Characterising and managing harmful algal blooms that cause production loss on Australian prawn farms

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Acknowledgments

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Executive Summary

Overview

Land-based marine aquaculture farms in Australia are now better placed to manage the impact of harmful algal blooms on production. The Australian Prawn Farming Association (APFA) partnered with the Commonwealth Fisheries Research and Development Corporation and the Queensland Department of Agriculture and Fisheries in a project to critically assess the composition of the algal blooms associated with marine pond aquaculture and identify characteristics of harmful species that are key to controlling their threat. With operations based at the Bribie Island Research Centre, prawn culture pond blooms from farms spanning the tropical to subtropical east coast of Australia were sampled and a small number of species potentially harmful to prawns and fish identified. One of these species, *Heterosigma* sp., known for its devastation of fish culture operations around the world, was isolated from ponds and maintained in laboratory cultures for evaluation of the mechanism by which it affects prawns and to investigate biological characteristics that could facilitate effective bloom control.

Background

Many thousands of tonnes of aquaculture production is lost each year around the world due to impacts of harmful algal blooms (HABs). Much of this loss occurs in off-shore fish farms, with the massive bloom of *Pseudochattonella marina* that swept along the coast of Chile in 2016 and killed 25 million fish, 20% of the country's production, being a catastrophic example. Despite being land based, Australian prawn and barramundi farms are also impacted by HABs as they use natural inshore waters, containing a community of planktonic flora and fauna, to fill ponds. In the several years prior to the commencement of this project prawn farms reported regular high stock mortality and prawn harvests reduced by an estimated 50 to 90% in multiple ponds. These mortality events were attributed to one species in particular, a Raphidophyte, initially suspected as being *Heterosigma akashiwo*, well-known around the world as a fish killer.

Chronic HAB impacts resulting in lower productivity loss, sometimes only evident by growth retardation though minor daily mortality may be observed, occur regularly throughout the prawn farming industry. These losses have been associated with multiple species but can be more difficult to consistently attribute cause to particular species. A less recognised but perhaps equally important potential impact of harmful blooms is an increased susceptibility of stock to bacterial and viral pathogens as a result of elevated stock stress resulting in lowered immune function.

Objectives

The project aimed to improve the capacity of the prawn aquaculture industry, and other marine pond based aquaculture operations, to reduce productivity losses caused by harmful algal blooms. Various activities were designed to contribute to this aim. To effectively manage the problem more information about the presence, prevalence and impacts of harmful algal species in ponds was needed. The first part of the project sought to collect this critical information from across the prawn farming industry. This included accurate taxonomic identification of the species tentatively named *Heterosigma akashiwo* which had not been properly identified. The project also sought to clarify how the *H. akashiwo*-like alga affected prawns, that is, its mechanism of harm, as this has implications for how farms may seek to mitigate its impact. Potential alternative bloom control options were explored, with preliminary tests to provide indication of on-farm feasibility. To assist building the capacity of farms to effectively manage harmful blooms the project conducted training of farm staff in species identification methods and provided a comprehensive reference guide.

Methodology

To survey prawn pond bloom algal species bloom samples were collected from farms in each of the three geographic zones of the Australian prawn farming industry, north, central and south, with strategic sampling biased towards ponds that were problematic for the manager. In total 78 bloom samples were analysed. Approximately half of these were subjected to a comprehensive analysis by a NATA-accredited

microalgae identification laboratory to confirm species identification with a high level of accuracy. The data collected was statistically analysed to identify any patterns in species occurrence and to explore links with reduced stock health events.

Four strains of *H. akashiwo*-like species were isolated from bloom samples collected from farms in the north, central and southern regions of Queensland. These strains were maintained in laboratory cultures and used for experimental work. Each *H. akashiwo*-like strain was identified by molecular taxonomy. Sequences of the internal transcribed spacer (ITS) region of the ribosomal RNA and the small subunit (SSU) rRNA were compared with sequences currently recorded on the NCBI database (GenBank).

A series of bioassay tests were conducted to demonstrate a toxin mediated impact on black tiger prawns *P. monodon* and provide material for histopathology investigation of the physiological disruption caused. Multiple prawn life stages were exposed to high *Heterosigma* sp. cell densities, including zoea larvae, early post-larvae, small juveniles and large juveniles. Fish larvae, cobia *Rachycentron canadum*, were also tested as it was expected to be highly sensitive to putative toxins. A toxicity assay using *in vitro* fish gill cells was also conducted at a separate laboratory.

The mucus production capacity of *Heterosigma* sp. was semi-quantitatively assessed using a test entailing sample acidification and a mucopolysaccharide-specific stain, alcian blue (Boekel, 1992). This work was supported by microscopic observation and image recording of cells with and without chemical stressors applied.

A review of the literature was undertaken to identify bloom control methods that could potentially be applied to aquaculture ponds. Several of these were tested on a small scale using laboratory *Heterosigma* sp. cultures.

Results and implications

The bloom survey analysed 91 prawn pond samples and identified a total of 137 microalgae to species or genus level, representing 96 different genera of dinoflagellates (26), diatoms (28), cyanophytes (12), raphidophytes (2) and other phytoflagellate groups. This represents the most comprehensive list of microalgae occurring in Australian prawn aquaculture ponds to have been compiled since the last farm survey conducted in the late 1990s (Stafford, 1999). It is notable that this previous survey did not identify the presence of *Heterosigma* sp., which was prominent in this survey.

Of all the microalgal species identified 19 could be considered to potentially pose a threat to the health of aquatic animals based on literature reports. This number may over-estimate those that present a true risk to prawn ponds; however, there is little information in the literature. The algal species of greatest threat for prawn farms fall into the ichthyotoxic category, meaning they are harmful to fish (though the definition can be expanded to mean harmful to other aquatic organisms as well), but are not a risk for human health. It should also be recognised that unlike shellfish, prawns do not filter-feed on micro-algae and therefore do not strongly accumulate toxins.

There is no indication that harmful bloom species presence in ponds varies consistently over the geographic extent of the prawn farming industry or throughout the grow-out cycle. This finding indicates that harmful species, for example *Heterosigma* sp., have potential to bloom on any farm at any time so vigilance and preparedness should be maintained. The level of risk for high impact blooms may, however, vary by location and throughout the grow-out cycle but there is insufficient information to make this assessment.

Only several algal species or groups were significantly associated ($P<0.01$) with observable reduced stock health status as assessed by feed intake reduction, mortality and abnormal behaviours. Two of these were expected, *Heterosigma* sp. and Gymnodinioid dinoflagellates, as the association is consistent with reported experiences of pond managers. Less obvious was the reduced stock health association with the small chain forming cyanophyte *Pseudanabaena limnetica* and nanoflagellates, a grouping of small motile species. These species may frequently be overlooked during quick bloom assessments conducted on farms. Conversely two species were strongly associated with a 'normal' healthy stock status, a small phytoflagellate of the Cryptophyte group, *Plagioselmis prolonga*, and a chain-forming diatom, *Cerataulina pelagica.*

Gill fouling, evident as a browning of the gill filaments, was strongly associated with *Heterosigma* sp. ($P<0.03$) as well as with several other species, including the euglenid *Eutreptiella* sp. which may excrete mucoid substances but is not known to be harmful. The presence of gill fouling is a strong predictor for each of these species so it is recommended that if gill fouling is observed then the bloom should be checked for presence of *Heterosigma* sp. and remedial action commenced if required.

Presumptive *Heterosigma* Strain A had an ITS sequence 100% identity match to *H. akashiwo* strain RP02EHU and is therefore most likely this species. The other three strains, B, C and D, are almost identical to each other and likely a single genetic strain. Their comparatively low ITS and SSU sequence match with *H. akashiwo* strains or a second species, *H. minor*, indicates that these strains may be a species new to science.

None of the four *Heterosigma* sp. isolates showed acute toxicity to any prawn life stages in laboratory tests; however, strong toxicity to fish was demonstrated for Strain A, *H. akashiwo*, in a live larvae test and *in vitro* gill cell assay. Prawns appear to have less sensitivity to *H. akashiwo* toxins than fish, so toxin-mediated prawn mortality in ponds may not be common; however, this mechanism cannot be ruled out. Toxin potency variability observed in these tests and as reported in the literature confound interpretation of laboratory results.

Severe fouling of prawn gills by mucus derived from *Heterosigma* sp. was demonstrated in the laboratory with gill appearance the same as that observed in prawn ponds. *Heterosigma* sp. cells have a great capacity for mucoid substance production and stressors, particularly rapid pH drop, can trigger spontaneous release of mucus. All cultured strains produced mucus; however, the apparent quantity produced was highly variable which explains why not all *Heterosigma* sp. blooms are associated with gill fouling. The conditions promoting high mucus production remain obscure.

Two bloom monitoring and management workshops were conducted in association with the annual Australian Prawn Farmers Association symposium in 2015 and 2017. Both were attended by farm managers and operational staff. Additionally the project contributed to the development of a web-based reference guide for identification of microalgae. This is the most comprehensive resource on the topic available to farms.

Recommendations

- The online Algae Directory should be maintained indefinitely and regularly revised so it continues to be a useful resource for the industry. There would also be significant advantages to ensuring that the industry has ongoing access to an algal species identification service.
- Management of pond blooms is relatively imprecise and practical tools for higher levels of control over bloom composition should be explored. Productivity losses and bloom remediation costs remain a burden on industry.
- The role of the Gymnodinioid group of dinoflagellates in adverse health events should be further investigated. The bloom survey identified Gymnodinioid species as being strongly linked to health events. New DNA technology for species identification could be a powerful tool for such work.
- The industry needs to continue monitoring and recording of bloom composition and stock health status within individual farms or coordinated across the industry as this is critical to assessing the true impact of particular species and identifying new threats. The risk profile for individual farms can only be ascertained through continuous monitoring, recording and analysis.
- No regional or temporal differences in harmful species presence are evident so every farm needs to maintain vigilance and preparedness to respond to an event. However the likelihood of problematic blooms occurred may vary markedly among farms and throughout the season.
- Significant *H. akashiwo* toxin impacts may be prevented by early detection and effective response before the blooms transition to a more toxic phase. *H. akashiwo* toxicity is variable and may vary significantly over the course of a bloom cycle with highest potency levels towards the end of blooms.

Keywords

aquaculture, black tiger prawn, *Penaeus monodon*, microalgae, harmful algal bloom, *Heterosigma akashiwo*, toxicity, mucus,

1. Introduction

Impact of Harmful Algal Blooms

Harmful algal blooms (HABs) in natural and impounded waters are a common cause of stock losses for off-shore finfish cage farming and onshore pond aquaculture systems around the world. Off-shore fish farms can be devastated by extensive HABs that sweep through the area with few options for farms to mitigate the impact. For example, the 2016 massive *Pseudochattonella marina* bloom that swept along the coast of Chile, likely promoted by higher than normal water temperatures, killed 25 million salmon, 20% of the country's production (Cabello and Godfrey, 2016; Clément et al., 2016). Because pond-based farms have enclosed, discrete water bodies there is potential for the operator to exercise some influence over the composition of the algal bloom. In Australia, marine pond production is dominated by Penaeid prawns (*Penaeus* spp.) with 4,000 to 5,000t production per year. Barramundi (*Lates calcarifer*), groupers (*Epinephelus* spp.) and cobia (*Rachycentron canadum*) are also produced in marine ponds. Despite a high level of pond management on these farms substantial losses linked to HABs have been documented by farm operators in recent years.

HABs cause productivity loss to prawn farms due to both stock mortality and growth retardation. For several years prior to the commencement of this project three farms reported large declines in production due to persistent HABs. In these cases typical pond production rates of 8-10t/ha were reduced by 50 to 90% in multiple ponds leading to total farm losses with an estimated value of more than $1 million per farm. In one of the worst affected farms a particularly harmful bloom species, *Heterosigma* sp., recurred persistently over three years and in each year losses up to the value of $750k were sustained. In examples of lower, chronic HAB impact, six farms reported multiple ponds experiencing an extended period of slow growth linked to a characteristic algal bloom. In affected ponds stock mortality rate was not significantly elevated but the reduced growth led to production losses of 5 to 10%, equivalent to approximately 400 to 800kg/ha with a value of $6,000-$12,000/ha.

A less recognised potential impact of HABs is an increased susceptibility of stock to bacterial and viral pathogens as a result of acute or chronic stress caused by the algal bloom. Stress can critically lower immune function of prawns and contribute to disease outbreak (Underwood et al., 2013; Joseph and Philip, 2007; Tseng and Chen, 2004). In such situations the role of a HAB may go unrecognised as a predisposing factor for the disease agent. Emergence of HABs in ponds also increases the cost of production because the mitigation and pond remediation response undertaken by the farm may extend for weeks.

Pond aquaculture and HABs

Creation and maintenance of a stable, healthy microbial community, including phytoplankton, zooplankton and bacteria, is essential for maximising productivity of an aquaculture pond. Even before filling the pond with water the operator takes steps to achieve this. However, the pond environment is subject to diverse influences beyond the operator's control, including weather events and the suite of organisms present in the source water used to fill and flush ponds. These influences can drive a pond's microbial community in a direction contrary to that required to support a healthy, fast growing prawn stock.

Farm operators employ a range of strategies aimed at establishing and sustaining a desired algal bloom community in the ponds. However, if an undesirable bloom does emerge there are few options to remediate the pond to drive the pond dynamic back towards a healthy state. Increased water exchange or 'flushing' is the most common response to an undesirable algal bloom condition but this action will destabilise the pond environment and the effectiveness of this action is variable (DPI&F, 2006). Timely identification of the problem is critical to mitigating adverse impacts of a HAB emergence. Ideally, interventions should be exercised prior to the prawn stock exhibiting symptoms of stress, when the impact of mitigation and pond remediation response is most likely to be effective. The set of processes undertaken by the pond operator to establish a healthy pond microflora and sustain it

represents perhaps the most critical component of the 'art' of pond aquaculture, an art for which Australian prawn farms have achieved a high level of knowledge and skill under the local conditions.

For this project, and more generally, HABs are defined as a dominance or abnormally high density in the plankton community of a single, or up to several, algal species that have the capacity to directly affect prawn health by chemical or physical mechanisms. Algae release a range of chemical compounds, a small proportion of which can interrupt the physiology or compromise tissue integrity of aquatic animals. Examples of physical harm are fouling of the gills by algal mucoid exudates or mechanical damage to gill membranes by sharp cell processes. In this project HABs were distinguished from undesirable blooms by their ability to directly affect the health of the stock. Undesirable blooms are those that pond operators find difficult to manage, are unstable and prone to crashing, or that do not promote the preferred water quality conditions. Such bloom species affect pond stock indirectly through their influence on pond environmental conditions. Australian prawn farms are well equipped to mitigate undesirable blooms and the impact of such occurrences is usually low, causing pond de-stabilisation, minor growth retardation or, at worst, minor stock loss.

The incidence of HABs along Australia's and the world's coastlines is increasing (Hallegraeff, 2014; Anderson et al., 2012) and with increasing utilisation of inshore areas for fish and shellfish production devastating impacts on aquaculture operations are regularly reported. Consequently there is a huge international scientific effort being directed to understanding and managing HABs. In Australia it has become evident that southern shellfish and salmon growers have experienced increased incidences of HAB events in recent years and these industries have placed a high priority on managing the HAB threat. Although prawn farmers also utilise inshore waters, the contained nature of the pond water body confers an advantage over aquaculture operations in open waters when it comes to options for HAB control. For instance, a prawn farm can avoid pumping water from the inshore environment if a harmful algal species is prevalent.

The problem of harmful algal blooms impacting production on Australian prawn farms has likely been around since the introduction of pond based aquaculture and it is apparent that the issue has been considerably under-reported by the prawn farming industry. Consequently, the industry had not previously highlighted HABs as a priority issue. There are several possible reasons for this:

- The occurrence of problematic HABs is inconsistent and unpredictable within and among farms and within production cycles and across seasons. This characteristic makes them difficult to investigate and collect data.
- Farms have tended to deal with their own episodic issues in isolation.
- Episodes of major stock losses on farms were potentially misdiagnosed and attributed to other causes, particularly disease, when in fact disease was a secondary consequence of the primary cause: a HAB bloom. It is well known that acute stress typically weakens prawn's immune defence and the animal load of viruses such as gill-associated virus (GAV) or Mourilyan virus (MoV) increases.

Harmful microalgal species

HAB species have been allocated to three categories depending on their potential impact on the environment and human health (Hallegraeff, 2014; UNESCO, 2004):

1. Strongly blooming species that cause deterioration of water quality over a wide area, e.g., oxygen depletion, which impacts the health of aquatic fauna and flora.
2. Species harmful to aquatic animals but that do not affect humans.
3. Species producing toxins that can impact human health.

The species suspected of harming prawns on farms in Australia fall into the second category; that is they can have a serious impact on prawn health but do not represent a health concern for humans from consumption of affected prawns. Algal species in this category are referred to as being ichthyotoxic.

Additionally, unlike shellfish, prawns do not filter-feed on micro-algae and therefore do not strongly accumulate toxins.

The 10 main micro-algae taxonomic groups present in Australian inshore waters have harmful species representatives (Hallegraeff, 2015; Ajani et al., 2011)Species known or suspected of causing harm in Australian prawn ponds belong to three groups: dinoflagellates, raphidophytes and cyanobacteria (Stafford, 1999) (personal communication with prawn farm operators). A raphidophyte species, detected on several north Queensland farms and tentatively identified as *Heterosigma* sp. prior to commencement of this project, is of particular concern due to the relatively low density at which it affects stock and the rapid progression from no symptoms to onset of mortality (Hallegraeff, 2015; Tobin et al., 2013) (Matt West, APFA, pers. comm.). This alga has demonstrated potential to cause catastrophic losses in Australian prawn ponds and was the primary stimulus for the launch of this project. It was considered such a significant threat that an additional project was undertaken by industry to develop a pond monitoring device that could provide automated warning of a *Heterosigma* bloom (FRDC 2011/728 "Enhancing survival in aquaculture specifically *P. monodon* by creating a *Heterosigma* algal identification unit").

Heterosigma sp. characteristics

Until recently the *Heterosigma* genus was considered to be monospecific, represented by *H. akashiwo*, with an impressively wide distribution from cool temperate waters in the northern hemisphere, through tropical waters to temperate waters in the southern hemisphere (Engesmo et al., 2016). Significant phenotypic differences among geographical strains exist, though rDNA sequence similarities among all strains remain high at greater than 99.8% (Engesmo et al., 2016; Fredrickson et al., 2011). Recently a second species has been proposed, H. *minor*, though it is currently only identified from a restricted area, in the waters of Virginia, USA (Engesmo et al., 2016). At the time of commencement of this project the taxonomy of the tentatively labelled *Heterosigma akashiwo* observed in Australian prawn ponds had not been confirmed as it had not been submitted for specialist laboratory identification. Attempts had been made to send live samples from farm to laboratory for identification, but due to the relative fragility of *Heterosigma* sp. the cells consistently disintegrated during transport.

