# Marine and Tropical Sciences Research Facility Milestone Report, October 2007

**Program 6:** **Understanding threats and impacts of invasive pests on ecosystems**

**Project 2.6.1:** **Identification and impact of invasive pests in the Great Barrier Reef**

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**Research Plan:** Toxic microalgae in the GBR ecosystem; establishment of cultures, identification aids, biogeography and potential for toxin production (Duration of project, including field and laboratory study schedules)

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**The content of this report is correct at the time of writing (October 2007). Subsequent amendments to the intended project outputs have since been made.**

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## Aims

The aims of this project are to:

- Establish cultures of dinoflagellates and other microalgae from samples taken from port and reef sediments and macroalgal surfaces.
- Determine the morphological and genetic identity of microalgae via high resolution light microscopy (LM), scanning electron microscopy (SEM) and molecular techniques.
- Develop specific probes from type cultures in order to develop fast and efficient molecular techniques to assess the presence and abundance of toxic microalgal species in environmental samples.
- Validate the specificity of the molecular tools for toxic microalgae.
- Monitor the distribution and abundance of toxic microalgae on the Great Barrier Reef using molecular techniques.
- Determine toxin profiles for cultured ciguateric dinoflagellates.
- Produce an atlas of microalgae occurring in the Great Barrier Reef ecosystem (port and reef sediments, macroalgae and the water column) to enable morphological identification of toxic species and estimations of their seasonality and abundance.

## Significance

The Great Barrier Reef (GBR) ecosystem is an area of high productivity, which provides a habitat for a wide diversity of organisms. As well as being of ecological importance, it is economically important in terms of fisheries and tourism. Microalgae such as dinoflagellates are important components of the GBR ecosystem, and yet almost nothing is known about them.

The proposed research aims to identify and monitor the distribution of microalgae, and especially toxic dinoflagellates, in this area and determine which biological and environmental factors cause an increase in their abundance and toxin production. Morphological and various molecular techniques will be developed side-by-side to facilitate rapid identification of species of microalgae.

An atlas for morphological identification of toxic and non-toxic microalgae, their seasonality and abundance patterns will also be produced to enable recognition of changes in community structure, which may occur with introductions of organisms through various vectors or as a consequence of climate change or other environmental factors. The information and techniques produced during this project will provide a sound basis for the development and implementation of effective resource management by stakeholders.

# Introduction

In the past few decades, incidences of toxic microalgal outbreaks have increased as have the geographical ranges of many species (Hallegraeff 2003). Likely reasons for the spread of these species are the transport to non-native locations via ships' ballast water, aquaculture and increased tourism. It is estimated that up to 10,000 species are transported in ballast water each day (Carlton 1999), taken up from native ports and released upon arriving at their destination. As ocean travel increases, so does the distribution of marine microalgae across the globe. Many species of dinoflagellates are able to survive in ballast water by forming cysts or using modes of nutrition other than photosynthesis (Doblin *et al.* 2004).

The ABWMAC schedule of introduced species has listed *Alexandrium minutum*, *A. catenella*, *A. tamarense* and *Gymnodinium catenatum* as target pest species, which are likely to be spread through ballast water transport. These species have a large global distribution and bloom in coastal waters, producing saxitoxin and saxitoxin analogues which cause paralytic shellfish poisoning (PSP) in humans. Toxic dinoflagellate cysts have been detected in the ballast waters of ships in Australian ports and successfully germinated (Hallegraeff and Bolch 1992, Hallegraeff 1998), showing evidence for introduction by this route. Ballast water from a ship in Eden, Australia was estimated to contain over 300 million *Alexandrium* cysts (Hallegraeff and Bolch 1992). One-off marine pest baseline surveys have been carried out previously in coastal habitats of Townsville and Hays Point (CSIRO 1998, Neil *et al.* 2001), which included identification of toxic microalgae from within the water column and sediments. Identification was by morphological methods only and cysts of the genus *Gymnodinium* were found. Toxic dinoflagellate cysts are likely to accumulate in sheltered areas with soft, silty bottoms (CSIRO 1998), fitting the description of the habitat within the Port of Townsville. If ballast water introductions occur in this area, cysts may remain viable in the sediment for five to ten years (Anderson *et al.* 1995).

