CAPIM, The University of Melbourne)

Dr Susie Wood (Cawthron Institute, New Zealand)

Dr Jonathan Puddick (Cawthron Institute, New Zealand)

Support staff and volunteers:

For assistance with fish collection, dosing, dissections:

Rod Watson, Steve Kruger, Rachael Manassa, Brooke Sullivan, Andre Limsowtin and Erin Cummings

For assistance with sample preparation for LC-MS/MS:

Laura Biessy, Jacob Thompson-Laing and Carrie Page

For expert advice on LC-MS/MS analysis:

Roel van Ginkel

Intellectual Property

All information associated with the experimental research assessing nodularin uptake, accumulation, tissue distribution and elimination in finfish has been disclosed and is to be made public through publication in an internationally peer-reviewed scientific journal.

Appendix E: Factsheet disseminated to commercial fishers at start of project

Research Program Leader

Dr Jackie Myers

Jackie represents CAPIM and has over ten years experience in Project Management in estuarine and freshwater research. This includes investigations into triggers of *Nodularia spumigena* blooms, toxin production, and projects involving toxin movement into seafood.

In the current project Jackie will review monitoring and testing systems, research toxin removal in fish in the laboratory and during blooms to provide recommendations to commercial operators and government agencies at the end of the project.

Jackie will be collecting as much information as possible from all parties involved in the seafood industry in Gippsland Lakes that are affected by algal blooms.

You can contact Jackie at any time and stay updated with the project as it unfolds at **www.capim.com.au**. Go to the "Research Projects" section of the website then "Gippsland Lakes Project".

CAPIM

For more information go to:
www.capim.com.au

Or contact
Jackie Myers

Phone: 03 5258 0257
E-mail: jhmyers@unimelb.edu.au

The Centre for Aquatic Pollution Identification and Management (CAPIM) is part of the Department of Zoology at the University of Melbourne. It is comprised of scientific researchers from different disciplines, coming together to address and understand the impact of pollution on water environments. CAPIM is a world-class research centre and has worked together with government and industry since January 2010.

Development of management recommendations to assist in advisories around seafood safety during toxic bloom events in Gippsland Lakes is supported by funding from the FRDC on behalf of the Australian Government.

CAPIM

Developing Recommendations To Assist Advisories Regarding Seafood Safety During Toxic Bloom Events

*** Gippsland Lakes ***

Information for Commercial Fishing Operators

Centre for Aquatic Pollution Identification and Management (CAPIM)

Minimising Disruption to Fishing During Algal Blooms

In recent years, Gippsland Lakes has been subjected to an increasing number of algal blooms, causing disruption to the fishing industry. Closures have had a detrimental economic impact upon the industry and have restricted market access for commercial fishers.

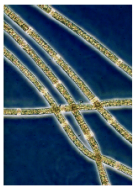
This brochure has been developed specifically for commercial operators, to advise of a scientific research project which is being conducted in the area from now until the end of 2015.

The project is being funded by the Fisheries Research Development Corporation (FRDC). The outcome will include a set of management recommendations to assist with the development of advisories regarding seafood safety during toxic bloom events in Gippsland Lakes.

Consultation with Seafood Industry Victoria about the concerns of commercial fishers with current monitoring practices has led directly to this project.

The project is being conducted by Dr Jackie Myers from the Centre for Aquatic Pollution Identification and Management (CAPIM) which is based at The University of Melbourne. CAPIM specializes in water quality management. Jackie has extensive experience in this field and will be consulting with all stakeholders, including commercial fishers.

What will the Project Achieve?



Nodularia spumigena. Photo by Maria Laamanen and Kaarina Sivonen.

The purpose of the project is to develop methods for minimising economic losses and disruption to commercial and recreational fishers during bloom events.

It will do this in two ways. Firstly, by developing recommendations for a more efficient and cost effective approach to scientific analysis of fish, thereby reducing timeframes for delivering advice and information to fishers.

Secondly, by conducting a review of protocols and practices Australia-wide and overseas, thereby identifying ways to improve monitoring and testing procedures during bloom events in Victoria.

What activities will occur in Gippsland?

Jackie will be visiting Gippsland Lakes over the 2013 Summer and periodically throughout the project. During these visits, samples of different fish species will be collected from a number of sites.

Currently only black bream are being used as a representative species in monitoring programs, though others may also be suitable for these purposes.

How will scientific analysis help?

Samples will be analysed to determine the tissue distribution and elimination profile of the toxin, Nodularin. This process will help determine the appropriate fish samples which should be taken and the frequency in which they ought to be collected.

The analysis will also measure the extent of fish processing required during bloom events which directly impacts market accessibility.

What is required for the project to work?

1) Jackie needs to know when a bloom event is occurring to ensure samples are collected at the right time.
2) Being able to access site locations to collect samples will be critical to the testing process.
3) Gaining a thorough understanding of the current protocols in place, how they are carried out in practice and the extent of impacts and implications to stakeholders.

How can Commercial Operators be involved?

Access to sites and fish will be critical. If you are willing or able to assist Jackie in getting to the locations by boat to collect samples, or if you are able to provide samples to her, it would be very helpful.

Any information you can share with Jackie about your view of the current monitoring and testing processes would be greatly appreciated. Understanding how the current monitoring systems work in practice and the impacts they have upon you and your business, is important to the project and its outcomes.

Project Updates / Contact Details

Jackie can be contacted at (03) 5258 0257 or by email at jhmyers@unimelb.edu.au.

You can view updates of this project at **www.capim.com.au.** Go to the "Research Projects", then "Gippsland Lakes Project".

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