The raphidophyte *Heterosigma* sp. can impact the health of aquatic animals via several mechanisms, and in any particular harmful bloom event the primary cause of animal mortality is not always clear (Dorantes-Aranda et al., 2015). The mechanism may vary among bloom events and impacted species. *H. akashiwo* is a well-known fish killer, causing losses of both wild and cage-cultured fish around the world (Dorantes-Aranda et al., 2015). Four potential causal mechanisms for fish mortality have been identified (Dorantes-Aranda et al., 2015; Strom et al., 2013; Cochlan et al., 2012; Marshall et al., 2003):

1. Gill fouling with mucus produced by the alga leading to chronic or acute asphyxiation. In fish the gills can also produce copious mucus in reaction to an irritant which exacerbates the fouling.
2. Production of neurotoxin, brevetoxin or brevetoxin-like compounds, has been demonstrated for some *H. akashiwo* strains.
3. Production of reactive oxygen species such as superoxide, hydroxide, and hydrogen peroxide radicals can cause gill damage.
4. Release of toxic polyunsaturated fatty acids (PUFAs), including the free fatty acid form of EPA, particularly in combination with reactive oxygen species and neurotoxins, can create a "toxin cocktail" able to produce rapid lethal effects.

Different investigations into the mechanism of harm by *H. akashiwo* have provided differing outcomes (Ling and Trick, 2010; Rensel, 2007; Twiner et al., 2004; 2001) and the precise cause of fish kills attributed to the species are often unclear. Another characteristic that makes *Heterosigma* sp. a particularly insidious organism is that stressed cells can respond by producing more toxin and copious mucus (Ikeda et al., 2016; Matheson et al., 2014; Cochlan et al., 2012; Powers et al., 2012). Damaging or rupturing cells will also release cellular contents, including harmful compounds, into the water.

Attempting to destroy a *Heterosigma* sp. bloom in a pond therefore has potential to accelerate stock losses.

Evidence accumulated by farms prior to commencement of this project provided a strong indication that gill fouling caused by excess mucoid substances associated with *Heterosigma* sp. blooms was the most obvious mechanism for prawn mortality. However inconsistencies in the characteristics of blooms and their observed impact, indicate that an additional mechanism, mediated by a toxin, could be involved.

Evidence from a number of studies indicate that *Heterosigma* sp. blooms are promoted by moderate salinities, moderate to high temperature and elevated nutrient levels (Kok et al., 2015; Bronicheski, 2014; Matheson, 2014; Butrón et al., 2012; Mohamed and Al-Shehri, 2012), characteristics typical of prawn production ponds. However, *Heterosigma* sp. blooms are unpredictable and may be difficult to detect in a pond even at cell densities sufficient to cause symptoms in prawns. As a result some prawn mortality or stress events directly resulting from the presence of *Heterosigma* sp. may be attributed to 'brown gill syndrome', referring to symptomatic fouling of the gills with an undiagnosed root cause.

Project development

This project arose from the prawn farming industry recognising that across the industry the apparent impact of HABs on productivity had reached an intolerable level. *Heterosigma* sp. was predominantly responsible for a dramatic rise in stock losses in the five years leading up to 2013. Prior to this there was little information that linked the species to mortality events though this may have been due to misdiagnosis of the cause. From the inception of the project it became clear that knowledge and understanding of HABs in general across the prawn farming industry was lacking and there was much more to be learned about the characteristics of *Heterosigma* sp. that could contribute to improved mitigation. The project therefore had a strong focus on the presumptive *Heterosigma akashiwo*, and aimed to formally identify the alga, investigate how it affected prawns and assess methods to control bloom development in ponds. The project also sought to catalogue the presence and prevalence of other potentially harmful algal species in farm ponds and assess their impact through an industry-wide prawn farm surveillance program.

2. Objectives

1. Identify the *Heterosigma* sp.-like flagellate responsible for large stock losses on north Queensland prawn farms and survey other farms for the same or similar flagellates.
2. Identify dinoflagellate and cyanobacteria HAB species present on farms using sampling and identification protocols developed in objective 1.
3. Resolve the mechanism by which *Heterosigma* sp. causes morbidity and mortality in prawns.
4. Identify practical *Heterosigma* sp. bloom control measures and conduct verification tests on selected options.
5. Provide industry training in HAB species identification and produce a harmful algae reference guide.

3. Method

3.1. Identification of the presumptive *Heterosigma* sp.

3.1.1. Collection and preparation of presumptive *Heterosigma* sp. samples

The sampling process from farm bloom to molecular identification followed the sequence:

i. Sampling of farm bloom. Presumptive *Heterosigma* sp. blooms were opportunistically sampled from farms in different regions. Samples were collected from pond zones of low turbulence and if possible at the peak of the bloom rather than the decline phase.

ii. Transport to the Bribie Island Research Centre (BIRC) algal laboratory. Of the four samples successfully transported to BIRC, one was sent via courier from North Queensland and three were carried by hand via air travel from north and central Queensland and via road from south Queensland.

iii. Isolation of presumptive *Heterosigma* sp. The serial dilution method of isolation was used with 96- and 24-well assay plates over four weeks to achieve a non-axenic uni-algal culture. The isolates were transferred to new culture media every seven to ten days. The medium used for isolation was 0.22µm filtered GSe/2 at 30 g L^{-1} salinity. Incubation was at 24-26° C under 14:10 light:dark cycle at 6,500 lux supplied by cool white fluorescent tubes.

iv. Amplification of the culture. Cultures assessed as uni-algal by light microscopy examination were selected from 24-well assay plates and used for inoculating 70mL cultures. These cultures were held under the same culture conditions as above.

v. Isolate identification at molecular laboratory. Samples of live isolate cultures were transported to the laboratory at the University of Technology Sydney (UTS) for sequencing and identification via GenBank library comparison.

Prior to commencement of the project attempts to transport bloom samples to laboratory had resulted in full disintegration of cells upon arrival, demonstrating the relative fragility of the genus. Bloom sampling methodologies and sample transport from farm to laboratory took into account the relative fragility of *Heterosigma* sp. cells and attempted to minimise physical disturbance to samples and transit time.

As experience in handling and manipulating samples and cultures was gained during the conduct of the project it was determined that pond bloom samples are far more tolerant of transport to the laboratory when taken from blooms where *Heterosigma sp*. is the strongly dominant species, with few if any other algal species present. Once a mono-species culture had been generated in the laboratory live samples remained viable after courier transport durations of around two days.

Four presumptive *Heterosigma* sp. uni-algal cultures derived from different farms, representing three geographic regions of Queensland (north, central, and south), were established at BIRC and maintained for an extended period (Table 1). These were used to supply material for molecular identification as well as for toxicity experiments.

Table 1. Details of *Heterosigma* sp. strains isolated and cultured at the Bribie Island Research Centre.

Strain	Region[1]	Farm species / sample source
A	North	Barramundi / production pond
B	South	Prawn / discharge channel
C	Central	Prawn / discharge channel
D	North	Prawn / production pond

[1] See Figure 1 for description of sampling regions.

3.1.2. Molecular identification of four strains of presumptive *Heterosigma* sp.

Samples of four presumptive *Heterosigma* sp. geographic strains derived from uni-algal non-axenic cultures were received at UTS and molecular analyses were coordinated by coordinated by Assoc. Prof. Shauna Murray. One of the isolates was subsequently found to include a contaminating tiny algal cell, as identified by molecular tests, which had not been observed directly and must have been present at extremely low levels.

DNA was extracted from the four cultures using a CTAB method (Doyle and Doyle, 1990). PCR was performed on the internal transcribed spacer (ITS) region of the ribosomal RNA and the small subunit ribosomal (SSU) rRNA using universal eukaryotic primers.

PCR products were cleaned and sent for sequencing in both directions at Macrogen (Korea). Twelve individual sequences were assembled to obtain six contiguous sequences, three of ITS and three of SSU; that is, one of each gene for each culture isolate. These six sequences were compared with all sequences currently known on the NCBI database (GenBank) using BLAST searches.

3.2. Identification of HAB species present on prawn farms

3.2.1. Pond sampling program

Prior to the commencement of the first main prawn grow-out season for the project, 12 farms had agreed to actively assist with an ongoing pond sampling program. A sampling kit was sent to these volunteer farms which included the components necessary to take pond water samples and preserve them for subsequent forwarding to BIRC. The kit included:

- 500mL sample bottles
- Marker pen
- *Pro forma* sampling record sheet
- Preservative (Lugol's iodine) and SDS information
- Sampling methodology guide
- All components contained in a large, sturdy plastic bucket with lid that can be thrown into the farm vehicle and taken to the pond to be sampled.

Lugol's preserved samples can be stored for weeks at the farm, or several months in a refrigerator, without deterioration, prior to forwarding to BIRC or being collected by a project representative when visiting the farm.

Farms were asked to sample ponds when prawn health events or unusual blooms were observed as well as to record pond symptoms and other information of sampled ponds, particularly prawn feeding status, abnormal prawn behaviour, mortality and gill fouling ('dirty gill'). In addition to this *ad hoc* sampling, additional pond sampling was conducted during visits to 14 farms by the project investigator

between November 2014 and December 2015. Farm visit sampling was predominantly of ponds considered sub-optimal by the manager but ponds considered normal or 'healthy' were also sampled. Sampled ponds were photographed to record bloom colour. Pond samples were received from farms from far northern to southern Queensland and represented the three main sampling regions (Figure 1).

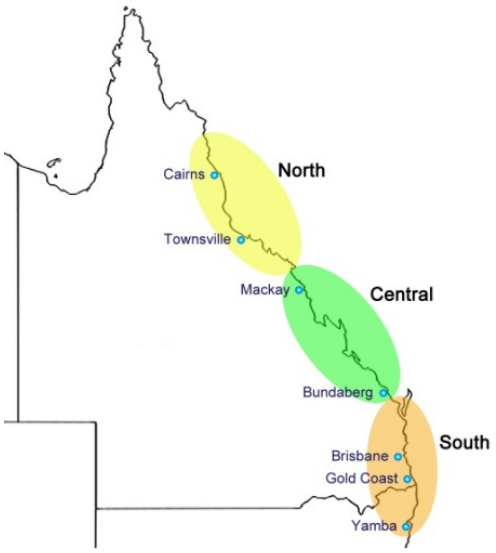

Figure 1. Geographic regions of the Australian prawn farming industry referred to in this report. Each region includes at least five operating prawn farms.

3.2.2. Pond bloom composition analysis

All samples received were microscopically assessed at BIRC using a simplified method to assess relative abundance of microalgae identified to the lowest taxon possible on a scale of 1 to 5, where 1 is rare and 5 is strongly dominant. Data on bloom composition, prawn stock and pond characteristics were recorded in a Microsoft Access database. High-resolution images, and video of algal cells if live samples were obtained, were recorded during microscopic screening to contribute to the online algae identification database constructed for the APFA as part of Seafood CRC project 2012/729 and for farm algae identification training.

A total of 91 pond bloom samples from 14 prawn farms were processed at BIRC. Of these, 34 samples were forwarded to Microalgal Services, a NATA-accredited algae identification laboratory expert in harmful algae identification. This laboratory identified to the lowest possible taxon and enumerated all algae larger than picoplankton size (0.2 to 2 μm) present at greater than 1,000 cells per litre using light microscopy. Samples from ponds showing undesirable characteristics as assessed by the pond manager, typically those exhibiting abnormal prawn behaviour or lowered growth rate, were forwarded to Microalgal Services.

All algae identified as belonging to a known harmful species or genus were categorised, for the purposes of this project, as 'potentially harmful' for prawns based on evidence provided by literature reports linking the alga to adverse events for any impacted species recorded in the environment, aquaculture systems or the laboratory. This designation was used regardless of whether or not any coincident prawn health symptoms were observed at the time of sampling. In most instances it is not known whether the alga has significance to prawn health due to the lack of evidence available for potentially harmful species.

Pond bloom data were subjected to statistical analyses to identify temporal and spatial occurrence patterns and associations with stock condition. The statistical program Genstat Ed.16 was used for all analyses. To analyse the relationship between bloom composition and prawn stock health condition,

pond operator assessment of stock health status and gill fouling was used. The relative intensity of each of the two stock condition characters was ranked as per Table 2. Correlation analyses were used to identify significant relationships among identified algae species and stock health status and gill fouling as well as among the algae species. The occurrence of algal species across the production season and by region (Figure 1) was analysed by ANOVA to identify patterns. The main production season was divided into three periods, early (September to November), mid (December to February) and late (March to May) for the analyses. Multiple regression analyses sought to identify individual and grouped algal taxa that were associated with apparently healthy prawn stock as well as with ponds exhibiting undesirable characteristics. Both absolute alga abundance, cells per mL, and alga relative abundance, percent of total algal cell count, were used in the analyses.

Table 2. Ranking system for two prawn condition indicators used in potential algal impact statistical analyses.

Rank	Health status	Gill fouling
0	No abnormal behaviour	Gills clean and clear
1	Reduced feed intake or other behavioural abnormalities (e.g., prawns aggregating at pond edge)	Low fouling – typically observed as light brown coloured filaments
2	Low level mortality per day	Moderate fouling – darker brown than above but gill filaments visible
3	High level mortality per day	High fouling – dark brown with accretion of material such that gill filaments are obscured.

3.3. *Heterosigma* sp. mechanism of harm

3.3.1. *Heterosigma* sp. cultures

Investigation of the mechanism of *Heterosigma* sp. bloom impact on the prawn population in ponds was originally designed around opportunistic monitoring and prawn sampling during mortality events on farms. On-farm controlled exposure experiments of prawns, conducted in small vessels using pond sourced blooms, were planned as part of the study. In the several years leading up to the commencement of the project severe events occurred frequently enough to expect sufficient data would be generated from on-farm work. In the first year of the project, however, the number and severity of pond *Heterosigma* sp. events were greatly reduced and it became clear that this strategy would not be successful. The methodology for investigating the harmful mechanism of *Heterosigma* sp. was therefore revised to utilise laboratory isolated cultures derived from farm pond blooms. Throughout the project four *Heterosigma* sp. isolates were collected and maintained at BIRC and all four were used in toxicity bioassays and mucus production investigations.

Heterosigma sp. cultures were scaled up from 70mL stock cultures to supply sufficient volume for small scale laboratory experiments using 500mL, 2L and 4L conical flasks and 10L carboys (Figure 2). Culture conditions were the following:

- Growth medium: Natural seawater filtered to 1µm and autoclaved. Diluted to 30 gL^{-1} salinity using RO water. 0.22 µm filtered GSe nutrient mix added aseptically post-autoclave.
- Light conditions: 9,500 lux illuminance supplied by an array of cool white fluorescent tubes. 14h:10h light:dark cycle.

- Temperature: 24 to 26º C
- Culture duration: 8 to 12 days. End of exponential growth phase typically around 10 days.
- Aeration: Strong bubbling of 1 µm-filtered air via open-end 3mm glass tube. CO_2 -enriched air to maintain culture pH at 7.2 to 7.5 was also tested.

The GSe nutrient formulation was selected as the medium for use in all cultures as it includes soil extract which was considered may help retain a culture physiology closer to that in ponds where a variety of organic and inorganic compounds are present. The standard F2 culture medium, most commonly used, does not have this mix of compounds.

Figure 2. *Heterosigma* sp. culture in 2L conical flasks containing 1.6L of GSe medium.

3.3.2. Live animal toxicity bioassays

Six bioassay tests were conducted with the objective of demonstrating the presence of a non-mucus mechanism of harm and describing physical symptoms associated with the putative toxin. Experimental treatments that significantly impacted the health of test animals would be a source of symptomatic samples for histopathology. The experiments included different life-cycle stages of tiger prawns (*Penaeus monodon*), and larval stage cobia (*Rachycentron canadum*) (Table 3). Fish larvae were included as it was expected they would have greater sensitivity to the ichthyotoxins that have been purported for *Heterosigma* sp. blooms. The fish larvae were at the stage when gill structures were evident but not fully formed.

All tests were conducted in a temperature- and light-controlled laboratory. Light was provided by a single 5,000K fluorescent tube from the side of the test containers providing illuminance of 3,000-4,600 lux on the side facing the light source and the light cycle was 14h light:10h dark. Two types of test containers were used: 5L glass jars for tests 1 to 3 and 5L plastic containers for tests 4 to 6 (Figure 3). Moderate aeration was supplied via an open ended 1mL pipette, sufficient to provide gentle water movement that prevented *Heterosigma* sp. aggregations from forming and inhibited particulate debris settlement.

Table 3. Bioassays conducted investigating the toxicity of *Heterosigma* sp.

Test No.	Date	Bioassay species	Life stage	*Heterosigma* sp. strain tested
1	16/02/16	*P. monodon*	juvenile (av. 12.1g)	A
2	21/03/16	*P. monodon*	juvenile (av. 0.6g)	A
3	8/04/16	*R. canadum*	larvae (20-25mm)	A and B
4	29/08/16	*P. monodon*	zoea larvae (Z3)	A, B, C and D
5	19/09/16	*P. monodon*	post-larvae (day 15)	A (stressors applied)
6	28/11/16	*R. canadum*	larvae (16-22mm)	A, B, C and D

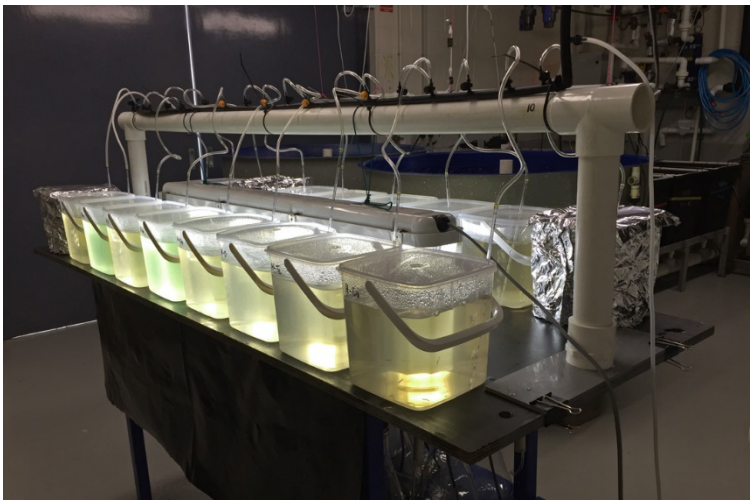

Figure 3. Toxicity bioassay experimental system used for tests 4 to 6.

Water quality parameters were monitored daily. Across all tests, temperature ranged between 24.5 and 28.5º C (within tests the range was less). Unless manipulated for treatment purposes pH ranged from 7.8 to 8.2, salinity 33 to 36 g L^{-1}, dissolved oxygen > 75% and ammonia (NH_3-N) < 0.1 mg L^{-1} though one test container in test #1 peaked at 0.3 mg L^{-1}.

All test containers were regularly examined for mortalities and animal behaviour. Observations were typically made hourly following the commencement of the test and at least twice daily after the first overnight period.

In all bioassay tests *Heterosigma* sp. was sourced from laboratory cultures at late exponential growth phase. The cell density in the *Heterosigma* sp. treatments varied among tests but in all cases initial concentrations exceeded the maximum recorded for prawn culture ponds that were associated with significant prawn mortality (37,000 cells mL^{-1}). In all but one test *Nannochloropsis oculata*, an algal species commonly used in fish hatcheries, was used as a control alga. *N. oculata* culture was sourced from 5,000L mass cultures at late exponential growth phase and the cell density of was adjusted to approximately match the optical density of the *Heterosigma* sp. treatments. Wide differences in cell biomass between the species meant that applying standard cell densities across treatments would provide a less valid comparison. *Chaetoceros muelleri* was used as the control algal species in Test #4 with zoea stage prawn larvae. This species is commonly used as a feed in prawn hatcheries and was

sourced from 10L cultures being used for this purpose at BIRC. Cell counts were conducted daily using a Sedgewick rafter slide (*Heterosigma* sp. and *C. muelleri*) and haemocytometer (*N. oculata*).