Under the correct environmental conditions these cysts can germinate, which, upon successful vegetative reproduction may initiate a bloom. During blooms, toxic dinoflagellates may be found at cell densities greater than 20 million per litre (Hackett *et al.* 2005).

The phenomenon of toxic algal blooms, which cause shellfish poisonings, was virtually unknown in Australia until 1986 when large concentrations of saxitoxin were discovered in wild mussels in Port Phillip Bay, and saxitoxin derivatives in shellfish farms in Tasmania. The organisms responsible were thought to be *Alexandrium catenella* and *Gymnodinium catenatum*, respectively (Hallegraeff 1992).

As *Alexandrium* spp. and *Gymnodinium* spp. have not been found in cyst records or previous plankton monitoring schemes in Australia, it is likely that they have been introduced to these areas (Hallegraeff 1992). The first verification of a toxic algal outbreak in New Zealand occurred in 1993 in the Bay of Plenty (Chang *et al.* 1997), when cases of neurotoxic shellfish poisoning (NSP) were reported. Investigation into the toxin profiles of cockles and oysters during this event discovered evidence of brevetoxin contamination within their tissues (Ishida *et al.* 1996). Subsequent outbreaks have occurred since with considerable costs to the shellfish industry, leading to the initiation of extensive monitoring programs to aid in the early detection of these blooms (Rhodes *et al.* 2001).

The concept of introduced and invasive species is often not clearly defined (Falk-Petersen *et al.* 2006). Introduced species are defined as species that are not indigenous to a given area, and instead have been accidentally or deliberately transported to this new location by human activity (Binggeli 1994), whilst invasive species are defined as species that establish a new range in which they proliferate, persist and spread to become economically or ecologically

harmful (Mack *et al.* 2000). Evidence suggests that the presence of many of the toxic microalgae which cause shellfish poisoning in Australian waters is due to human introductions from other parts of the globe (Hallegraeff 1992). According to the rule of 10ths developed by Williamson and Fitter (1996), the majority of introductions are unlikely to establish within the new range, but those that do may proliferate and become invasive. Some species of benthic toxic microalgae are thought to be native to the GBR ecosystem. These are species such as *Gambierdiscus toxicus*, *Ostreopsis* spp. and *Coolia* spp., which associate largely with macroalgae on coral reefs and are thought to be responsible for ciguatera poisoning in humans. In Australia, most reported ciguatera poisonings occur from fish caught on the GBR (Lewis 2006). Ciguatoxins are lipophilic, bioaccumulate in tissues of fish, and are passed on along the food chain. Top predators such as moray eels and barracudas can accumulate large quantities of these toxins. It has been suggested that more than 400 species of fish can be ciguateric (Lehane and Lewis 2000).

Coral reefs are subject to frequent disturbances such as high temperatures and irradiance, crown of thorns outbreaks and cyclones, causing coral mortality and a reduction in coral cover, which, especially in areas where eutrophication and overfishing occurs, may cause a phase shift from coral-dominated to macroalgae-dominated reefs (McCook 1999). Terrestrial discharge onto the Great Barrier Reef includes sediment, fertilisers, pesticides and herbicides alongside other contaminants. Aerial mapping has documented plume extensions of up to fifty kilometres from the coast (Devlin and Brodie 2005). As coral bleaching increases, so does macroalgal cover on the reef (Shulman and Robertson 1996).

This in turn is likely to enhance proliferation of macroalgal-associated microalgae by increasing available substrate for colonisation. Carlson and Tindall (1985) found that on average 76% of macroalgal-associated dinoflagellate communities were toxic. Although many macroalgal-associated dinoflagellates are known to be native to the GBR region, they should be classed as invasive, if a range expansion occurred (Mack *et al.* 2000). This may be a cause for concern for human health in terms of ciguatera poisoning.