Methods and conditions specific to each of the six tests are outlined below.

Test 1. Juvenile prawns

Design:

Part 1. Two algal treatments, *Heterosigma* sp. strain A and *Nannochloropsis oculata*, randomly allocated to six culture containers (three replicates per treatment).

Part 2. Chemical treatments were applied to stress or lyse the algal cells, as detailed below, to induce a rapid release of cell contents and/or mucoid substances into the culture medium. Three chemical treatments applied to three pairs of test containers each consisting of a *Heterosigma* sp. and control treatment.

Test parameters:

- 3L culture volume
- Three prawns, average body weight (BWt) 12.1g, stocked into each test container
- No food provided
- Initial algal density: *Heterosigma* sp. 151,000 cells mL^{-1}
- Experimental duration: Part 1 - 25h. Part 2 - 3h
- Part 2 chemicals applied to stress and lyse the *Heterosigma* sp. Three chemicals were added individually to a treatment pair, one container of *Heterosigma* sp. and a control.
  1. Benzalkonium chloride (BKC) – a quaternary ammonium disinfectant (antimicrobial) commonly used for domestic and aquatic hatchery purposes. It also has surfactant properties.
  2. Lauryldimethylamine oxide (LDAO) – a readily biodegradable non-ionic/amphoteric surfactant.
  3. Acetic acid. An organic acid.
- Two additions of BKC and LDAO were made, the first 25h after commencement of the test to give a concentration of 0.67 and 3.0 mg L^{-1} respectively, and the second addition 1h later to give a cumulative concentration of 1.33 and 5.66 mg L^{-1} respectively. After 28h total test duration prawns were removed from the containers and stocked into holding tanks and survival and growth was monitored for one week.

Test 2. Small juvenile prawns

Design:

Two algal treatments, *Heterosigma* sp. strain A and *Nannochloropsis oculata*, randomly allocated to six culture containers (three replicates per treatment).

Test parameters:

- 3L culture volume
- Four prawns, average BWt 0.9g, stocked into two treatment pair containers and eight prawns, average BWt 0.3g, stocked into one treatment pair.
- Fed twice daily, at approximately 0900h and 1700h, to slight excess with a prawn ‘starter’ crumble.
- Uneaten feed and other debris removed by siphon each morning
- Initial algal density – *Heterosigma* sp. 172,000 cells mL^{-1}
- Experimental duration: four days

At the end of the experiment remaining prawns were examined on a light box designed to illuminate the gill chamber and reveal fouling. Animals from each algal treatment were pooled and stocked into 200L holding tanks and monitored for growth and survival for the next ten days.

Test 3. Cobia larvae

Design:

Three algal treatments, *Heterosigma* sp. strain A, *Heterosigma* sp. strain B, and *Nannochloropsis oculata*, randomly allocated to six culture containers (two replicates per treatment).

Test parameters:

- 20 cobia larvae, 20-28mm total length, stocked into each test container. The two test containers that experienced complete mortality after one day were restocked with 22 and 24 larvae.
- Test containers were examined every 10 to 30 minutes during the 3 hours post-stocking
- In the restocked treatment moribund larvae, that is, lying on the bottom and still ventilating, were removed and fixed for histology.
- 3L culture volume
- Fed Artemia metanauplii
- Initial algal density – *Heterosigma* sp. strain A 185,000 cells mL^{-1}, *Heterosigma* sp. strain B 73,000 cells mL^{-1}. After 24h Artemia greatly reduced cell density in the *Heterosigma* sp. B treatment and a second inoculum was added. *Heterosigma* sp. strain B cell volume was estimated to be almost twice the cell volume of *Heterosigma* sp. strain A.
- Experimental duration: two days

Test 4. Prawn larvae

Design:

- Five treatments, four *Heterosigma* sp. strains and one control alga, with three replicates per treatment
- Control treatment algal species - *Chaetoceros muelleri*

Test parameters:

- Culture volume: 3.5L
- Initial stocking density: 20 to 25 larvae per container
- Initial algae density: *Heterosigma* sp. – 37,000 to 52,000 cells mL^{-1}: *C. muelleri* – 143,000 to 201,000 cells mL^{-1}
- Duration: 48 hours
- Water quality parameters: Salinity 36.0 g L^{-1}; pH 8.1 to 8.3; water temperature 28.0 to 29.6°C

Test 5. Prawn post-larvae

Design:

- Six treatments (four culture stressors applied to *Heterosigma* sp. Strain A, and two controls, a positive control with unstressed *Heterosigma* sp. and a negative control without *Heterosigma* sp. Three replicates per treatment.
- Negative control treatment algal species – *Nannochloropsis oculata*

- Treatments arranged in a randomised block design of three groups of six treatments

Test parameters:

- Culture volume: 4.0L
- *Heterosigma* Strain A used.
- Stocking density: 20 postlarvae (PLs) per container
- PL 18 stage *P. monodon* harvested from 5,000L culture used to stock the experiment (average length 14.3mm, average weight 8.4mg)
- Post-larvae fed frozen newly hatched Artemia nauplii
- Duration: three days
- Initial algae density: *Heterosigma* sp.*:* 35,000 to 84,000 cells mL^{-1}. *N. oculata:* $1.71x10^6$ to $2.55x10^6$ cells mL^{-1}.
- *Heterosigma* sp. culture stressors
    1. Continuous darkness. Experimental container completely covered in aluminium foil to block light ingress during the experiment.
    2. High temperature shock. The required volume of *Heterosigma* sp. culture inoculum sealed in a glass flask and immersed in a warm water bath at 35° C for 1 hour prior to addition to the experimental containers. The temperature shock treatment profile was derived from Dingman and Lawrence (2012).
    3. Low pH shock. Acetic acid, a weak organic acid, added to the experimental containers at a level sufficient to lower pH to 6.5-7.0. Due to the high buffering capacity of seawater, the pH rebounded to around 7.7 overnight. A small volume of 10% acetic acid solution (<0.5mL) was added in the morning and afternoon to re-lower pH to the target range.
    4. Copper chloride. 20% $CuCl_2$ solution added to test containers to give a final copper ion concentration of 0.1 mg L^{-1}. This copper level was selected to be below the toxic threshold for PLs but sufficient to chronically stress *Heterosigma* sp. (Boyd, 2015; Nookala et al., 2014; Chen and Lin, 2001; Bambang et al., 1995).

Test 6. Cobia larvae

Design:

- Six treatments (*Heterosigma* sp. Strains A, B, C, D at standard density, Strain A at double standard density, and control algae species) with three replicates per treatment
- Control treatment algal species – *Nannochloropsis oculata*
- Treatments arranged in a completely randomised design

Test parameters:

- Culture volume: 2.0L
- Stocking density: 11 larvae per container
- Initial larvae size: 16-22mm total length
- Newly hatched *Artemia* nauplii at 1 to 2 mL^{-1} maintained in all cultures by daily addition as necessary
- Initial algae density: For *Heterosigma* sp. strains inoculum volume was calculated to achieve 150,000 cells mL^{-1} for standard density and 300,000 cells mL^{-1} for double density treatments. Cell density decreased due to consumption by *Artemia* and every morning fresh *Heterosigma* sp. culture was added to achieve the target density. *N. oculata* density was $3x10^6$ cells mL^{-1}; chosen to be a similar optical density to the standard *Heterosigma* sp. density
- Duration: three days
- Larval mortalities and survivors counted in the morning and afternoon each day
- Debris siphoned from the container bottom each day

- A mucoid substances test was conducted on day 2 using the acid-reaction method (see section 3.3.4). This test is a crude indicator of the abundance of mucus-forming compounds in the water.

3.3.3. *In vitro* fish cell line toxicity bioassays

A live sample of *Heterosigma* Strain A was sent to Professor Hallegraeff at the Institute of Marine and Antarctic Studies (IMAS) laboratory in Tasmania where it was maintained in culture. The toxicity of this strain was tested by PhD candidate, Andreas Seger, as part of an Australian Research Council (ARC)-funded research project led by Prof. Hallegraeff, "Understanding fish-killing mechanisms by harmful algal blooms: Towards the design of effective mitigation strategies".

The bioassay followed a method developed by Dorantes-Aranda (2012) based on the use of a rainbow trout, *Oncorhynchus mykiss*, gill cell line to investigate potentially ichthyotoxic factors derived from harmful algae. Fish gill cells were attached as a monolayer to a cell culture plate following methods described by Dorantes-Aranda et al. (2011) and exposed to live and lysed cells of *Heterosigma* Strain A at concentrations of 100,000 and 200,000 cells mL^{-1} and without the alga present. Cell viability post-exposure was measured using a viability stain. Cell viability was monitored over 24 hours and the percent cell viability in the alga treatments was calculated as a proportion of the viable cells remaining in the control.

3.3.4. Mucoid substance production and gill fouling

The production of mucoid substances by *Heterosigma* sp. and its impact on prawns was investigated via three avenues: data from bloom sampling and farm reports, observations of *Heterosigma* sp. cultures manipulated in the laboratory and prawn exposure tests.

Farm surveillance data

The industry-wide pond bloom survey, during which data were collected on the presence and cell density of *Heterosigma* sp. as well as prawn condition, provided the opportunity to statistically analyse the relationship between *Heterosigma* sp. and prawn health. Regression analyses were applied to all pond samples where *Heterosigma* sp. was present, using intensity of gill fouling (Table 2) and cell concentration data.

Laboratory mucus tests

The status of mucoid substances in laboratory cultures was regularly monitored by performing a simple acid-reaction test on live cultures. This test is a variation of the waterborne mucus test first developed by Australian Prawn Farms (Matt West, pers. comm.) which is commonly referred to as the snot test. The acid-reaction test used hydrochloric acid to acidify the culture and alcian blue to stain mucus aggregates. Tests were conducted as per the following protocols.

- Small culture volumes: Add 10µL 0.25M HCl per mL, mix then add 1 µL 1% alcian blue. Cultures observed under light microscopy at 10 minutes after staining. 24-well assay plates were used for conducting multiple tests simultaneously.
- Larger culture volumes: Add 100µL 0.25M HCl to a 10mL sample of the culture or bloom in a 15mL sample jar that was capped and mixed with a swirling motion. When a strong reaction occurred a large, brown aggregate formed and there is no need for staining. Otherwise the sample was stained by adding 10µL of 1% alcian blue and a sub-sample of the culture was observed under a microscope.

The acid-reaction test as used above is non-quantitative and used only to provide an indication of mucoid substance presence and coarse-scale relative quantity. A modification to the test was developed to provide a quantitative estimate for laboratory investigation of factors affecting mucus production. No analyses were undertaken with the modified acid-reaction test however, as the mucus investigation was not completed due to termination of the project when the white spot syndrome virus (WSSV) outbreak occurred in prawns farms in southern Queensland.

Alcian blue was also used to observe the mucus release behaviour and properties of individual *Heterosigma* sp. cells. Microscopic examination showed that cells were acutely stressed by acidification in the presence of alcian blue. The spontaneous release of mucus was recorded on video.

The relative contribution of extracellular and intracellular alcian blue positive mucoid substances of laboratory *Heterosigma* sp. cultures was examined. A 10mL sample of 2L conical flask cultures of *Heterosigma* sp. strains, at 10 days culture duration, were first tested with the acid-reaction test. Cell-free medium was then generated from each culture by centrifuging at 3050rpm (2200G) for 10 minutes, as per Kok et al. (2015), then re-centrifuging the supernatant at 4000rpm for 10 minutes. The cell-free supernatant, verified by microscope examination, was subjected to the acid-reaction test in 1mL volume in 24-well assay plates. The relative quantity of blue stained aggregates was assessed microscopically. The method did not lend itself to quantification via image analysis or spectral absorbance.

Experimental fouling of prawn gills

When each toxicity bioassay was conducted (see section 3.3.2) the gill chambers of the test animals were closely examined for the presence of foreign material. Additionally in Test 1, using advanced *P. monodon* juveniles, three chemicals were added to the cultures to stress or lyse the *Heterosigma* sp. Strain A cells. The prawn gill chambers were then re-examined.

3.4. *Heterosigma* sp. bloom control measures

Potential options for preventing or controlling *Heterosigma* sp. bloom development in aquaculture ponds were identified from the literature and from discussion with farm operators. The potential for application of options was assessed against seven criteria (text box below) to produce a short list for further scrutiny within the project. The short-list included several physical and chemical options that received preliminary testing at the laboratory scale. Two types of chemical surfactants were tested to determine the concentration range that may be effective for bloom control and ultrasound which can physically disrupt cells of some algal species.

Criteria used to assess *Heterosigma* sp. bloom control options

1. Potential for specificity against *Heterosigma* sp. – actual activity to be subject of testing.
2. Unlikely to have an impact on prawn health or growth – to be subsequently confirmed.
3. Viable cost – fully assessed once more performance information acquired.
4. Farm-ready application – handling safety and specialised equipment requirements not exceeding current farm capability or capacity
5. Readily degradable – chemical is non-cumulative, breaking down into benign end products within a timeframe of days.
6. High potential for Australian Pesticides and Veterinary Medicines Authority (APVMA) approval but ideally not required.
7. Not have any negative implications for effluent discharge. (i.e., no environmental concern).

Inhibition by surfactant compounds

Two types of chemical surfactants, lauryldimethylamine oxide (LDAO) and cocamidopropyl betaine (CAPB; derived from coconut oil), were tested against uni-algal *Heterosigma* sp. cultures. Initial chemical tests were undertaken with 1mL subsamples of exponential growth phase *Heterosigma* sp. culture in 24-well assay plates. Addition of a wide range of concentrations of active chemical and observation over 3h provided data on acute toxicity. The effect of the chemical on *Heterosigma* sp. cells was graded into three categories: 1. No effect. Cells swimming with normal motion. 2. Some effect. Cells mostly not actively swimming but moving slightly indicating they are still alive. 3. Acute effect. No cells moving and an increasing proportion of cells exhibiting a ruptured membrane. This testing was conducted on the two surfactants of interest, LDAO and CAPD, as well as benzalkonium chloride (BKC), a common antimicrobial with surfactant properties with known strong activity against microalgae.

Based on the estimates of acute effective concentration from the 24-well assay plate samples, two tests were conducted to provide further data for estimating the concentrations effective for inhibiting *Heterosigma* sp. bloom growth and also destruction of cells. In both tests aerated 2L conical flasks containing 30 g L^{-1} F2 growth medium and maintained under conditions as previously described were inoculated with exponential growth phase Strain A to approximately 20,000 cells mL^{-1}. In the first test LDAO and CAPD were added at 5, 10 and 20 mg L^{-1} into a culture flask and BKC was added at 1 mg L^{-1} to a flask. In the second test LDAO and CAPD were added at 2, 4 and 6 mg L^{-1} and BKC added at 0.5 mg L^{-1}. In both tests one flask did not receive any chemical. Test cultures were examined microscopically 30 to 45 minutes post-treatment and cell motility and form was assessed.

Experiments that were planned to expand on these preliminary tests were not conducted because the White Spot Disease outbreak diverted staff resources from the project.

Ultrasound exposure

An investigation of the sensitivity of *Heterosigma* sp. to ultrasound frequencies was launched; however, on-going issues with the submersible transducers meant that only a preliminary small-scale test was performed. Two 2L beakers containing *Heterosigma* sp. culture were exposed to high-intensity ultrasound at frequencies ranging from 19 to 65 KHz for 5 and 15 minutes. The status of the cells was monitored at 5 min, 1h and 18h post-treatment by microscopic examination of cell appearance and behaviour.

4 Results and Discussion

4.1 Identification of the presumptive *Heterosigma* sp.

4.1.1 Molecular identification

Sequencing of the four project isolated strains and comparisons with known *Heterosigma* sp. sequences listed in the GenBank database revealed that there are most likely two species of *Heterosigma* occurring in Queensland aquaculture ponds (Table 4). While the sequencing of this strain A was confounded by the identification of an unrelated flagellate the 100% match of the ITS sequence is considered sufficient for confirmation that it is *Heterosigma akashiwo*. This species is ubiquitous with a recorded distribution throughout coastal temperate and tropical waters from far northern to far southern latitudes (Engesmo et al., 2016). This species is a well-known cause of fish kills for both aquaculture and fisheries resulting in huge economic losses. Consistent with the reputation of *H. akashiwo* as a potent fish killer, Strain A was sourced from a barramundi pond experiencing stock mortality. Gross symptoms of dead and moribund stock did not provide evidence for the mechanism of impact. In particular the gills showed no evidence of fouling indicating that one of the recognised *H. akashiwo* mechanisms of harm, asphyxiation by extra-cellular mucus aggregates, was unlikely. It was therefore probable that one or more ichthyotoxins were a significant pathway for the alga's impact.

The other three strains, B, C and D, are almost identical and likely a single genetic strain. Their comparatively low ITS and SSU sequence match with catalogued *H. akashiwo* strains indicate that they are sufficiently different to be a separate species. The *H. akashiwo* strain they most closely align with, ARCHA0504-1, is a newly proposed *Heterosigma* species, denoted *H. minor* sp. nov. (Engesmo et al., 2016). This further supports the likelihood that the Queensland strains, B, C and D, are not *H. akashiwo*. Their substantial difference from *H. minor* sp. nov. indicates they may be a species new to science. A phylogenetic tree was compiled to illustrate the relationship of the Queensland strains with the *H. akashiwo* strains listed in the GenBank database (Appendix 1).

Table 4. Sequencing results for the four *Heterosigma* sp. strains isolated from Queensland farms.

Strain	Sequence size	Sequence closest identity match on the GenBank database (% sequence match)
A	ITS sequence -739 bp	*H. akashiwo* strain RP02EHU (100%)[1]
	SSU sequence - 2342 bp	*Incisomonas marina* (100%).[3]
B	ITS sequence - 538 bp	*H. akashiwo* strain ARCHA0504-1 (85%)[2]
	SSU sequence - 2272 bp	*H. akashiwo* strain RP02EHU (98%)[1]
C	ITS sequence - 534 bp	*H. akashiwo* strain ARCHA0504-1 (84%)[2]
	SSU sequence - 2272 bp	*H. akashiwo* strain RP02EHU (98%)[1]
D	ITS sequence - 535 bp	*H. akashiwo* strain RP02EHU (85%)[1]
	SSU sequence - 1050bp	*H. akashiwo* strain RP02EHU (99%)[1]

[1] strain RP02EHU was isolated from Spain. Ref. http://www.ncbi.nlm.nih.gov/nuccore/858971814

[2] strain ARCHA0504-1 was isolated from USA. Ref. http://www.ncbi.nlm.nih.gov/nucleotide/858971811

[3] *Incisomonas marina* is a completely unrelated very small heterotrophic flagellate, and shows that the Strain A culture was not uni-algal. Microscopic examination indicated that the contaminant was present at only minor background levels.

Until this project collected samples and undertook molecular identification to confirm the identity of *Heterosigma* sp. there was no published record of this genus occurring in Queensland, where the bulk of the Australian prawn farming industry is located. Previously it had been recorded only south of the New South Wales / Queensland border (Steve Brett, Microalgal Services, pers. comm.) (Ajani et al., 2011). It is most likely that this situation existed only because a sufficiently extensive microalgal survey of Queensland's coastal waters had not been conducted. However, it is interesting that the reasonably comprehensive survey of microalgae occurring on five prawn farms over two years up to 1999 (Stafford, 1999) did not identify *Heterosigma* sp. or any other Raphidophytes. Without historical data the presence or absence of *Heterosigma* sp. in Queensland prior to this project is only speculative.

Since the commencement of prawn farming in Queensland in the mid-1980s, there have been anecdotal reports of stock mortalities exhibiting severe gill fouling. Gill fouling is a common symptom of *Heterosigma* sp. blooms; however, other pond conditions can also produce the same symptoms so such occurrences are not definitive. Given the level of knowledge of bloom species in earlier years and the rudimentary diagnoses that followed such events it remains possible that *Heterosigma* sp. has impacted prawn aquaculture from its commencement and not just in the most recent decade. The earliest record found by the author of a harmful prawn pond bloom event almost certainly caused by *Heterosigma* sp. is from a south-east Queensland farm in 2005. In this case a photographic record was made that assists identification.

4.1.2 Cell morphology and behaviour

The appearance of cells observed in farm samples and in cultures of the isolates held at BIRC is consistent with the description of *H. akashiwo* in the literature; however, the BIRC laboratory cultures demonstrated remarkable plasticity with respect to cell shape and size. This attribute has also been noted for *H. akashiwo* cultures in other laboratories (Engesmo et al., 2016). The observed changes in median cell appearance were not consistently correlated with time or age of culture. Over a period of weeks post-isolation *Heterosigma* sp. in small-volume cultures would become more elongate as it entered the stationary growth phase. This cell shape change was coincident with a transition from cells being predominantly active at the surface of the medium to a strong tendency to aggregate at the bottom of the vessel. However, over time this characteristic was lost and such shape and behavioural changes became less predictable. Occasionally two distinct populations of cells were apparent in a single culture, distinguished by a strongly bimodal distribution in cell size and shape.

When first isolated, Strain A was consistently a different shape to the other three strains, being rounder compared with the more ovoid cells of Strains B, C and D. However, over a period of months Strain A became almost indistinguishable in shape from the other strains. On average Strain A was consistently smaller than the other three strains (Table 5).

Table 5. Average cell size of three *Heterosigma* sp. strains isolated from marine aquaculture ponds in Queensland.

Strain	Length[1] (µm)	Width[1] (µm)	Estimated volume[2] (µm^3)
A	15.1	10.0	590
B	21.9	12.1	1,090
C	19.4	12.8	1,130

[1] Cells were measured at the exponential growth phase of cultures and represent the most dominant cell form of the strain.

[2] Volume estimate based on biovolume calculation method of Hillebrand et al. (1999). Cell depth estimated as ⅔ of the width for all cells.

Throughout the course of this project it became increasingly evident that *Heterosigma* sp. cell morphology is highly variable. This was observed as markedly different cell size and shape among samples from different farms and ponds and temporal changes in single isolate cultures maintained in the laboratory. Cells with such form plasticity are referred to as being pleomorphic. Five basic cell shapes were commonly observed (Figure 4). These shapes have been assigned descriptive names that facilitate discussion with the industry. Across all observations cell sizes ranged from 7 to 25µm length though most commonly cells were within the 13 to 20µm length range.

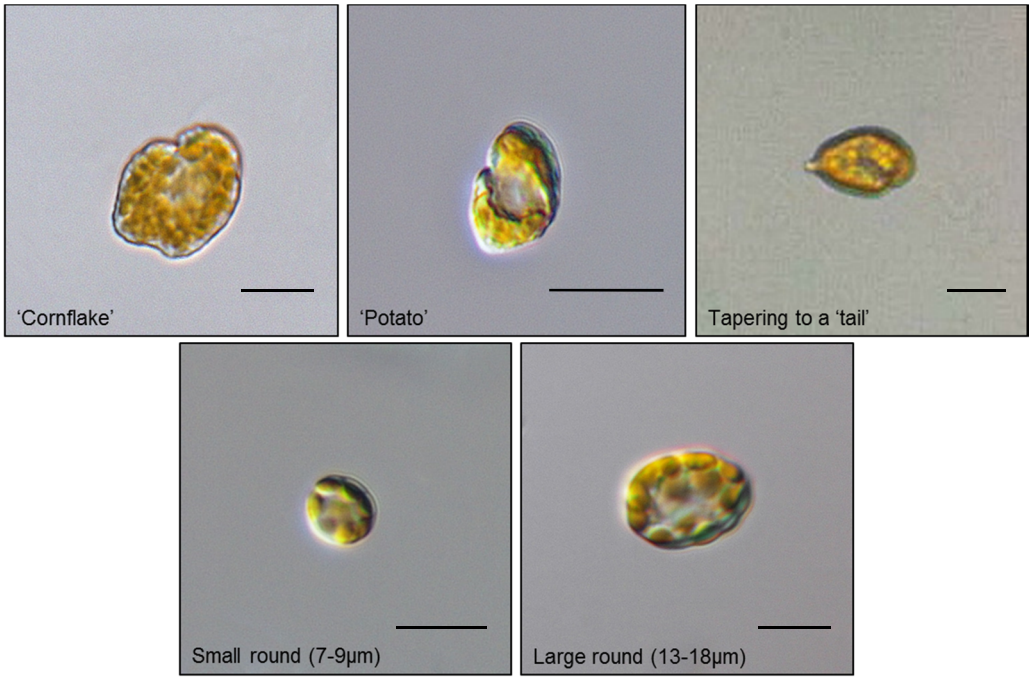

Figure 4. Five basic shapes of *Heterosigma* sp. motile cells observed. Scale bar is 10µm.

The 'cornflake' cell shape was mainly observed in pond samples, and in laboratory culture quickly disappeared. In cultures the 'potato' and 'tailed' shape were the most common.

In addition to varying cell morphology, striking differences in cell behaviours were observed in small-volume non-aerated cultures. Initially exponential growth phase cultures were consistently pelagic in nature with strong photopositive response and very few cells present adjacent to the vessel bottom. As the culture cycle progressed to the stationary phase there was a rapid shift, over one to two days, to a strong epibenthic zone association and cells exhibited a poor photopositive response though remained highly motile.

Following isolation established cultures consistently exhibited epibenthic behaviour in association with a shift from potato shaped cells to the 'tailed' shape. After the cultures had been held for some months the association between epibenthic behaviour and 'tailed' shape disappeared, although stationary to depletion phase cultures continued to exhibit low photopositive response and cell aggregation in the epibenthic zone. It is likely the physical and behavioural changes are related to nutrient depletion in late cycle cultures (Powers et al., 2012).

The distinct pelagic/benthic behaviours observed in laboratory cultures at BIRC have also been reported for *H. akashiwo* elsewhere and is considered to be a natural behaviour related to the transition from the vegetative to the resting stage of cells (Tobin et al., 2013; Tobin et al., 2011). Diurnal vertical migration of *H. akashiwo* blooms has also been recorded for blooms in natural waters (Handy et al., 2005). It was suggested that these vertical migrations can be related to a nutrient uptake maximisation strategy where the cells aggregate near the bottom sediment surface at night, rather than indicating transition to a resting cell stage. In the BIRC cultures, even after an extended period

following the change to benthic oriented aggregation, a subsequent transition to non-motile resting stage cells was not observed. It has been shown that lowering temperature and light levels promotes the formation of resting cells (Tobin et al., 2013; Tobin et al., 2011) and in the BIRC laboratory light and temperature were kept constant.

The life-history of *Heterosigma* sp. includes an encysted stage that can remain viable in sediments for months (Han and Park, 2012; Imai and Itakura, 1999) (Figure 5). It is this characteristic that is considered to be crucial to rapid development of blooms in natural waters (Esenkulova and Haigh, 2014). Synchronous excystment triggered by an environmental cue, for example, and increase in temperature or light, can give rise to a large concentration of vegetative cells within a short period (Shikata et al., 2007; Imai and Itakura, 1999).

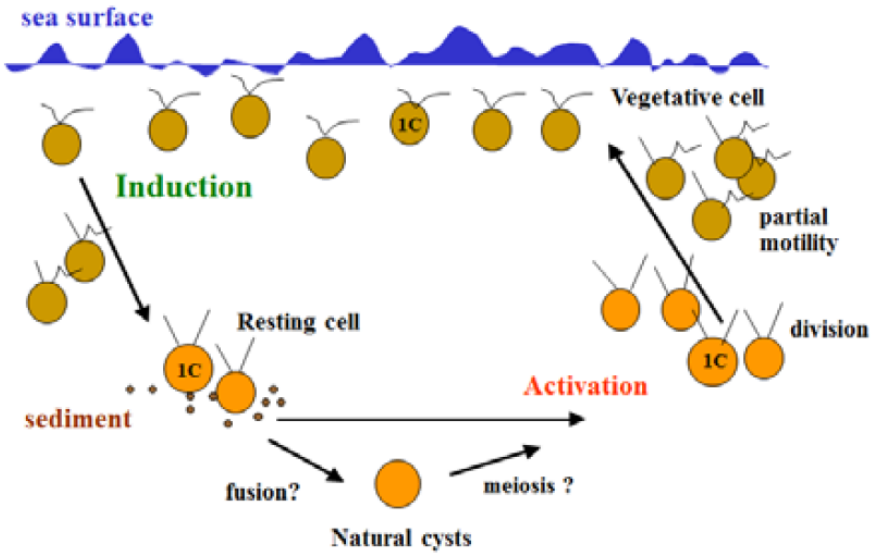

Figure 5. Hypothetical life-history of *Heterosigma akashiwo* showing different cell stages. Diagram from Han and Park (2012).

Cysts typically adhere to small particles such as sand or clay and can remain viable even if buried in sediments (Imai et al., 1993). Factors promoting encystment include nutrient depletion, reduced temperature and reduced light intensity (Itakura et al., 1996). It is highly likely that encystment is occurring in prawn ponds whether or not a strong bloom is observed. This would provide a viable seed population for an indeterminate period after the original bloom has disappeared and promote rapid bloom development if conditions trigger excystment. It is not likely to be possible for farms to prevent *Heterosigma* sp. cysts from forming and remaining in the pond following a bloom; however, it is crucial that cysts are prevented from persisting between production cycles. *Heterosigma* sp. cysts, like those of dinoflagellates, are relatively resistant to a wide range of aquatic environmental conditions, being protected by a mucus coat (Smayda, 1997), but are not well resistant to desiccation and high temperature (>40° C) (Acomi and Ghita, 2012; Gregg et al., 2009; Hallegraeff et al., 1997). It is anticipated that sufficient dry-out of pond bottom sediments will destroy cysts in between culture cycles though this has not been demonstrated.

4.2 Identification of HAB species present on prawn farms

4.2.1 Algal species identified

A total of 91 prawn pond bloom samples were analysed, from which 137 microalgae were identified to species or genus level, representing 96 different genera of dinoflagellates (26), diatoms (28), cyanophytes (12), raphidophytes (2) and other phytoflagellate groups (Table 6). This represents the most comprehensive list of microalgae occurring in Australian prawn aquaculture ponds to have been compiled, though some species identified in the last extensive farm survey conducted in 1999 (Stafford, 1999) were not identified in the current one and vice versa.

Nineteen algal species considered to pose a potential risk of harm to aquatic animals were identified in pond samples (Table 7). An alga's potential for harm was assessed by reference to literature reports which confirmed a negative impact on fish or aquatic invertebrates in wild or cultured populations. For most species on this list potential for harm to prawns is unknown. Additionally, several species were quite rare, identified from only a single sample and only occurred at very low concentration. It is not known whether these particular microalgae have the capability to bloom to sufficient concentration to pose a real risk in prawn ponds. Additionally, in assessing the potential risk to prawn health posed by the algae listed it is critical to note that it is generally recognised that there is considerable variation in toxin production and/or potential for harm among strains within a known harmful species and among species of a genus that includes harmful species. Additionally a species' potential to cause harm can be dramatically affected by biotic and abiotic conditions so that it is typically not possible to predict whether a certain species identified poses a real and immediate threat or remains a potential threat to animal health. Observations of prawn condition and behaviour are therefore critical to assessing the true impact of a particular blooming species, though causation will still be inferred from weight of evidence until controlled exposure tests are performed.

There is little evidence in the literature for links between potentially harmful algal species and prawn health impacts for most of the species listed in Table 7. A review of harmful algae-mediated adverse outcomes in penaeid prawn culture ponds that includes reports between 1993 to 2002 in Asia and South America identified few reports but revealed that apart from red-tide blooms causing anoxia due to extremely dense blooms, very few microalgal species were linked to stock health events (Alonso-Rodríguez and Páez-Osuna, 2003) (Table 8). Of the eight algal species in this table only one, *Prorocentrum minimum*, is currently considered to pose a potential threat in Australian prawn ponds. Keawtawee et al. (2012) provide additional evidence of a causal association between dinoflagellate density, particularly *Ceratium* sp. and Gymnodinioid species, and reduced *P. monodon* growth in culture ponds in Thailand. There are also reports of *H. akashiwo* occurring in prawn production ponds in Asia, for example in China (Yu et al., 1999) and Thailand (Lirdwitayaprasit et al., 1996), although occurrence and impact data are scarce.

Table 6. Microalgal genera identified in prawn culture pond bloom samples during the project.

Diatoms	**Dinoflagellates**	**Cyanobacteria**	**Chlorophytes**	**Dictyochophytes**
Amphora	*Akashiwo*	*Anabaena*	*Chlorella*	*Pseudochattonella*
Asterionellopsis	*Alexandrium*	*Aphanocapsa*	*Crucigenia*	
Bacillaria	*Amphidinium*	*Chroococcus*	*Didymogenes*	
Bacteriastrum	*Azadinium*	*Cyanothece*	*Dunaliella*	**Chrysophytes**
Bellerochea	*Dinophysis*	*Limnothrix*	*Monoraphidium*	*Ochromonas*
Cerataulina	*Diplopsalis*	*Merismopedia*	*Oocystis*	
Ceratoneis	*Fragilidium*	*Myxobactron*	*Picochlorum*	
Chaetoceros	*Gonyaulax*	*Oscillatoria*	*Stichecoccus*	**Euglenoids**
Cocconeis	*Gymnodinium*	*Phormidium*		*Eutreptiella*
Coscinodiscus	*Gyrodinium*	*Pseudanabaena*		
Cyclotella	*Heterocapsa*	*Romeria*	**Prasinophytes**	
Dactyliosolen	*Karlodinium*	*Synechocystis*	*Nephroselmis*	**Others**
Entomoneis	*Katodinium*		*Pterosperma*	*Amoeba*
Eucampia	*Nematodinium*		*Pyramimonas*	*Apedinella*
Fragilaria	*Oxyphysis*	**Cryptomonads**	*Tetraselmis*	*Ciliates*
Guinardia	*Oxyrrhis*	*Campylomonas*		*Mesodinium*
Leptocylindrus	*Oxytoxum*	*Chroomonas*		
Melosira	*Peridium*	*Hemiselmis*	**Prymnesiophytes**	
Minidiscus	*Peridinium*	*Komma*	*Chrysochromulina*	
Minutocellis	*Polykrikos*	*Leucocryptos*	*Emiliania*	
Navicula	*Prorocentrum*	*Plagioselmis*	*Prymnesium*	
Nitzschia	*Protoperidinium*	*Rhinomonas*		
Pleurosigma	*Pyrophacus*	*Rhodomonas*		
Pseudo-nitzschia	*Scrippsiella*	*Teleaulax*	**Raphidophytes**	
Rhizosolenia	*Takayama*	*Urgorri*	*Fibrocapsa*	
Skeletonema	*Torodinium*		*Heterosigma*	
Thalassionema				
Thalassiosira				

Table 7. Potentially harmful microalgae species identified in prawn pond bloom samples.

Genus/Species	*No. samples*	*Max. conc. (cells mL^{-1})*	*Potential for harm (from literature)*
Raphidophytes			
Fibrocapsa japonica	*1*	150	Blooms have been associated with fish mortality.
Heterosigma sp.	27	57,000	Blooms have been associated with fish mortality.
Pseudochattonella verruculosa	1	500	Now classified as a Dictyochophyte. Has been associated with fish mortality.
Dinoflagellates			
Alexandrium tamarense	1	10	Linked to massive fish kills. Possible ichthyotoxins. (PSP, saxitoxin)
Azadinium sp.	1	<5	Some species of *Azadinium* can produce Azaspiracid toxins.
Cochlodinium sp.	3	5,800	Blooms have been associated with fish kills.
Dinophysis caudata	1	5	Can produce okadaic acid, dinophysis-toxin and pectenotoxins.
Heterocapsa rotundata	22	15,000	*S*hown to be toxic to Artemia.
Karenia mikimotoi	1	5	*Karenia* spp. have been associated with fish kills.
Karlodinium sp.	12	10,200	Blooms of *Karlodinium* have been associated with fish kills.
Noctiluca scintillans	3	40	Not toxic but can irritate fish gills by release of high ammonia.
Prorocentrum cordatum (=*P. minimum*)	18	26,000	Blooms linked to fish and prawn mortality in ponds.
Prorocentrum rathymum (=*P. mexicanum*)	2	50	Hemolytictoxin and fast-acting toxins detected in culture of the species.
Takayama sp.	7	3,600	Some *Takayama* spp. associated with fish and invertebrate kills.
Gymnodinioid *spp.*	42	11,000	Diverse group of species that are difficult to identify.
Diatoms			
Pseudonitzschia of the *'delicatissima'* group	3	1,200	The *'delicatissima'* group contains species that can produce domoic acid. (neurotoxin)
Pseudonitzschia pungens/ multiseries	1	35	*Pseudo-nitzschia multiseries* can produce domoic acid. (neurotoxin)
Prymnesiophytes			
Chrysochromulina spp.	30	120,000	Some *Chrysochromulina* spp. have been associated with mortality of fish and invertebrates.
Cyanobacteria			
Oscillatoria spp.	36	1,600	Blooms have been associated with prawn mortality and low growth.

Table 8. Harmful algae events occurring in Penaeid prawn ponds in Asia and South America reported during the period 1993 to 2002. Data derived from Alonso-Rodríguez and Páez-Osuna (2003). Data source references are listed in this paper.