The numbers of diagnosed ciguatera poisonings are thought to be vastly underestimated as the symptoms vary greatly and typically include both gastrointestinal and neurological symptoms (Lewis 2001). It is suggested that up to 50,000 cases occur annually (Lehane and Lewis 2000). Many countries in the tropics have established monitoring programs to determine the abundance of *Gambierdiscus toxicus* thought to be the principle causative agent, although not all strains of this dinoflagellate are toxic. The symptoms of ciguatera are diverse (Lehane and Lewis 2000, Lewis 2001), and Legrand and others (1990) found multiple toxins in the viscera of a moray eel, suggestive of the involvement of multiple toxins possibly produced by different genera and species in the poisoning event. The toxic dinoflagellate genera *Ostreopsis* and *Coolia*, which coexist with *G. toxicus*, are less well studied, but have been implicated as causative agents in ciguatera poisoning (Tindall *et al.* 1990, Morton *et al.* 1992). Species from the genus *Ostreopsis* have been found to produce ostreotoxins and palytoxin analogues (Meunier *et al.* 1997, Lenoir *et al.* 2004), whilst those from the genus *Coolia* produce cooliatoxins (Holmes *et al.* 1995). Further research into toxin production by these species is necessary in order to evaluate the degree of their involvement in ciguatera poisoning.

# Methodology

## 1. Produce an atlas of microalgae present in the GBR ecosystem for morphological identification of toxic species and estimations of their seasonality and abundance

Very little is known about community structure, distribution and abundance of sediment- and macroalgal-associated microalgae within the GBR ecosystem. Yet, knowledge regarding microalgal communities and the seasonality of their abundance patterns is essential to identify introductions and to judge the degree of range expansions of native species in response to changed ecological conditions on the reef. Gillespie *et al.* (1985) investigated a range of sites across Queensland, but restricted the investigation to the distribution of *Gambierdiscus toxicus*. In order for stakeholders to identify problem areas and arrive at informed management decisions, a season- and abundance pattern-based identification atlas of GBR microalgae must be compiled.

***Method***

Live samples will be taken at three-monthly intervals (season) from port-, reef sediments and from macroalgal surfaces. Niskin bottle samples will also be collected at the same time to evaluate the presence and abundance of planktonic microalgae. The samples will be split to allow for the isolation and establishment of cultures (see Section 2), while the other half will be preserved for LM and SEM documentation of microalgal species present in the different habitats and for quantification of seasonal abundance patterns. Information regarding the temporal-, spatial distributions and substrate associations of toxic microalgae will be graphically represented using maps, to identify problem areas. An identification key for toxic dinoflagellates found within the GBR ecosystem will be compiled to assist in sound taxonomic identification. The atlas and identification key can be used as a management tool by GBR-based government agencies and port authorities to conduct future monitoring of identified high-risk areas.

## 2. Establish cultures of dinoflagellates from samples taken from port-, reef sediments and macroalgae

Previous studies have been conducted to establish the presence of toxic dinoflagellates in the ports of Townsville and Hays Point and of *Gambierdiscus toxicus* in Queensland generally (Gillespie *et al.* 1985). These studies relied solely on morphological identification of the species present and cultures were never established. Samples collected in section 1 will be used for the establishment of type cultures of toxic and non-toxic dinoflagellates to enable the development of species-specific genetic probes.

***Method***

Sediments from sites within the port of Townsville and other local ports such as Cairns and Mackay will be sampled by means of a grab sampler. The sediments will be sonicated to dislodge cysts from sediment grains, screened through a 150 µm sieve to remove large debris and concentrated on a 20 µm sieve (Hallegraeff *et al.* 1988). In addition, samples will be taken from reef sites by SCUBA, sampling macroalgae, coral rubble and the surrounding seawater. Benthic samples will be shaken vigorously to dislodge any associated dinoflagellates from macroalgal surfaces and the seawater will be filtered as previously described.

Toxic and non-toxic (for molecular probe validation) dinoflagellates will be isolated by micropipetting and identified by LM and SEM. Dinoflagellates will be cultured in a variety of media (f/2, K, ES and L1) and at different temperatures (24°C and 28°C), to optimise

culturing success. Established cultures will be sub-cultured at regular intervals for culture maintenance.