Harmful algal species	Max. cells mL^{-1}	Penaeid species	Country	Impact observed
Cyanophyte (blue-green algae)				
Synechocystis diplococcus	3,400	*P. vannamei*	Mexico	Reduced growth
Schizothrix calcicola	140,000	*P. vannamei*	Mexico	Reduced growth
Dinoflagellate				
Noctiluca scintillans	2,000	*P. orientalis*	China	Mortality (ammonia)
Alexandrium tamarense	10,000	*P. monodon*	China	Mortality (toxin)
Prorocentrum minimum	34,000	*P. stylirostris*	Mexico	Reduced growth
Gymnodinium catenatum	no data	*P. vannamei*	Mexico	Mortality (toxin)
Diatom				
Nitzschia navis-varingica	no data	*P. monodon*	Vietnam	Mortality (toxin)
Raphidophyte				
Chattonella spp.	no data	*P. monodon*	Malaysia	Mortality (mucus)

4.2.2 Algal occurrence patterns

There is no indication that bloom species presence varies over the geographic extent of the prawn farming industry, particularly in relation to potentially harmful species (Table 9). Comparison of the north, central and south regions (Figure 1) reveals species with more than three occurrences recorded spanned the full geographic range of the industry. It should be noted that sampling numbers were limited and not equal across the three regions and pond sampling followed a biased strategy, as previously discussed, so the maximum cell counts expressed in Table 9Table 9 cannot be interpreted as potential differences in a species' potential for forming strong blooms across the regions. A slight bias towards higher cell densities was detected for several algal groups: dinoflagellates and *P. cordatum* in the Central region ($P<0.02$), *Heterocapsa* sp. in the North and Central regions ($P<0.05$) and *Oscillatoria* spp. in the North and South regions ($P<0.05$). However, substantially more sampling would be required to confirm these patterns due to the limited number of samples in this study and biases inherent in the data set.

There was no temporal pattern identified in the occurrence of algal species or groups when comparing bloom composition in the early (September to November), mid (December to February) and late (March to May) periods of the main production season. Patterns in species occurrence and abundance may be expected as general trends in bloom composition and density, consistent with nutrient and other environmental changes in pond conditions as the culture cycle progresses (Case et al., 2008). The highly volatile nature of phytoplankton blooms in ponds over short time scales of days to weeks (Boyd, 2009; Burford et al., 2003) would likely obscure general patterns when sampling is limited.

Table 9. Occurrence of potentially harmful algae across the three geographic regions of the Australian prawn farming industry.

Alga	Max. cell conc. (cells mL^{-1})		
	North	Central	South
Heterosigma sp.	37,200	200	57,000
Heterocapsa rotundata	15,250	54,200	250
Karlodinium sp.	10,200	3,400	330
Prorocentrum cordatum	26,000	100	1,100
Takayama sp.	3,600	1200	5
Gymnodinioid spp.	11,000	6,300	2,400
Chrysochromulina spp.	120,000	4,800	2,200
Oscillatoria spp.	1,600	present[1]	900

[1] cell counts not performed

The lack of a *Heterosigma* sp. occurrence time pattern is also consistent with the range of water temperatures recorded at the time of blooms. The lowest temperature recorded for a pond *Heterosigma* sp. bloom was 22° C and the highest 30.4° C. However multiple-year monitoring of its occurrence on one central region farm has found that highest prevalence tends to occur in the summer months (December to February) when temperatures are over 27° C. There is insufficient data for the influence of salinity on *Heterosigma* sp. blooming on Australian farms to be identified; however, literature reports indicate that *H. akashiwo* is euryhaline (Table 10) and able to grow in almost the full range of salinities experienced on farms.

Table 10. Range of environmental parameters over which natural *H. akashiwo* blooms have occurred or cultures grown and presence of ichthyotoxins measured.

	Growth / Viable bloom	Ichthyotoxicity confirmed	Reference
Temperature	10 - 30.7 °C	10 - 30 °C	(Ikeda et al., 2016; Branco et al., 2014; Bronicheski, 2014; Mohamed and Al-Shehri, 2012; Martinez et al., 2010; Ono et al., 2000)
Salinity	1.6 - 40 psu[1]	10 - 32 psu[1]	(Ikeda et al., 2016; Branco et al., 2014; Bronicheski, 2014; Mohamed and Al-Shehri, 2012; Martinez et al., 2010; Haque and Onoue, 2002)
pH	7.4 - >8.2	-	(Ikeda et al., 2016; Bronicheski, 2014; Matheson, 2014; Kim et al., 2013; Mohamed and Al-Shehri, 2012)
Irradiance	90-1200 μmol^{-2} s^{-1}	10-200 μmol^{-2} s^{-1}	(Kok et al., 2015; Branco et al., 2014; Butrón et al., 2012; Ono et al., 2000)

[1] psu is approximately equivalent to g L^{-1} (ppt)

Differences among farms or farming regions in average abundance of specific algal species may affect the harmful algae event risk profile for a farm. However, this study suggests that some level of risk appears to be present in all years and at all farms. Every farm therefore needs to maintain vigilance

and preparedness to respond to an event. Farms have also reported strong variation between years in the frequency and intensity or problematic blooms, particularly in relation to *Heterosigma* sp. and Gymnodinioid dinoflagellates. There has been some suggestion that periods following extreme weather events such as cyclones may experience higher than average harmful bloom problems although there is insufficient data to make an assessment. Currently there is no way of predicting good or bad bloom years.

4.2.3 Algal link to reduced prawn health status

The correlation analyses conducted on the bloom survey data identified only several algal species or groups that were significantly associated with observable health impacts on the prawns as assessed by feed intake reduction and abnormal behaviours. The Gymnodinioid group of dinoflagellates was strongly associated with adverse health impacts, as was the cyanophyte *Pseudanabaena limnetica,* small flagellates (<10µm) and *Heterosigma* sp. (Table 11). It is difficult to identify Gymnodinioid dinoflagellate species from preserved bloom samples due to cellular distortion so this higher-level grouping was used in the analysis, although it is recognised that the group contains several related genera and multiple species. The Gymnodinioid result is consistent with the experiences of prawn farmers who often associated '*Gymnodinium*' dominated blooms with reduced feed intake by prawns. Literature reports indicate that several species of this group are toxic to aquatic fauna but impacts on prawns have not been investigated (Yu et al., 2017; Place et al., 2012; Mooney et al., 2010; Van Wagoner et al., 2010; Salas et al., 2005).

P. limnetica is a very small, 1 to 3µm wide, chain-forming blue-green alga that is likely overlooked during quick bloom assessments on farms. It is not known for being toxic to aquatic fauna or adversely affecting cultured stock health in any way. Its strong association with adverse events may not reflect cause and effect; rather, its high abundance may be a marker of unfavourable pond conditions. The same situation could be true for small flagellates, a uni-cellular nanoplankton grouping that includes a number of species.

The correlation analyses also identified species that were consistently associated with ponds with a 'normal' stock status; that is, stock feeding at expected rates and not exhibiting abnormal behaviours. A small phytoflagellate of the Cryptophyte group, *Plagioselmis prolonga*, and a chain-forming diatom, *Cerataulina pelagica*, were strongly associated with a positive stock health status (Table 11Table 11). The real meaning of the relationship identified remains to be demonstrated but a simple explanation is that these two species tend to dominate blooms when pond conditions are less favourable for other species that are associated with lower stock health. Interestingly, blooms of *C. pelagica* are known to cause gill irritation, gill fouling and mortalities in fish and shellfish (Taylor et al., 1985). Diatoms in general are considered to be the more desired bloom inhabitants by prawn farmers as they are most often associated with a healthy plankton community and high stock performance, though bloom instability can be an issue (DPI&F, 2006).

Table 11. Algal species and groups associated with reduced stock health status and with a normal, positive, health status and proportion of health status variability explained by the presence of the alga. All relationships are significant (P<0.01).

Species	Group	% Health impact explained
Negative Health status		
Gymnodinioid spp.	dinoflagellate	25.5
Pseudanabaena limnetica	cyanophyte	25.0
Small flagellates	nanoflagellate	17.4
Heterosigma sp.	raphidophyte	13.6
Positive health status		
Plagioselmis prolonga	small flagellate	38.0
Cerataulina pelagica	diatom	17.1

Fouling of prawn gills was considered as a separate health issue for pond stock. Fouling, usually observed as a browning of the gill filaments, is not necessarily linked to reduced growth or mortality, however at the more extreme levels reduces gas transfer leading to asphyxiation. Mortality related to gill fouling may be particularly evident when prawns are moulting. Gill fouling can be caused by pond conditions other than the composition of the bloom (DPI&F, 2006) but in this project the percentage of gill fouling event variation explained by certain species strongly support that it is a dominant cause (Table 12).

Table 12. Algal groups associated with gill fouling and proportion of gill fouling status variability explained by the presence of the alga. All relationships are significant (P<0.03).

Species	Group	% Gill fouling explained
Increased Gill Fouling		
Eutreptiella sp.	euglenid	42.2
Heterosigma sp.	raphidophyte	34.7
Prorocentrum cordatum	dinoflagellate	27.2
Minutocellis scriptus	diatom	27.2
Prorocentrum triestinum	dinoflagellate	17.3
Reduced Gill Fouling		
Heterocapsa spp.	dinoflagellate	20.3

The euglenid *Eutreptiella* sp. was a common inhabitant of blooms; species of this genus are known to excrete mucoid substances (Walne et al., 1986) and in natural waters can also create dense blooms in eutrophic conditions (Stonik, 2007), causing environmental anoxia. *Heterosigma* sp. is well known to excrete mucous compounds into the water (Lopes Daniella et al., 2012; Yamasaki et al., 2009; Engesmo et al., 2007) and mucous asphyxiation is one of the primary mechanisms for mortality associated with blooms (Hallegraeff, 2015).

Statistical investigation of associations among algal species identified that the dinoflagellate *Prorocentrum cordatum* (= *P. minimum*) had a strong tendency to co-occur with *Heterosigma* sp. ($P<0.01$; correlation coefficient = 0.51). This association has also been noted in natural waters and perhaps reflects similar optimal environmental conditions for the two species (Yamasaki et al., 2010). *P. cordatum* is not known to produce mucus, but its inclusion in the 'gill fouling' list is most likely due to it commonly being present when *Heterosigma* sp. is present.

Information available in the literature does not provide an indication of why the diatom *Minutocellis scriptus* may be statistically associated with gill fouling. Similarly it is also not clear why the dinoflagellate *Prorocentrum triestinum* is associated with occurrence of gill fouling and the dinoflagellate *Heterocapsa* spp. with the absence of gill fouling (Table 12).

The bloom composition survey was conducted using targeted sampling and this may have implications for the interpretation of algal associations with particular pond states. There was a strong bias in the sampling and data collection, with a focus on ponds exhibiting undesirable stock symptoms as assessed by the pond manager. During farm visits by Department of Agriculture and Fisheries (DAF) project staff samples were also taken from ponds considered normal by the manager but again they were selectively sampled. As such the statistical associations expressed above, while serving as useful indications of relationship patterns, should be considered with some degree of caution. Additionally the results relate only to associations and do not by themselves provide any information on cause and effect relationships.

The relative abundances of four of the most commonly occurring potentially harmful algae are provided in Table 13. The significant relationships identified between the alga and health indicators were further broken down by algal abundance to elucidate possible thresholds for appearance of symptoms. Data records across the range of algal abundances are limited and no dose response could be statistically determined. The figures in Table 13 provide an indication of the association between the health indicators and all occurrences of the alga as well as when present at higher abundance levels. They reveal that there was a high variation in stock symptoms and algal abundance indicating the likelihood of factors other than cell density that influence stock health.

Table 13. Occurrence data for the most commonly occurring potentially harmful algae species and their relationship with observed stock health indicators.

	No. occurrences at each abundance rank[1]						No. times alga co-occurred with adverse event			
							Health impact[2]		Gill fouling[3]	
	1	2	3	4	5	Total	All (Abund 1-5)	High only (Abund 3-5)	All (Abund 1-5)	High only (Abund 3-5)
Heterosigma sp.	1	5	8	7	0	21	15 (75%)	12 (86%)	8 (62%)	7 (78%)
Gymnodinioid	0	7	8	6	0	21	14 (67%)	9 (64%)	4 (36%)	4 (44%)
P. cordatum	0	6	2	4	0	12	10 (83%)	5 (83%)	5 (83%)	2 (67%)
Oscillatoria spp.	0	18	4	6	1	29	10 (36%)	4 (36%)	4 (20%)	1 (13%)

[1] Relative abundance: 1=rare; 2=present; 3=common; 4=high; 5=very high/strongly dominant
[2] Stock exhibiting marked reduction in feed intake, abnormal behaviours or mortalities
[3] Gill fouling evidenced by brown colouration of the gill filaments

A total of 33 blooms were sampled from ponds exhibiting some level of reduced health status; 20, 9 and 4 records for health impact rankings 1 (minor), 2 (moderate) and 3 (high) respectively. Similarly a total of 13 sampled ponds displayed gill fouling predominantly at a low intensity with only one pond affected at a high level. A health impact and gill fouling co-occurred in 7 of the 13 ponds where gill fouling was recorded, indicating that the two health indicators were not mutually dependent. Anecdotal reports from pond managers indicate that gill fouling typically needs to be intensive for symptoms of asphyxia to be evident in the stock. It is likely, however, that prevailing dissolved

oxygen concentration has a significant influence on level of prawn distress in relation to gill fouling extent.

Reports from farms that have repeatedly experienced *Heterosigma* sp. blooms is that gill fouling by mucous aggregations is the primary cause of mortality. And if strong fouling occurs during a period of moulting then mortality rate can be exacerbated. As gill fouling simply restricts the rate of oxygen transfer through the gill filaments the higher the environmental oxygen concentration the higher the oxygen flux across the gills. The best option for farms to mitigate impacts during such events is to ensure the oxygen level is maintained at a high level at all times.

4.3 *Heterosigma* sp. mechanism of harm

4.3.1. *Heterosigma* sp. cultures

Over a period of eight months between September 2015 and May 2016 four geographic *Heterosigma* sp. strains were isolated from pond blooms and maintained in culture for the purposes of investigating the species' mechanism of harm. Cultures were successfully maintained over a long period in a variety of vessels, 24-well assay plates with 1mL of culture medium per well, 150mL, 2L and 4L glass conical flasks and 10L plastic carboys. Culture volumes over 1L were aerated. During initial isolation procedures *Heterosigma* sp. cell viability seemed to be highly variable among replicate cultures; however, once the cultures had become at least near uni-algal, *Heterosigma* sp. culture growth became consistent and cell density with time relatively predictable. Small volume cultures were extremely robust with live and highly motile cells persisting for weeks once the culture reached the stationary phase. This robustness attained following isolation facilitated the successful transport of live samples to laboratories in Tasmania and New South Wales where previously samples sent direct from the farm had failed to remain viable.

Aerated cultures followed the standard growth curve with optimal culture cycle duration being around 10 to 12 days at 24° C and high light (9,000-10,000 lux) (Figure 6). The size of Strain A cells was consistently smaller than the other three strains (Table 5) so biomass increase with time was more similar among the strains than cell number. Maximal growth rates occurred during the first four days of culture with calculated specific growth rates (μ day^{-1}) typically in the range 0.7 to 0.9 with occasional higher peak growth rates (μ_{max}) of 1.0 to 1.2, for all strains. These growth rates are similar to those obtained in laboratory cultures of other *H. akashiwo* strains (Kok et al., 2015; Herndon and Cochlan, 2007) and indicate that *Heterosigma* sp. are one of the fastest growing algal groups found in ponds.

Bioassays of strain toxicity used day 9 to 12 day old cultures, the end of the exponential growth phase, when cell densities were typically in the range $7x10^5$ to $1x10^6$ cells mL^{-1}. Cells numbers continued to increase for days after this period before stabilising and declining.

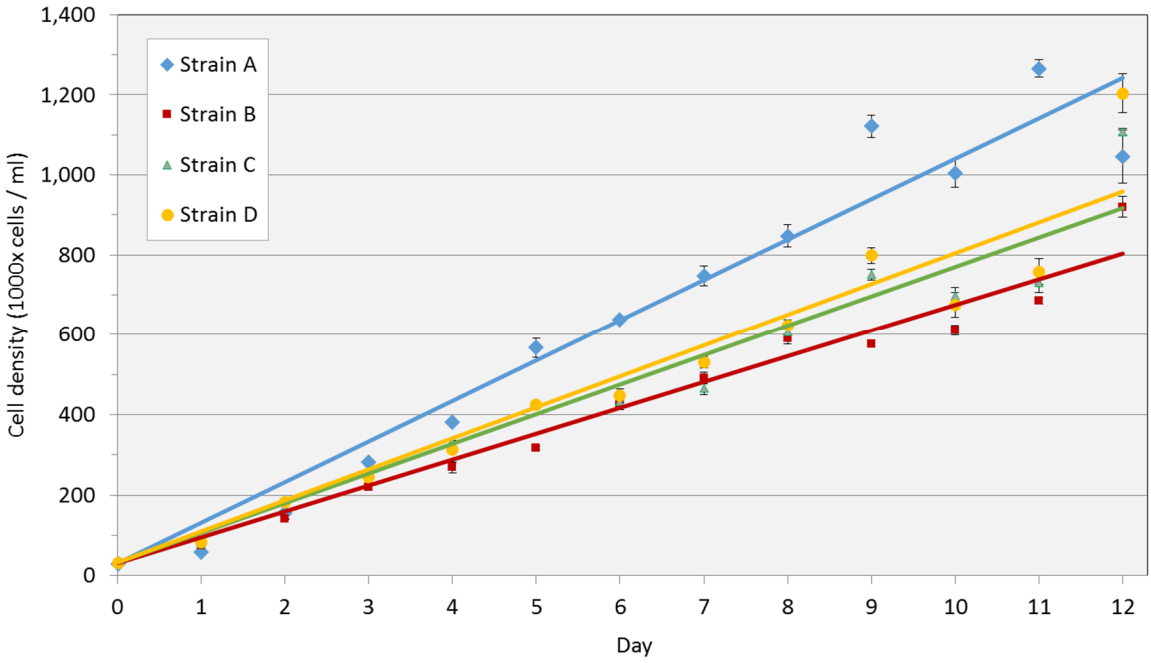

Figure 6. Growth (mean±SE) of *Heterosigma* sp. strains in aerated 2L flasks. Strain A was identified as *H. akashiwo* and Strains B-D are an unidentified *Heterosigma* species

4.3.2. Toxicity bioassays

Live animal bioassays at BIRC

The first two bioassay tests investigated the first *Heterosigma* sp. strain isolated, Strain A, subsequently identified as *H. akashiwo*, and cultured to sufficient volume. This strain was derived from a barramundi pond experiencing a strong bloom and high fish mortality. The intention of the tests was to repeatedly conduct the experiment to generate sufficient animals with acute symptoms to undertake histopathology assessment and identify abnormalities such as tissue disruption by comparison of control and exposed animals. When the project commenced, symptomatic prawn samples for pathology analysis were to be generated on farms, either collected directly from affected ponds or from on-farm tanks where exposure and collection could be better controlled. It became clear that this strategy was not reliable due in part to the unpredictability of blooms and the improved impact of mitigation employed by farms as the project progressed. It was therefore necessary to investigate the mechanism of harm using laboratory cultures.

Test 1. Large juvenile prawns

Bioassay test 1, using large juveniles, had no mortality in either the exposed or control groups, and exposed prawns exhibited no signs of health impacts or animal distress over the 25h duration of the first part of the test. In all containers prawn behaviour remained consistent, mostly quiescent with occasional swimming. With the addition of anti-algal chemicals after 25 hours *Heterosigma* sp. cells displayed dramatic changes within minutes but there was no apparent change in the behaviour of prawns in any containers. The prawns also remained strongly reactive to interaction with a probe. At 3h post-chemical addition benzalkonium chloride (BKC) at a final concentration of 1.3 mg L^{-1} had caused all *Heterosigma sp.* cells to rupture, whereas cells exposed to lauryl-dimethylamine oxide (LDAO) at a concentration of 5.7 mg L^{-1} were non-motile and rounded but intact. Acetic acid addition dropped pH to 4.5 which caused cells to rupture rapidly.

Test 1 does not present any ambiguity regarding a lack of response of prawns to high-level exposure to *Heterosigma* sp. This is particularly significant since this strain was previously implicated in a fish

kill in which gross symptoms and behaviour of morbid fish indicated reaction to an ichthyotoxin. The expectation is that if a toxin was present in the test medium and *Heterosigma* sp. cells then the prawns would have been exposed to a maximal level of the toxin upon rupturing of the cells. The lack of apparent acute affect is also significant given the extremely high density of the *Heterosigma* sp. exposure, ~150,000 cells mL^{-1}, which is approximately three times higher than the maximum density recorded for a prawn pond. One week after the test all prawns, which were held in clear water conditions with continuous filtered water renewal, were alive and appeared normal with clear gills. There was no sign of melanisation of the gills that may indicate localised disruption.

Test 2. Small juvenile prawns

The second bioassay test was designed to provide further data for the potential toxicity of *Heterosigma* sp. A smaller animal size was used as there may be size-dependent variation in sensitivity to any *Heterosigma* sp. ichthyotoxins. The smaller prawns also allowed for an extended experimental period without need for culture medium renewal.

In test 2 survival was high across all treatments with only two prawns dying in the control algae treatment (Table 14). There was no indication of prawn physiological or respiratory stress based on consistently normal behaviour, feeding and successful moulting. Only limited moulting occurred, once in the control and three times in the *Heterosigma* Strain A treatment (Table 14). Moulting is a physiologically demanding process for prawns, and even though only three moulted in the *Heterosigma* Strain A. treatments, the fact that they successfully completed the process further supports the apparent lack of chemical or other stress imposed by the alga exposure. Similar in design to Test 1 the second test was on a small scale, but it too did not provide any indication of harm to prawns caused by high-level exposure to *Heterosigma* Strain A.

Table 14. Bioassay Test 2 prawn status after exposure to *Heterosigma* Strain A and *N. oculata* for four days.

	Heterosigma Strain A			*N. oculata*		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
No. stocked	4	4	8	4	4	8
Survival (%)	100	100	100	100	75 (1 died)	87 (1 died)
No. moults[1]	1	2	0	1	0	0
Behaviour[2]	normal[4]	normal[4]	normal[4]	normal[4]	normal[4]	normal[4]
Gill fouling[3]	nil	nil	nil	nil	nil	nil

[1] based on number of exuvia removed.

[2] comparison with expected activity patterns and vigour, including response to physical interaction.

[3] assessed by observation of gill chamber under strong backlighting. Nil = no discolouration.

[4] prawns mostly quiescent with occasional swimming and exhibiting a strong reaction to interaction.

Test 3. Cobia larvae

Following bioassay tests 1 and 2, which indicated that *Heterosigma* Strain A was not harmful to prawns, a potentially more sensitive animal was tested. *Heterosigma* sp. is best known for fish kills (Cochlan et al., 2012; Smayda, 2006) so to demonstrate the presence of a toxin in the Strain A isolate cobia, *Rachycentron canadum*, larvae were used. In addition to Strain A, an additional *Heterosigma* sp. strain, Strain B, isolated from a southern Queensland prawn farm, was tested. This strain was collected from the effluent channel on the farm and no adverse symptoms of fauna in the channel or farm ponds were observed at the time.

A strong reaction to *Heterosigma* Strain A exposure occurred, with abnormal swimming and larval morbidity occurring within three hours, and within 20 hours 100% mortality had occurred (Table 15). In contrast, larvae exposed to *Heterosigma* Strain B maintained high survival over 20 hours with only four mortalities (Table 15). Over the same period of time only one larva died in the control cultures.

After complete mortality in the Strain A treatment the containers were restocked at 24 hours. Once again mortality was rapid with severe symptoms obvious within four hours (Table 15). The experiment was then terminated and moribund larvae collected from the Strain A treatment and live larvae from the Strain B and *N. oculata* treatments.

Histopathology of larvae from all treatments identified that all fish larvae had an epitheliocystis infection with symptoms evident in the gills and as abnormalities in major organs. This diagnosis was also confirmed for the source population of larvae that remained in the bulk rearing tank. Due to the systemic effect of the epitheliocystis infection it was not possible to attribute any tissue pathology to the impact of *Heterosigma* Strain A. At most it can be assessed that no pathologies different to those of the source larval population or the Strain B and *N. oculata* treatment larvae were obvious for the moribund Strain A exposed larvae.

Despite the pre-existing epitheliocystis infection in the larval population the fact that it was common across all treatments means it does not detract from the result that *Heterosigma* Strain A is highly toxic to the fish larvae and Strain B is not. The epitheliocystis infection may have compromised the resistance of larvae to challenge and caused a more pronounced effect for Strain A; however, if that is the case then larvae in the Strain B treatment would also have been highly sensitive to any harmful compounds.

Table 15. Survival rate of cobia, *R. canadum*, larvae exposed to two strains of *Heterosigma* sp. and *N. oculata*.

Survival (%)	Strain A		Strain B		*N. oculata*	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
5h	20	25	100	100	100	100
20h	0	0	100	80	95	100
28h (4h[1])	5[1]	65[1]	100	65	95	100

[1] New larvae stocked at 24h for the Strain A treatment only. At 28h = 4h exposure.

Test 4. Prawn zoea

There was no significant difference in zoeal survival across all treatments with averages ranging from 49 to 73% (P=0.27) (Table 16). Development of the larvae, however, was clearly inhibited in the Strain A treatment, with no larvae progressing to the mysis stage after 48 hours compared with all larvae progressing in the other treatments.

The zoeal larval stage of *P. monodon* is a filter feeder that consumes microalgae and it was expected that if zoea fed on *Heterosigma* sp. then it would be subject to an extremely high exposure level. Ingested cells would lyse and release cell constituents directly into the gut. Rapid catastrophic mortality of prawn zoea larvae has been similarly demonstrated for *P. vannamei* fed the raphidophyte *Chattonella* sp. (Pérez-Morales et al., 2017). *Chattonella* sp. is a raphidophyte and close relative of *Heterosigma* sp. and has a similar potential toxin profile. An indication that prawn zoea larvae are feeding is the presence of faecal trails that remain attached and extend up to many body lengths behind as it swims through the water. Well-formed faecal trails were observed in all algal treatments in this experiment, providing evidence that the larvae were feeding on the algae at a relatively high rate and therefore highly exposed to the *Heterosigma* sp. strains.

Table 16. Survival and growth of *P. monodon* larvae grown in media containing different *Heterosigma* sp. strains (A to D) and *C. muelleri*. Average survival among treatments is not significantly different (P=0.27). Final larval stage is percent of total population remaining at the end of the test and is used as a measure of growth.

	C. muelleri	Strain A	Strain B	Strain C	Strain D
Survival average (%)	71	49	65	70	73
Survival range (%)	62-80	36-71	62-71	49-89	67-80
Final larval stage (%)	Z3 0 Mysis 100	Z3 100 Mysis 0	Z3 0 Mysis 100	Z3 0 Mysis 100	Z3 0 Mysis 100

As the zoeal larval stage is considered a very sensitive bioassay the lack of any clear impact on prawn larvae survival is the best evidence that under standard culture conditions the four *Heterosigma* sp. strains held in collection do not exhibit acute toxicity to prawns. The apparent growth inhibition observed in the Strain A treatment does, however, support the potential for presence of low-impact toxic or anti-nutritional factors. This relatively low-grade impact does not support the existence of a potent toxin as generally described in the literature based on mass fish kills, particularly given the high level of exposure to such compounds experienced by the larvae.

Test 5. Prawn post-larvae exposed to stressed Heterosigma sp. culture

Post-larval survival was high in all treatments except for the low-pH treatment which was significantly different from all others (P<0.01) (Table 17). However daily survival and mortality counts, indicate that after the first 24 hours PL survival stabilised for the remaining two days with 86% of PLs that survived to 24h surviving until the end of the experiment. There is potential for an initial excessive shock to the larvae at stocking that directly led to an immediate loss. At the time of stocking there was a 0.4 to 0.5 difference in pH value from source tank to test container which was considered to be within the tolerable range for PL18 stage prawns. A role for *Heterosigma* sp. in the elevated mortality of this treatment cannot be discounted but the continued high survival after the first 24 hours does not support this contention, particularly since the medium was re-adjusted to pH 6.5-7.0 on following days by acid addition.

Table 17. Mean *P. monodon* post-larval survival for algal treatments. Survival is a mean of three replicates. Values with different superscripts are significantly different (P<0.01).

	Treatment					
Survival (%)	*Dark*	Temp. shock	Low pH	Copper	*Heterosigma* control	*N. oculata* control
Mean	98.3 [a]	95.0 [a]	61.7 [b]	93.3 [a]	83.3 [a]	96.7 [a]
Range	95-100	90-100	55-70	85-100	70-95	90-100

The condition of the gills remained clear throughout the experiment in all test containers. Mucus accumulating in the gills is obvious as browning of the gill chamber region due to entrapment of algal cells and debris.

The health status of post-larvae surviving at the end of the experiment was assessed only by gross observation of behaviour in comparison to that expected of this life stage under laboratory conditions, i.e. mostly quiescent with occasional swimming particularly in response to stimulation with a probe.

This experiment does not support the contention that *Heterosigma* sp. subjected to sub-optimal conditions expected to impact its normal growth or cause cellular stress can enhance its toxicity to

prawns. The strain used in this experiment was chosen from the four strains held in culture at BIRC as it was the only one to show any effect on prawn larvae, retarded growth, in the bioassay conducted previously (Test 4). This strain had also shown a high level of toxicity to fish larvae in a bioassay conducted several months earlier (Test 3).

There is a body of evidence internationally that *Heterosigma* sp. naturally displays variation in toxicity, presumably through modulating toxin production and/or release. It is also evident that a variety of circumstances or environmental conditions can promote *Heterosigma* sp. toxicity including bloom growth status (Cochlan et al., 2012; Powers et al., 2012), water parameters (Ikeda et al., 2016) and nutrient concentration (Matheson et al., 2014; Cochlan et al., 2012). Combined, these works indicate that conditions reducing growth rate, or rate of cell division, can promote toxicity. This conclusion is supported by the results of Ono et al. (2000) who found that for *Heterosigma* sp. cultures in a laboratory, temperature and light levels that gave the lowest growth rate also had the highest toxicity to juvenile red sea bream (*Pagrus major*).

Heat shock had been shown to cause significant physiological change to *Heterosigma* sp. cultures, including stalling of growth, initiation of encystment and programmed cell death (PCD) (Dingman and Lawrence, 2012). The heat shock applied in this experiment replicated the treatment applied by Dingman and Lawrence (2012) that caused a *Heterosigma* sp. culture in the logarithmic growth phase to remain viable but cease growth for 24 hours.

In the current experiment *Heterosigma* sp. grew slowly in all treatments compared with growth rates typically seen in laboratory cultures, indicating that conditions were sub-optimal. This in itself could potentially induce toxicity if, simply, growth retardation is a trigger. Of the stress treatments only the continuous darkness treatment had a clear effect on *Heterosigma* sp. growth compared with the unstressed *Heterosigma* sp. control treatment. In the dark treatment *Heterosigma* sp. increased slightly in the first 24h but then declined in cell density from 55,000 cells mL^{-1} to 26,800 cells mL^{-1} in the final 24h. This dramatic decline in cell number most likely indicates a high level of cell stress as well as the release of cellular contents into the water column. If cell stress could trigger toxicity in the strain used then it could reasonably be expected to have been exhibited in this treatment.

Test 6. Cobia larvae

Larval survival varied significantly ($P<0.05$) among the treatments (Table 18; **Error! Reference source not found.**); however, the relational pattern is difficult to interpret and does not give confidence that *Heterosigma* sp. toxicity is implicated in the cause of mortality. Strain A at the standard *Heterosigma* sp. density of 150,000 cells mL^{-1} was associated with the lowest larval survival, significantly less than the control, indicating potential expression of a *Heterosigma* sp. toxic affect. However, the Strain A double dose of treatment had the same survival as the control and significantly greater than the standard dose treatment. Additionally, the only treatments with survival significantly different from the control had higher survival, indicating potential benefit of the higher *Heterosigma* sp. exposure.

Table 18. Mean survival of cobia larvae (n=11) after 3 days exposure to *Heterosigma* Strains A to D and control alga *N. oculata*. Values with the same superscript are not significantly different (P>0.05).

	Control	*Heterosigma* strain				
	N. oculata	Strain A	Strain A 2x	Strain B	Strain C	Strain D
Survival (%)	36.4[a]	18.2[a]	69.7[bc]	66.7[c]	24.2[a]	39.4[ab]

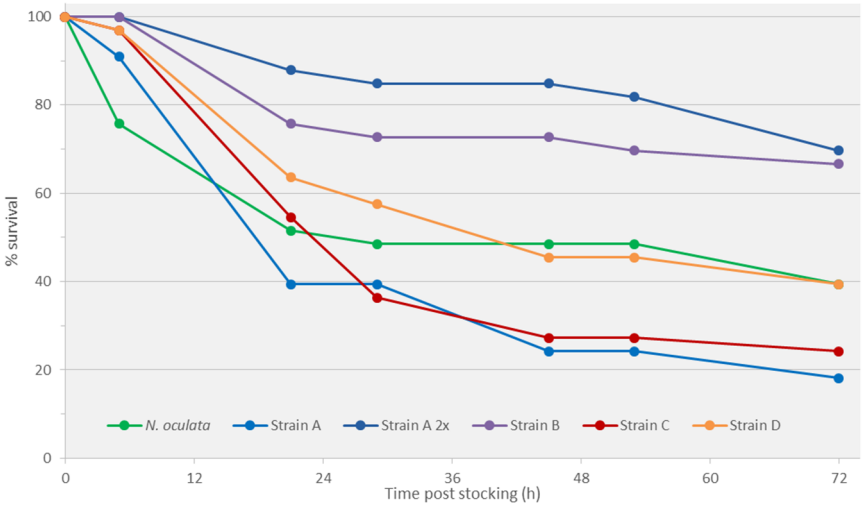

Figure 7. Survival of cobia larvae exposed to four strains of *Heterosigma* and the control alga, *N. oculata*. Statistical significance of the treatment mean survival differences are indicated in Table 18.

In vitro fish cell line bioassay

The fish cell line bioassay was completed by a PhD candidate, Andreas Seger, at the IMAS laboratory in Tasmania as part of an ARC-funded research project titled "Understanding fish-killing mechanisms by harmful algal blooms: Towards the design of effective mitigation strategies". Professor Gustaaf Hallegraeff is the principle investigator and supervisor of Andreas Seger. The full extent of related work has not yet been published and only the results of bioassay tests conducted with the *Heterosigma* Strain A culture supplied to the IMAS laboratory are referred to here.

Both live and lysed preparations of Strain A significantly (P<0.05) reduced gill cell viability, compared with non-exposed cells, demonstrating the presence of toxic factors (Figure 8). The mechanism of toxicity has not yet been determined but according to Prof. Hallegraeff and Andreas Seger the most likely candidate is the "release of a cocktail of reactive oxygen species" particularly oxidised free polyunsaturated fatty acids, though other chemical mechanisms cannot be ruled out.

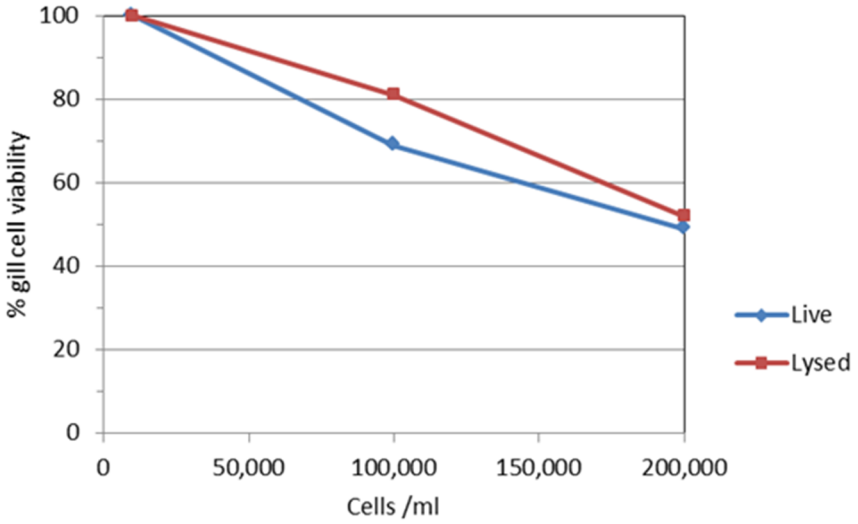

Figure 8. Fish gill cell viability after exposure to live and lysed *Heterosigma* Strain A at different cell densities for 24h. Gill cell viability is calculated against a control gill cell treatment not exposed to *Heterosigma* sp.

4.3.3. Ichthyotoxicity discussion

There have been several reports from prawn farms that in the presence of a *Heterosigma* sp. bloom prawns without significant gill fouling have exhibited disorientation and mortality, providing anecdotal evidence that *Heterosigma* sp. may release harm causing toxins. Evidence of *Heterosigma* sp. toxicity to *P. monodon* is yet to be conclusively demonstrated in controlled animal tests and there is also little evidence in the scientific literature for an ichthyotoxin mechanism for Penaeid prawns. The ability for *H. akashiwo* to affect a Pandalid prawn, *Pandalus danae* has been demonstrated in experiments similar to that conducted in this project. The study by Littik (1996) showed that 24h exposure to the *H. akashiwo* strain at 65,000 to 85,000 cells mL^{-1} did not cause elevated *P. danae* mortality. In contrast to this project the prawns in the (Littik, 1996) study, however, showed some gross change in response to *H. akashiwo* exposure with darkening of the exoskeleton and haemolymph. There was also structural damage to the gills including fusion and necrosis of the lamellae. In the same body of work Littik (1996) also found that exposing rainbow trout *Oncorhynchus mykiss* fingerlings to *H. akashiwo* resulted in 100% mortality of the fish, providing evidence for presence of a potent ichthyotoxin in the strain used. This is the same outcome as in the first round of bioassays in this project where cobia larvae exhibited rapid mortality with exposure to Strain A but prawns showed no effect. There is therefore strong evidence that prawns are far less sensitive to the putative toxic factors attributed to *Heterosigma* sp. than at least some fish species.

Planktonic crustaceans, particularly the brine shrimp *Artemia salina*, a branchiopod crustacean, has shown moderate susceptibility to *Heterosigma* sp. toxins of both whole cultures and cell extracts. Toxicity bioassays conducted by Powers et al. (2012) found that nauplii and adult brine shrimp displayed moderate mortality when exposed to *H. akashiwo* at 15,000 cells mL^{-1} over 48 hours. In another study using *A. salina* nauplii as a bioassay for extracts of *H. akashiwo* blooms and cultures a moderate impact was again identified with very high cell density equivalent to 48h LC50 values of around 100,000 cells mL^{-1} (Mohamed and Al-Shehri, 2012).