3. **Determine the morphological and genetic identity of cultured dinoflagellates via LM, SEM and molecular techniques**

Prior to the development of molecular techniques, microalgae could only be identified to species level based on their morphological characteristics. More recent studies have shown both genetic and biochemical variation within morphotypes (Chinain *et al.* 1997, Bolch *et al.* 1999). This is especially true for *Alexandrium tamarense*, *A. catenella* and *A. fundyense* (the "*tamarensis* complex"). At present it is unclear whether the *A. 'tamarensis* complex' consists of separate species or different strains of the same species. Molecular analyses of the large and small subunit rDNA have found that the genetic variation did not coincide with morphotypically defined species, but that they aligned with geographically distinct groups (Scholin and Anderson 1994, Scholin *et al.* 1994), suggesting that microalgae may exhibit phenotypic plasticity. Many species of *Alexandrium* form cysts which can not be identified to species level based on morphology (Kamikawa *et al.* 2005, Kamikawa *et al.* 2007). Cysts in the sediment are seeds for bloom events and successful and accurate monitoring of cyst number and species affiliation is important.

Establishment of type cultures, defined both morphologically and genetically, will ensure accurate identification and prevent false positives. Providing identification via molecular techniques for samples from a variety of different sites in the GBR will allow superimposition of a genetic-diversity distribution pattern on the distribution pattern based on morphology (Section 1). This approach will be invaluable to judge the degree of phenotypic plasticity associated with habitat type.

***Method***

Cultures will be re-identified morphologically by LM and SEM. Primers DINOF and EUK4816R (Oldach *et al.* 2000) or 18ScomF1 and Dino18sR1 (Lin *et al.* 2006) will be used to amplify 18S-rDNA from subsamples of each of the cultures. Other regions of DNA such as the D1 region of the LSU-rDNA, ITS1 region, psbA will also be amplified, as they have been successful in differentiating species and strains in previous studies (Chinain *et al.* 1999, Godhe *et al.* 2006, Ki amd Han 2007). Products will be run on a gel and sequenced. Alignment of sequences in GenBank will determine their genetic identity and aid in establishing biogeographical patterns.

4. **Development and validation of a rapid and efficient technique for determining the presence of and quantification of toxic dinoflagellates from environmental samples**

In addition to problems discussed previously, the identification and quantification of toxic dinoflagellates based on morphological characteristics is time consuming and requires taxonomical expertise. This is costly in terms of manpower and may lead to misidentification of some morphologically similar species. The unknown degree of morphological plasticity in response to environmental factors (Lilly *et al.* 2005) is an additional hurdle that can only be overcome using genetic tools. The successful development and validation of a rapid and efficient molecular technique to quantitatively monitor the presence of toxic dinoflagellates both spatially and temporally will provide an essential tool for the management of the GBR ecosystem and will be essential in sound policy development to manage toxic microalgal outbreaks.

The method will be modified from a sandwich hybridisation assay developed by Diercks *et al.* (in press) to detect *Alexandrium minutum* from environmental samples. The main problem they encountered was that the level of detection was extremely low (10,000 cells and even less for environmental samples). Detection was determined colourimetrically using a photometer. In this investigation we aim to overcome this by using a different substrate (luminol) for the horse radish peroxidase, which can be detected and quantified by chemiluminescence.

### *Method*

A successful universal dinoflagellate primer from the previous investigation will be used as the capture probe for hybridisation. Reporter probes will be developed for each species or strain of toxic dinoflagellate that was cultured from the previous investigation, using sequences previously determined through amplification. BLAST analysis will be used to determine specificity of the probes against other available target sequences.

These probes will aim to capture rDNA. Although rDNA is found in lower quantities in vegetative cells than rRNA, the quantity between vegetative and encysted dinoflagellates is constant and does not vary in response to environmental changes. Cysts, which are dormant and unlikely to contain large quantities of rRNA (Kamikawa *et al.* 2007), would not be identifiable using the sandwich hybridisation assay developed by the Dierck *et al.* (*in press*), which used rRNA as the target nucleic acid. Horseradish peroxidase and a luminol substrate will be used for detection and quantification of target rDNA via chemiluminescence, which will increase the sensitivity of the sandwich hybridisation method. If sensitivity needs to be increased further, amplification of DNA, prior to sandwich hybridisation may be necessary.

Once the probes have been designed, they will be validated against false positives, by testing with cultures of the organisms they are specific to and with other genera and species established in culture alongside (see Section 2).

Once probes have been validated, screening of environmental samples will begin (see section 5). Calibration curves will be established using known cell densities and amounts of rDNA. These will be important to allow for quantification of toxic dinoflagellates in field samples (see Section 5).

## 5. Determine the distribution and abundance of toxic dinoflagellates in field samples from the GBR by molecular techniques.

Most studies undertaken within the GBR ecosystem have used morphological identification to quantify dinoflagellates (Gillespie *et al.* 1985, Neil *et al.* 2001). Studies using molecular techniques generally aimed to identify the presence of only one or two species (Gray *et al.* 2003, Doblin *et al.* 2004, Kamikawa *et al.* 2007, Diercks *et al.* in press). The modified sandwich hybridisation assay (section 4) in combination with developed specific probes for a number of different toxic dinoflagellate species will be applied to field samples to identify and quantify the occurrence of toxic dinoflagellate species and strains on the GBR efficiently, enabling production of a map of species distribution (see section 3).

The distribution of toxic dinoflagellates varies temporally and spatially. Understanding their distribution is important in determining areas of high risk. Where a disturbance event has occurred on the reef, such as a bleaching event, a phase shift may occur with reefs, at least temporarily, becoming dominated by macroalgae. Water quality and herbivore biomass are important in structuring which macroalgae dominate on the reef (Gillespie *et*

*al.* 1985, Albert *et al. in press*). Analysis of dinoflagellate communities associated with different substrates and species of macroalgae is important to determine the risk associated with these phase shift events. Very little is known regarding the ecology and special distribution of toxic dinoflagellates on the GBR. The establishment of depth profiles, seasonality and substrate associations of different toxic species on the reef will allow the identification of areas susceptible to toxic algae outbreaks.

### *Method*

Samples will be collected by SCUBA from areas of the GBR such as Magnetic, Orpheus, Lizard the Whitsunday Islands and other sites during sampling trips across the GBR. Sediments from coastal areas will be sampled by means of a grab sampler. Each sample will be designated dependant on location, season, substrate it was taken from (coral rubble, species of macroalgae) and depth. Dinoflagellates will be separated and sieved as previously described (Section 1), and stored until analysis. Using 96-well plates, 96 samples can be processed at a time. Each plate will be probed with a species-specific detection probe. Results will be mapped to determine the distribution and relative abundance of toxic dinoflagellates on the GBR. Sampling will be conducted seasonally to gain insights into the temporal distribution of the toxic microalgae. Seasonality of microalgal assemblages at different depths and associated with different substrates will be analysed via multidimensional scaling using PRIMER v6 (Clarke and Warwicke 1994).

## 6. Determine toxin profiles for cultured ciguateric dinoflagellates.

Many strains of *Gambierdiscus toxicus* have been found not to be toxic (Holmes *et al.* 1991). The absence of ciguatoxin for a *G. toxicus* collected from a site with high abundance of this species was confirmed by Gillespie *et al.* (1985). Queensland is high risk for ciguatera poisonings (Lewis 2001) and the capture of narrow-barred Spanish mackerel and barracuda has been prohibited in Platypus Bay, due to the high number of ciguateric fish (Lehane and Lewis 2000). Although *G. toxicus* is thought to be the main causative agent of ciguatera poisoning (Lewis 2001), other toxic genera such as *Coolia* and *Ostreopsis* are found to coexist with *G. toxicus* (Steidinger 1993), and have been indicated as causative agents (Morton *et al.* 1992).