The algal culture and bioassay test conditions of this project may not have replicated the conditions conducive to *Heterosigma* sp. fully expressing its toxicity capacity and that this may occur occasionally in prawn ponds. In light of what is currently known about *Heterosigma* sp. biology and its inherent variability, the potential for toxin mediated harm to prawns cannot be ruled out on the

basis of the current results. Scientific literature indicates there is the possibility of brevetoxins or neurotoxins (Astuya et al., 2015) in some strains of *H. akashiwo* as well as potential for release of a cocktail of oxidative compounds and toxic fatty acids (Marshall et al., 2003; Twiner et al., 2001; Twiner and Trick, 2000) that are the agents responsible for mass fish kills.

Results of bioassay tests using the early prawn life stages, Tests 4 and 5, did not reveal any toxic effect sufficient to impact survival. The stunting of larval prawn growth by *Heterosigma* Strain A may be an indication of the presence of some harmful effect, though could equally indicate a feeding or nutrition issue as there was no other feed source available in these cultures. If the larvae did not filter feed on or adequately digest the Strain A cells then growth would be similarly affected. Regardless of the mechanism the different response of the prawn larvae to Strain A compared with the other strains indicates a significant difference between them which may, in light of the taxonomic identification of the strains, reflect a consistent difference between *H. akashiwo* and the unnamed *Heterosigma* species.

Results of the three bioassay tests after Test 3, which indicated high Strain A toxicity to cobia larvae may have been confounded by a loss or reduction in the toxicity of *Heterosigma* Strain A. In Test 3 cobia larvae mortality upon exposure was rapid. These fish may have had low resistance to such challenge due to a pre-existing condition although the alga-associated impact was clear. *In vitro* experiments at IMAS also support the presence of toxic factors for this Strain. In the subsequent bioassay test with cobia, months later, the results are difficult to interpret and do not clearly implicate a toxin. It may therefore be possible that the Strain A culture had either temporarily or permanently reduced its toxicity. High levels of toxicity variability has been reported for *Heterosigma* sp. (Mohamed and Al-Shehri, 2012; Fredrickson et al., 2011; Ono et al., 2000) and this includes physiological drift and reduction of potency over time in culture (Cochlan et al., 2012).

A range of circumstances have been reported to be linked with relative toxicity of *Heterosigma akashiwo* strains, including; bloom growth status (Cochlan et al. 2012; Powers et al. 2012), water parameters (Ikeda et al., 2016; Haque and Onoue, 2002; Ono et al., 2000) and nutrient concentration (Cochlan et al. 2012; Matheson et al. 2014). Review of works related to *H. akashiwo* toxicity indicates conditions that reduce the growth rate, or rate of cell division, can promote toxicity. This conclusion is supported by the results of Ono et al. (2000) who found that for laboratory cultures temperature and light levels that gave the lowest growth rate also had the highest toxicity to juvenile red sea bream (*P. major*). In the current bioassay test the *Heterosigma* sp. inocula came from cultures at the late exponential growth phase which according to (Cochlan et al., 2012) is when the toxicity may start to elevate. Additionally bioassay test 5 investigated the potential for non-ideal or stressful physical and chemical conditions to activate or increase toxicity but no toxic affect was evident for the prawn post-larvae.

Environmental conditions under which *Heterosigma* sp. blooms, grow or remain viable and exhibit toxicity are wide (Table 10) and include the full range of physico-chemical parameters experienced in prawn production ponds. It is generally recognised that elevated nutrient conditions promote development of *H. akashiwo* blooms in natural waters (Kok et al., 2015; Matheson, 2014; Mohamed and Al-Shehri, 2012; Herndon and Cochlan, 2007; Jiang et al., 2006) and therefore the eutrophic conditions in aquaculture ponds would also favour *Heterosigma* sp. growth. Even when present in prawn ponds *Heterosigma* sp. do not always go on to form significant blooms, and blooms do not typically persist for extended periods, indicating that despite conditions favourable for its growth other factors are constraints. It is most likely that biotic factors, micro-organisms of the pond mesocosm, inhibit *Heterosigma* sp. bloom development. Algicidal bacteria and *Heterosigma* sp.-specific viruses are known to impact bloom development and persistence (Lawrence et al., 2006; Lawrence et al., 2002; Nagasaki et al., 1999; Kim et al., 1998).

H. akashiwo bloom toxicity can vary significantly over the course of a cycle and highest potency levels tend to occur towards the end of blooms after the period of maximal cell proliferation (Cochlan et al., 2012; Powers et al., 2012). This is a critical characteristic for aquaculture operations, particularly fish farms due to the high toxin sensitivity of fish, as it means that significant toxin impacts may be prevented by early detection and effective response before the blooms transition to a more toxic phase.

Sensitive methods for detecting low cell densities of *Heterosigma* sp. would therefore be critical for impact mitigation. The 'snot' test developed at Australian Prawn Farms has so far been the most successful early-detection tool used by farmers. It is based on the presence of mucus in the water column and false negatives can occur. Prototypes of the '*Heterosigma* algal identification unit' that commenced development in a Seafood CRC project in 2011 have been tested on farm ponds but the device has not yet made it to commercial availability. Regular microscopic examination of blooms remains the most reliable method of *Heterosigma* sp. presence detection. Workshops conducted by the project have contributed to training of farm staff in sample preparation and identification.

4.3.4. Mucoid substance production and gill fouling

The link between *Heterosigma* sp. blooms, mucus aggregate formation in the water column and gill fouling in prawn ponds (Figure 9) is well supported by farm records (Matt West, pers. comm.). The results of the industry bloom surveillance program provided further evidence of this strong link (see section 4.2.3). Statistical analysis of the bloom identification data determined a strongly significant relationship between *Heterosigma* sp. abundance and gill fouling ($P<0.03$) with *Heterosigma* sp. presence explaining a large proportion, 39%, of the variability observed in gill fouling occurrence in ponds (Table 12). However, the data also indicate that relatively dense blooms can occur without evidence of mucus aggregates and gill fouling, demonstrating a high level of variation of mucus production.

Figure 9. Pond cultured prawn exhibiting fouled gills at the highest intensity ranking used in statistical analyses, i.e. rank = 3; dark brown with accretion of material such that gill filaments are obscured.

Variability in mucus or mucus aggregate formation has implications for the use of the 'snot' test as a *Heterosigma* sp. presence identification tool. This test has proved to be a valuable method as it is quick and simple to perform pond-side; however, it should be recognised that false-negative results can and do occur. Microscopic examination should also be performed to support the 'snot' test. The acid-reaction test as used in this project is based on the same principles as the 'snot' test and can be used as an alternative.

Regular mucus testing of cultures during the project with the acid-reaction test provided direct evidence of the apparent variability in mucus production by *Heterosigma* sp. For example, despite all four strains cultured at BIRC having at times exhibited significant mucus production only one strain, Strain C, exhibited mucus production during bioassay test 6. Additionally, in this case there was no evidence of gill fouling or mucus aggregate formation in Strain C, or other treatments, but the culture returned a strong positive to the acid-reaction test (see section 3.3.4).

It is apparent that conditions influence the formation of mucus aggregates even when sufficient mucoid substances are present. If the mucoid substances remained intracellular and not released into the medium then this could explain why gill fouling was not observed. Alternatively, dissolved mucoid substances only precipitate and aggregate under certain conditions that did not occur in the cultures. Acidic conditions, such as those created in the acid-reaction test, appear to be highly conducive to mucus aggregate formation.

The acid-reaction test is a quick and simple test that provides a relative indication of the total quantity of transparent polymeric substances present in the culture. As all *Heterosigma* sp. cells lyse in the test when used on whole culture or bloom samples it will include both intracellular and extracellular polymeric substances. It therefore represents the potential for gill fouling, rather than an indicator of gill fouling extent. For laboratory *Heterosigma* sp. cultures acid-reaction tests performed on small volumes of whole and centrifuged cell-free culture medium indicated that the dominant portion of the mucoid substances may typically be associated with the cells rather than as free, dissolved compounds. It is hypothesised that the rapid acidification causes the mucocysts to spontaneously discharge. Under acidic conditions the transparent polymeric substances precipitate and form macro-aggregates entrapping cellular debris and become clearly visible (Figure 10).

The acid-reaction test was also conducted on prawn pond water samples without *Heterosigma* sp. with negative results. This is consistent with 'snot' test results reported by Australian Prawn Farms which indicate false positives, that is, mucus test positive results in the absence of *Heterosigma* sp., is rare or possibly have not occurred over hundreds of tests. For some tests the strength of the mucus test result is higher than expected in a dose-dependent relationship, when samples have contained a very low density of *Heterosigma* sp. cells.

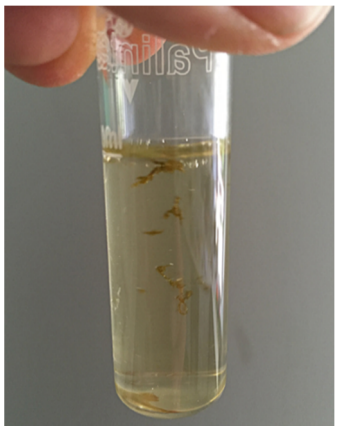

Figure 10. Acid-reaction test performed with laboratory grown *Heterosigma* sp. culture. Mucus aggregates are brown due to entrapped cellular debris.

The term mucus, as used in this report, is also referred to in the microbial or aquatic context as muco-polysaccharides or transparent polymeric substances. These substances commonly occur in the aquatic environment as products of microbiota, including bacteria, fungi and algae (Bar-Zeev et al., 2012; Bhaskar et al., 2005; Decho, 1990). Many species excrete these substances into the surrounding medium so can be referred to as exopolymeric substances. *Heterosigma* sp. produce acid polysaccharides (Lopes Daniella et al., 2012; Yokote et al., 1985) which selectively stain with alcian blue (Thornton et al., 2007), a characteristic which assists direct observation as the compounds are transparent. Such staining was used to provide visual confirmation of the capacity of *Heterosigma* sp. to release muco-polysaccharides into the surrounding medium.

Heterosigma sp. has mucocysts lining the cell membrane (Jeong et al., 2010) (Figure 11, A) and when these spontaneously discharge their contents a comparatively large volume of alcian blue stained mucus is evident (Figure 11, B). It was found that stressing cells by rapidly dropping the pH to around

5 to 5.5 would cause cells to spontaneously eject mucus without killing the cells. Lower pH values, around 4 to 4.5, release mucus into the medium but cause cells to rupture and release all contents. It is not known if there are specific conditions encountered by cells during pond blooms that exacerbates mucus release from cells. It could be chemical, for example pH change, or it could be mechanical, such as when cells come into contact with gill filaments when the prawn is ventilating. In the latter case mucus excreted would be in direct contact with the gills and potentially more readily cause fouling.

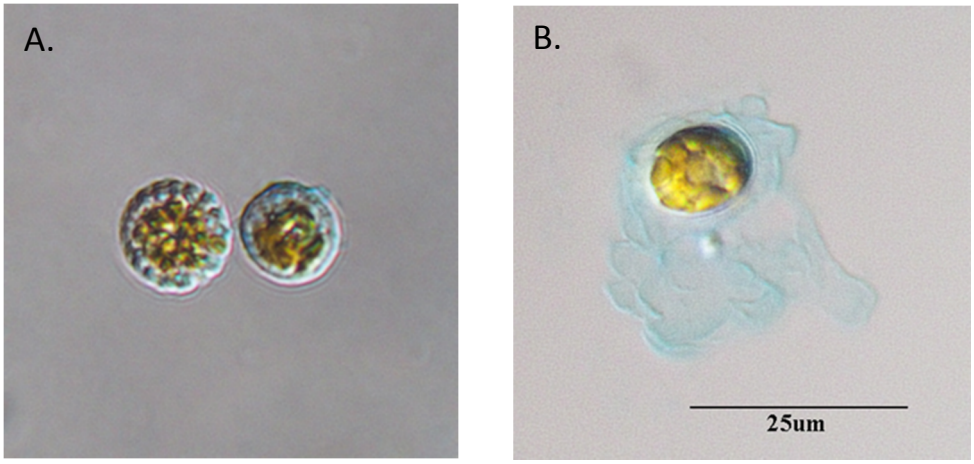

Figure 11. A. *Heterosigma* sp. cells showing alcian blue stained mucocysts inside the cell membrane. Cells were stressed by exposure to LDAO. B. Live *Heterosigma* sp. cell within an alcian blue stained 'cloud' of polymeric substance, mucus, ejected following exposure to low pH.

Neither gill fouling or mucus aggregates were observed during the toxicity bioassay tests conducted with prawns and fish larvae despite high cell densities (see section 4.3.2), even when cultures were stressed by physical and chemical conditions (Test 5; Figure 12). Gill fouling and suspended mucus aggregates were only demonstrated when cells were subjected to severe cell stress leading to rupture by rapid pH drop (Figure 13). It is interesting that stressing and rupturing cells using BKC did not lead to gill fouling. Chemical interaction between dissolved polymeric substances and BKC may prevent formation of large, sticky mucus aggregates.

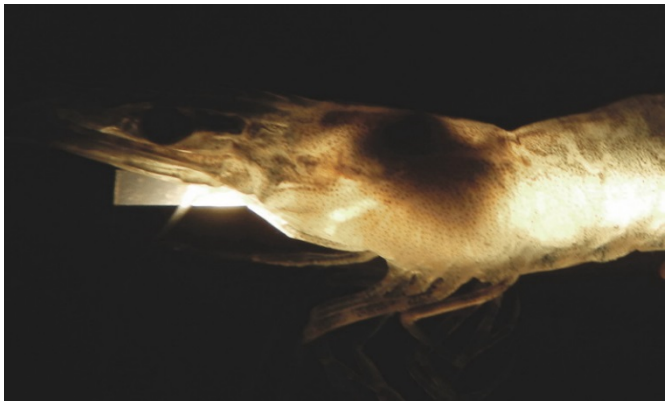

Figure 12. Small juvenile prawn exposed to high density *Heterosigma* Strain A for 4 days (bioassay Test 2), showing gill chamber with normal appearance. A backlight was used to make the gills clearly visible.

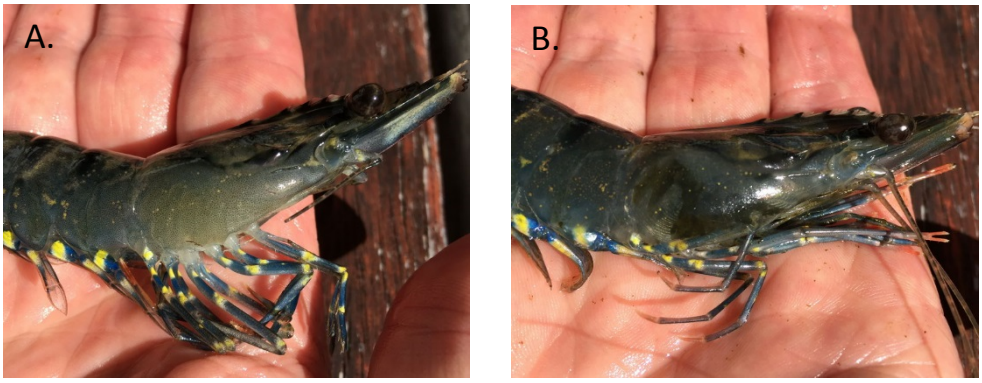

Figure 13. Prawn gills after exposure to high density *Heterosigma* sp. culture with algal cells ruptured by chemical addition. A. Benzalkonium chloride added at 1.33 mg L^{-1}, gills clear. B. Acetic acid added to pH 4.5, gill chamber extensively fouled with mucus and cellular debris. See **Error! Reference source not found.** for details.

4.4 *Heterosigma* sp. bloom control measures

The method currently used on prawn and barramundi pond farms to remediate undesirable or harmful blooms is elevated water exchange. This approach serves several purposes: dilution of cell density, dilution of toxic or harmful compounds and rapidly changing the plankton dynamics in the pond. The latter outcome is non-specific to the harmful species and can upset the stability of the pond over the short-term, potentially itself negatively impacting growth and production. High water exchange is not always an option for farms that have restricted access to source waters or source water quality is not suitable for use due to heavy rains or severe weather events. Increased pumping is also a significant cost burden.

Prawn and barramundi farms report that a regimen of high water exchange per day, 5 to 20% of volume, will ultimately be effective in mitigating the bloom impact and eliminating the offending algal species. Periods of high water exchange have been effective in a *Heterosigma* sp. bloom threat in as short as three days, however up to three weeks has also been required. Many variables will influence the dynamics of bloom composition transition.

It is therefore desirable to have other harmful bloom remediation options and ideally these would have a high degree of specificity to the target alga, limiting disruption and adverse impacts on the pond environment. This project focussed on control methods for *Heterosigma* sp. which was nominated as the species posing greatest threat to farm production. In a pond with an emerging *Heterosigma* sp. bloom a relatively low-intensity inhibition may tip the bloom balance away from its dominance in favour of more desirable species.

Ultrasound exposure

The relative physical fragility *Heterosigma* sp. cells may be a characteristic that can be exploited for a specific control method. Unlike many other microalgal species *Heterosigma* sp. does not have a cell well or other supporting structure (Engesmo et al., 2016) and the cell can be more readily ruptured by mechanical or chemical forces. Ultrasound waves propagated through water create high physical forces as pressure increases and decreases at more than 20kHz. There are various reports in the literature indicating the algicidal activity of ultrasound on cyanobacteria species that contain gas vesicles (Wu et al., 2011; Tang et al., 2004) and other algal groups (Nowotarski et al., 2011) but little indication of its effect on fragile flagellate species.

This project conducted small-scale preliminary trials using a proprietary ultrasound unit designed for treating water. *Heterosigma* sp. cell response was monitored following exposure to acute doses that would not be feasible for farm ponds but which provided information on the sensitivity of *Heterosigma* sp. to ultrasound. An exposure duration of five minutes was sufficient to induce cell immotility or ineffective swimming motion which persisted for more than one hour, though cells remained intact. After a further 16 hours most of the cell population had regained normal motility for both 5 and 15 minute exposure durations.

The effect of ultrasound on microorganisms is greatly affected by both intensity (wave amplitude; sound pressure) and frequency. The ultrasound generator used in this test was programmed to produce short durations of multiple narrow frequency bands in succession so that, over the course of approximately 90 seconds, cells were exposed to a wide range of frequencies. Identifying the most effective frequency within the range and extending its duration may improve *Heterosigma* sp. inhibition. In a pond where ultrasound intensity would be, for practical reasons, lower than that tested, it may be possible over longer exposure times to cause chronic inhibition that stops bloom progression. Additional testing at a tank scale would better indicate chronic exposure impacts but was not conducted due to equipment faults. Preliminary testing of prawn exposure to ultrasound, not reported here, indicated their relative insensitivity although more investigation is required.

Inhibition by surfactant compounds

Chemical control of harmful blooms is problematic for prawn farms for a number of reasons including safety for stock and environmental concerns. Harmful bloom prevention as part of normal pond management would be a preferred approach, but in light of the lack of options for control currently available to farms, consideration was given to the feasibility of a chemical option. Certain surfactant chemicals reportedly have some harmful bloom species specificity, have low toxicity to non-target organisms, including humans and stock, and are highly biodegradable in the aquatic environment (Mulligan, 2005).

Literature reports provide evidence for the significant activity of several groups of surfactant compounds against harmful algal bloom species; sophorolipids (Choi and Lee, 2012; Lee et al., 2008; Sun et al., 2004a; Baek et al., 2003), cocamidopropyl betaine (CAPB) and laurylamine (Sun et al., 2004b). CAPB and lauryl dimethyl amine oxide (LDAO) were chosen as candidates for testing in this project as both are readily biodegradable, have properties suitable for use in alkaline seawater environments and have reported minimum effective doses that may be practical for farms. Both compounds are common ingredients in household soaps and shampoos.

The lowest concentration of LDAO tested, 2 mg L^{-1}, caused a rapid decline in cell viability with almost all cells rupturing within 30 minutes (Table 19). CAPD did not show as dramatic impact within the same time period though cells were highly stressed as evidenced by cessation of swimming. No viable cells remained after 24 hours of continuous exposure to 2 mg L^{-1} of both LDAO and CAPD.

BKC is an antimicrobial commonly used in household cleaners and other disinfection applications because it is highly effective at very low concentrations. It has been used by prawn farms overseas to control infections and harmful algae species (Mohamed et al., 2000). The results of this test support the strong antimicrobial capacity of BKC with 0.5 mg L^{-1} causing total destruction of *Heterosigma* sp. cells over 24 hours (Table 19). However, there is little margin between minimum effective concentration for algal control and minimum dose toxic to stock. The 24-h LC_{50} concentration for *Heterosigma* sp. has been estimated at 0.12 mg L^{-1} and the 24-h LC_{50} toxic concentration for PL-30 stage *P. monodon* at 0.16 mg L^{-1} (Piyatiratitivorakul et al., 2002; Thuithaisong, 1998). There are also safety and environmental concerns about the use of this chemical (Ferk et al., 2007).

Table 19. Status of *Heterosigma* sp. cells post-treatment of culture medium with three chemicals. Ruptured cells are not viable. Onset of immotility is an indicator of cell stress. LDAO = lauryl dimethyl amine oxide; CAPD = cocamidopropyl betaine; BKC = benzalkonium chloride.

	High conc. range			Low conc. range				
		45 min			30 min		24 hour	
	mg L^{-1}	Motile[1] (%)	Ruptured (%)	mg L^{-1}	Motile[1] (%)	Ruptured (%)	Motile[1] (%)	Ruptured (%)
LDAO	*5*	0	100	*2*	0	95	0	100
	10	0	100	*4*	0	100	0	100
	20	0	100	*6*	0	100	0	100
CAPD	*5*	0	~60[2]	*2*	0	0	0	100
	10	0	~60[2]	*4*	0	0	0	100
	20	0	100	*6*	0	40	0	100
BKC	*1*	0	50	*0.5*	5	0	0	100
Control	-	10	0	-	95	0	95	0

[1] Motile cell estimate excludes cells exhibiting ineffective flagella movement, i.e. cells without directional motion.
[2] Difficult to discern extent of rupturing. Around two thirds of cells with irregular 'bumpy' outline indicating chloroplasts may have been at least partially free of the cell membrane.

An additional reason for using a surfactant compound to control *Heterosigma* sp. is that the surfactant property may also mitigate the impact of mucus aggregation and gill fouling. This could be a critical feature for any treatment method that may stress the *Heterosigma* sp. bloom and trigger spontaneous mucoid substance release.

The preliminary algicidal tests conducted during this project support the conclusion that from the perspective of effectiveness and dose LDAO and CAPD are viable options for control of *Heterosigma* sp. However, this is just one element of the range of considerations before a chemical can be appropriately used in an aquaculture pond. Further work defining treatment parameters, factors influencing effectiveness and toxicity to prawns was not conducted due to interruption of the project. Before proceeding further in this direction the industry should decide whether chemical control of harmful blooms is a desirable option. Information generated by this project provided useful information on two candidate compounds for consideration.

Other control options not tested - Clay

Selected naturally occurring clays or clays chemically modified to improve critical characteristics can effectively control bloom development of some HAB species through inducing flocculation and cell settlement or inducing cell lysis (Hagstrom et al., 2010; Beaulieu et al., 2005; Sengco et al., 2005). Clays may have some algal species specificity as cell surface characteristics, including surface charge, affect the binding of clay particles to the cell (Lee and EonSeon Jin, 2013; Yu et al., 1999). It may be possible to mix such clays in a pond and remove a harmful bloom without significant disruption to pond dynamics. A further advantage of these clays is that they may both control the alga and remove toxins from the water (Seger et al., 2015). Bentonite clays from a West Australian mine have shown effectiveness in controlling the toxic dinoflagellate *Karlodinium veneficum* (Hallegraeff, pers. comm.) and may be effective for *Heterosigma* sp. blooms.

5 Implications

Improvement to the capacity of prawn farms to effectively mitigate the impacts of *Heterosigma* sp. blooms that occurred concurrent with this project and as a result of its outcomes was evident in the 2016/2017 production season. An enhanced understanding of the HAB threat and prioritisation of regular microscopic bloom assessment, as well as the on-farm skill and knowledge to identify specific microalgal species, appear to be the primary factors responsible for the improvement. Through regular communications and staff training this project has contributed to harmful bloom awareness and understanding as well as farms' capacity to manage the threat. It has not filled all the gaps in knowledge for *Heterosigma* sp., for example factors promoting its bloom dominance or its mucus production, which may be intractable issues, but such knowledge is secondary in importance to the changes that have already been implemented on farms.

Considering the variability among strains currently labelled *Heterosigma* sp. based on morphological and molecular characteristics, and the variability in apparent toxicity to aquatic animals it is likely there is no consistent rule regarding potential for harm. This is evident from farm reports of high *Heterosigma* sp. densities accompanied by a lack of effects on prawns, while conversely, at other times, a relatively low *Heterosigma* sp. density may be accompanied by abnormal prawn behaviours and/or gill fouling. This variability also makes *Heterosigma* sp. a difficult candidate for investigation and experimentation. Clearly the identification of *Heterosigma* sp. by morphological characteristics grossly underestimates the complexity of strains and species of this genus and may obscure relationships between harmfulness to stock and environmental parameters.

6 Recommendations

- The online Algae Directory, the most comprehensive resource for bloom species identification and other related information available for the Australian prawn farming industry, should be maintained indefinitely and regularly revised so it has continued industry relevance. It is envisaged that this would have a minimal time commitment but the APFA will need to provide advice as to its expectations of this resource for the industry. Gross symptoms of disease and harmful algae exposure can appear similar and there may be a link between the two, so correct bloom characterisation should also have high priority particularly when associated with severe events.
- It would also be an advantage to continue a bloom species identification service to be available to industry. The benefits of this would be correct identification of potentially harmful species when stock health events occur which can be critical to the ultimate diagnosis and to the epidemiology of the event particularly in light of recent disease issues on prawn farms. Farms should have the capability for microscopic image capture to assist in quick species identification and for enduring farm reference. Microscopes that can produce reasonable images and video are relatively inexpensive.
- Management of pond blooms is relatively imprecise and practical tools for higher levels of control over bloom composition should be explored. Productivity losses and bloom remediation costs remain a burden on industry. Bloom control may become a greater production constraint if there is more extensive application of minimal water exchange pond management strategies throughout the industry in response to recent disease incursions.
- The role of the Gymnodinioid group of dinoflagellates in adverse health events should be further investigated. This project focussed on the impact of *Heterosigma* sp., however the bloom survey identified dinoflagellates, particularly Gymnodinioid species, as being strongly linked to adverse health events. Farms also report the regular association of *Gymnodinium*-like species and reduced feeding rate of stock. The Gymnodinioid group of dinoflagellates can be difficult to identify and likely only a small number of species in this group represent a threat. The industry would need assistance to investigate the role of certain species in health events and control options. New DNA technology for species identification could be a powerful tool for such work.
- This project identified the presence of a number of microalgal species that could potentially impact the health and growth of prawns in ponds however there is scant information available on which to make assessments. Therefore continuing monitoring and recording of bloom composition and stock health status either within individual farms or coordinated across the industry is critical to assessing the true impact of a particular species and identifying new threats. Though causation will still be inferred from weight of evidence until appropriate controlled exposure tests are performed. A species' potential to cause harm can be dramatically affected by biotic and abiotic conditions leading to high variability so it would likely be only long term datasets that could quantify the threat posed by some species.
- This project indicates that some level of risk appears to be present in all years and at all farms at any time of the season so every farm therefore needs to maintain vigilance and preparedness to respond to an event at all times. However differences among farms and throughout the season in average abundance of specific algal species may affect the harmful algae event risk profile for a farm. That is, the likelihood of a harmful bloom event may vary but the possibility is always present. The risk profile for individual farms can only be ascertained through their own monitoring and recording.
- The best option for farms to mitigate the most common impact of *Heterosigma* sp. blooms, gill fouling, is to ensure the oxygen level is maintained at a high level at all times. This can be achieved by increased pond aeration and water exchange. As gill fouling simply restricts the rate of oxygen transfer through the gill filaments the higher the environmental oxygen concentration the higher the oxygen flux across the gills. If strong fouling occurs during a period of moulting then mortality rate can be exacerbated.

- The potential for a toxin mediated *Heterosigma* sp. impact on prawns remains unresolved and further investigation is required given that some anecdotal evidence from farms is consistent with toxin symptoms. However such work is problematic as it is apparent that exposure tests under laboratory conditions may not be indicative of *Heterosigma* sp. dynamics in ponds. The primary method for investigating *Heterosigma* sp. toxins may be to opportunistically collect specimens from ponds exhibiting symptoms consistent with acute toxicity. Such sampling opportunities may not occur however as farms are proactively managing *Heterosigma* sp. blooms and preventing acute impacts.
- Significant *H. akashiwo* toxin impacts may be prevented by early detection and effective response before the blooms transition to a more toxic phase. Bloom toxicity can vary significantly over the course of a cycle and highest potency levels tend to occur towards the end of blooms after the period of maximal cell proliferation. This is a critical characteristic for aquaculture operations, particularly fish farms, due to the high toxin sensitivity of fish.

6.1 Further development

Marine pond aquaculture farms in Australia have or have access to sufficient knowledge and technical ability for identification of emerging potentially harmful algal blooms, though smaller farms may be restricted by the resources that can be applied to regular bloom assessment programs. Management of pond blooms is, however, still relatively imprecise with control over bloom composition falling short of that desirable. Ideally farms could reliably prevent the blooming of undesirable species and promote only algal groups considered beneficial to pond operation. The tools to achieve this are currently very limited. Even if bloom control ambitions are scaled back from the ideal, tools for controlling emergent blooms without unduly compromising the pond dynamics would be a great advantage and a priority for development. This would be particularly important for minimal water exchange pond management strategies which, following recent disease issues, particularly White Spot Disease, may be more extensively applied throughout the industry.

Although this project focussed on *Heterosigma* sp., the bloom survey identified dinoflagellates, particularly Gymnodinioid species, as being strongly linked to adverse health events. Prawn farms also report that dinoflagellate species are often associated with reduced feeding and low-level mortality. The Gymnodinioid group of dinoflagellates can be difficult to identify and harmful species may only make up a small proportion of this group. Farms would need assistance to investigate the role of certain species in health events. New DNA technology for species identification could be a powerful tool for such work.

7 Extension and Adoption

7.1 Extension

Communication and extension activities focussed on the end users of project outcomes, prawn farm managers and operational staff and to a lesser extent barramundi farmers. Farm visits by the principle investigator were an important component of information extension and training activities. Sixteen farms were visited over the course of the project during which harmful blooms and project developments were discussed with the manager and other staff and samples of blooms taken for the bloom survey.

Extension activities also occurred via two workshops and development of an online resource centre as outlined below.

Workshop for HAB monitoring and management 2015

This workshop was conducted in association with the Australian Prawn and Barramundi Farmers symposium on 29-31 July 2015 and ran for two hours on the 29 July. Workshop attendance was open to all industry operators and was free. There were 17 participants representing prawn farms, a barramundi farm and industry equipment suppliers.

Professor Gustaaf Hallegraeff, world renowned harmful algae expert from the Institute of Marine and Antarctic Studies (IMAS) in Tasmania was guest speaker and led a session on harmful species identification and impacts. The workshop also included demonstration examples of different types of microscopes for farm use and various methods for bloom sampling and sample manipulation techniques. Video and photographic images of live and preserved algal cells generated during the project assisted discussion of species identification. The workshop format allowed for ad hoc discussion among the participants and exploration of aspects of particular interest of the participants. Prof. Hallegraeff's knowledge and experience was of great benefit to discussions.

Workshop for HAB monitoring and management 2017

A one-hour workshop was held on the second day of the 2017 APFA Symposium held on the Gold Coast 1-2 August. The workshop was presented by David Mann and summarised the results and outcomes of the project with open discussion. The primary focus was on the link between various algal species occurring in ponds and reduced health status of stock and identification of Heterosigma sp. from bloom samples. A microscope with live Heterosigma sp. culture was included for identification training. Use of the online Algal Directory identification tool was also covered. There were 16 participants, predominantly from prawn farms.

Algae Directory

An online resource for farm bloom monitoring and species identification was created during the project and was used as a repository for information arising from the project that could be readily accessed by the industry. Information arising from this project, including images, video and text, and contributions from other sources can continue to be added by the web-site moderator, David Mann. See section 8 (Project materials developed) for further detail of the Algae Directory. The website is currently only accessible from the APFA home page and is password protected with the APFA responsible for distribution of the password.

Algae identification feedback

A by-product of the industry-wide pond bloom survey was that the project provided an algae identification service to the industry. This was a valuable avenue for training farm staff on correct identification of some of the less common bloom species. Farms tended to supply samples from ponds that were anomalous in some way and therefore significant to understanding the influence of various

bloom species on pond characteristics. The service provided farms with confirmation of identity of species present based on samples or images they had collected and examined themselves. In this way staff could expand their species identification repertoire and improve accuracy.

7.2 Adoption

The influence of project outcomes on farm practices and adoption of recommendations is difficult to assess though there is a marked difference in the industry's mitigation of bloom linked productivity loss between the start and the end of the project. The prawn farming industry in the 2017 season has a far better understanding of harmful algae blooms and impact mitigation is more effective than in 2013 at the start of the project. An important contribution to this change in industry capacity to manage the issue was the enhanced awareness that arose simply from regular communication on the issue with farms. It was apparent that conversation stimulated by the development of the project and presentation to the prawn farming industry prior to commencement contributed significantly to the general awareness of the issue across industry operators and emphasised the importance of paying more attention to this aspect of pond management. Matt West, APFA president, contributed significantly to broad discussion of the issue particularly in relation to *Heterosigma* sp. blooms and potential for productivity losses.

As an example of improvements to farm management of harmful blooms, several farms reported that they considered the 2016/17 season as the worst on record for *Heterosigma* sp. prevalence and persistence. However they also report little productivity loss. Early identification during regular bloom assessment by skilled staff and rapid response kept ponds asymptomatic.

A comprehensive catalogue of prawn pond bloom species will be a significant asset for scientists and the industry in future years as it is a historical record of current species along the Queensland coast. It will help identify changes over time, in particular the emergence of new harmful bloom threats.

7.3 Project coverage

This project, or related activities were not covered by any media articles.

8 Project materials developed

One of the key resources that the industry needs for monitoring algal blooms is a comprehensive reference for identifying species. The most appropriate format for such a resource is a webpage that enables ongoing revision to provide an enduring up-to-date comprehensive reference. A Seafood CRC-supported project led by Anni Conn was launched coincident with this HAB project for the production of an online 'Algae Directory' for marine pond microalgae identification. The two projects collaborated to design and populate the Directory which was linked to and accessible from the APFA website.

The initial content was drawn from the pre-existing microalgae reference, Stafford (1999), which was commonly used on farms. Information and images generated during the HAB project then contributed additional material as images, video and text.

The search functionality is based on photographic images that direct the user down through taxonomic groupings to finally arrive at the lowest possible taxa, species or genus, for the alga they are seeking to identify (Figure 14). Text descriptions provide further information on the algal group or species cell and bloom characteristics. There is also a special grouping of potentially harmful algae where the user can view all species that should be considered suspicious. The website also has provision for input of information by the end user, including text, images and video.

The site is currently moderated by David Mann for the APFA. Prior to the conclusion of this project the future of the website will need to be determined. It is an advantage to have someone with a knowledge of algal blooms responsible for inclusion of new information and responding to enquiries.

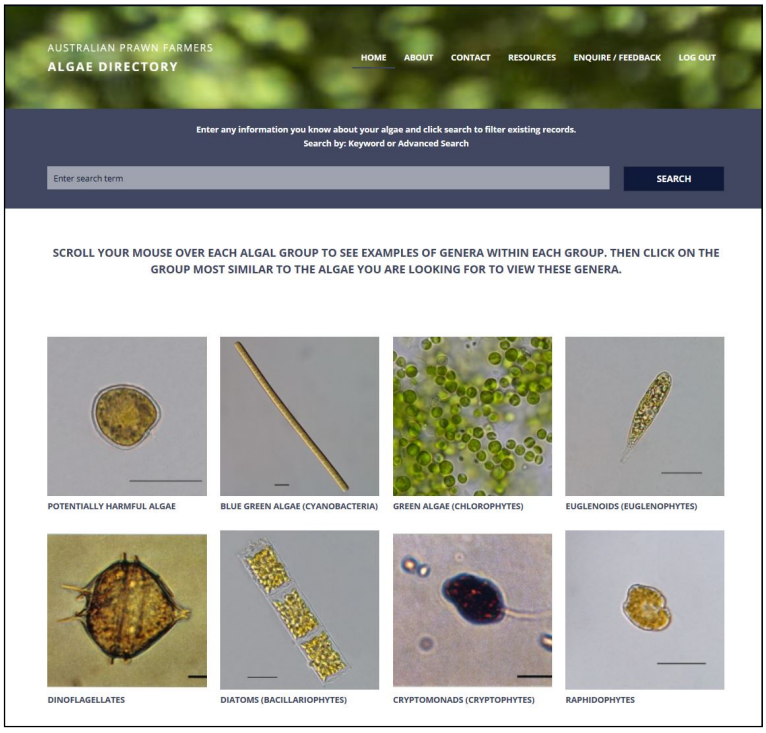

Figure 14. Opening page of the online Algae Directory showing apex images representing the main algal groups of the identification key.

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Appendices

Appendix 1. List of contributors to the project

	Role	Organisation
David Mann	Principle Investigator	DAF
Hazra Thaggard	Laboratory technician	DAF
Farm staff from multiple farms	Contributed farm data and information; assisted sampling	Particularly Australian Prawn Farms, Pacific Reef Fisheries and Seafarm.
Prof. Gustaaf Hallegraeff	HAB Expertise and training	University of Tasmania
Andreas Seger	Toxicity assay	University of Tasmania
Ass. Prof. Shauna Murray	Molecular taxonomy	University of Technology Sydney
Arjun Verma	Molecular taxonomy	University of Technology Sydney
Steve Brett	Algae identification	Microalgal Services

Appendix 2. Queensland strain *Heterosigma* sp. phylogenetic relationships

Reference sequences obtained from NCBI GenBank. Sequence alignment using ClustalW. Phylogenetic tree constructed using MEGA6.06, Tamura 3+G+I, bootstrap =1000

The numbers adjacent to each node represent a measure of statistical support for the node as a percentage, with 100 being maximal.

Higher values indicate stronger support that the sequences to the right of the node cluster together to the exclusion of any other.