The initiation and conditions necessary for toxin production are poorly understood, but they are likely to be energy-expensive (Wolfe 2000). The genera *Ostreopsis* and *Coolia* are particularly understudied in terms of their toxicity and very little is known about how environmental conditions affect toxin production and whether all strains are toxic. Quantification of the presence of these species may not be sufficient in determining risks to human health upon finfish consumption. Furthermore, although some research points to the production of ciguatoxins by *G. toxicus* directly, Holmes *et al.* (1991) found that this dinoflagellate produced precursors (gambiertoxins) which are oxidised to more toxic ciguatoxins upon consumption. The aim of this investigation is to characterise and quantify the toxins produced by *G. toxicus* and other ciguateric reef-associated toxic dinoflagellates by HPLC and to investigate whether toxin levels are affected by environmental conditions.

### *Method*

A calibration curve for direct cell counts and percentage transmission will be established. Subsamples of cultured dinoflagellates of the genus *Gambierdiscus*, *Ostreopsis* and *Coolia* be grown under different environmental conditions such light intensity, temperature and nutrients. Samples taken during exponential growth phase will be sonicated and extracted in ethanol (Holmes 1998, Lenoir *et al.* 2004). Reference toxins will be purchased and analysed alongside the active fractions of cell lysates using HPLC

and LC-MS analysis. Multiple independent culture replicates for the various culture conditions will be used to provide thorough estimates of variability of toxin production for each independent culture and each environmentally controlled culture condition. Should macroalgal-associated field samples contain large enough quantities of toxic dinoflagellates, they will be stored in methanol until analysis (Lenoir *et al.* 2004) and analysed to quantify mixtures of toxins found in environmental samples.

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## Tentative titles of expected publications arising from this research:

(Note that this document is a research plan – communications with stakeholders other than through research publications are not indicated here).

- Dinoflagellate cysts in port sediments within the GBR ecosystem.
- Toxic dinoflagellates in Queensland; determination of their biogeography using multiple molecular markers.
- A rapid and quantitative method of detecting multiple species of toxic dinoflagellates using chemiluminescent sandwich hybridisation in a microtiter plate assay.
- Unravelling the distribution of toxic microalgae on the GBR by chemiluminescent sandwich hybridisation in a microtiter plate assay.
- Community analysis of macroalgal-associated dinoflagellates; how depth and substrate affect their distribution.
- Seasonal variations in toxic dinoflagellate abundances on the GBR
- Evaluation of toxin profiles of ciguateric dinoflagellates from the GBR, Australia
- Variation in toxin production among strains of *Gambierdiscus toxicus* from the GBR, Australia.

## Timeline for activities outlined above. Time is allocated in two-month blocks.

| ACITITY | 2007 | | | 2008 | | | | | | 2009 | | | | | | 2010 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | JUL | SEPT | NOV | JAN | MAR | MAY | JUL | SEPT | NOV | JAN | MAR | MAY | JUL | SEPT | NOV | JAN | MAR | MAY | JUN |
| Develop research plan | | | | | | | | | | | | | | | | | | | |
| Literature review | | | | | | | | | | | | | | | | | | | |
| Workshops informing stakeholders of research | | | | | | | | | | | | | | | | | | | |
| Confirmation Seminar (PhD student) | | | | | | | | | | | | | | | | | | | |
| Preliminary sampling and culturing | | | | | | | | | | | | | | | | | | | |
| Identification – LM, SEM, molecular techniques | | | | | | | | | | | | | | | | | | | |
| Technique development – sandwich hybridisation, RT-PCR | | | | | | | | | | | | | | | | | | | |
| Validate techniques | | | | | | | | | | | | | | | | | | | |
| Sample collection | | | | | | | | | | | | | | | | | | | |
| Evaluate distribution of toxic dinoflagellates, temporal, spatial | | | | | | | | | | | | | | | | | | | |
| Ciguatera toxin profiles | | | | | | | | | | | | | | | | | | | |
| Pre-completion seminar (PhD student) | | | | | | | | | | | | | | | | | | | |