Review of genetic probe development for invasive marine species, with a focus on choice of target gene and on DNA amplification technology

Progress Report, Part 2, March 2009

David Blair, Paolo Momigliano, Samantha Garrard and Kirsten Heimann

School of Marine and Tropical Biology, James Cook University

Supported by the Australian Government's
Marine and Tropical Sciences Research Facility
Project 2.6.1 Understanding threats and impacts
of invasive pests on the Great Barrier Reef

This report should be cited as:
Blair, D., Momigliano, P., Garrard, S. and Heimann, K. (2009) *Review of genetic probe development for invasive marine species, with a focus on choice of target gene and on DNA amplification technology*. March Interim Report (Part 2) to the Marine and Tropical Sciences Research Facility. Reef and Rainforest Research Centre Limited, Cairns (33pp.).

Published by the Reef and Rainforest Research Centre on behalf of the Australian Government's Marine and Tropical Sciences Research Facility.

The Australian Government's Marine and Tropical Sciences Research Facility (MTSRF) supports world-class, public good research. The MTSRF is a major initiative of the Australian Government, designed to ensure that Australia's environmental challenges are addressed in an innovative, collaborative and sustainable way. The MTSRF investment is managed by the Department of the Environment, Water, Heritage and the Arts (DEWHA), and is supplemented by substantial cash and in-kind investments from research providers and interested third parties. The Reef and Rainforest Research Centre Limited (RRRC) is contracted by DEWHA to provide program management and communications services for the MTSRF.

This report is available for download from the
Reef and Rainforest Research Centre Limited website:
http://www.rrrc.org.au/mtsrf/theme_2/project_2_6_1.html

March 2009

Contents

1. Introduction and scope of this review

The focus of this review is the development and use of genetic probes for detection of marine invasive species. There is an emphasis on Australian waters, especially those of the Great Barrier Reef (GBR) Province, and the problems that might be encountered with alien species.

In Australia, most records of invasive species are from southern waters where the taxa involved are generally relatively easily identified large invertebrates (e.g. the starfish *Asterias amurensis*: see Table 1, page 16). Virtually all of these invaders are thought to have arrived as hull fouling organisms on ships or in ballast water (Hayes *et al.* 2005). Surprisingly little is known about invasive species in the tropical and subtropical waters of the GBR. The most worrying introduction in recent years was of the green mussel, *Perna viridis*, which appeared in Cairns' Trinity Inlet in 2001 (Stafford *et al.* 2007) but seems to have been successfully eradicated. However, given the interconnected nature of the world's oceans and the very limited baseline work done in the GBR region, it is impossible to assign indigenous status to present-day species of this region.

Identification of adult, relatively large invertebrates is generally easy; however microalgae and larval stages of planktonic and benthic organisms are much harder to identify with certainty based on morphological characteristics alone (Sawada *et al.* 2008). The strength of genetic probe techniques is to permit detection of larval stages in plankton, the benthos, and/or of juveniles. Genetic probes are rapidly becoming the method of choice for identifying microalgae. These include many species and strains that can produce toxins injurious to the ecosystem and to people ingesting contaminated fish (details are in previous reports from MTSRF Project 2.6.1 and are available for download from the MTSRF website[1]. Given the absence of baseline biodiversity work on microalgae in the GBR, it is not possible to say whether species found in our surveys are truly indigenous or whether they are recent arrivals due to anthropogenic effects or range expansion due to climate change. We are therefore treating them as though they are invasive species. Even if they are not, the kinds of genetic probes discussed here, coupled with sophisticated morphological analyses that we are undertaking at present, constitute the state-of-the-art approach to identifying these organisms.

Approaches and policies of the Australian Government to the problem of marine invasive species can be found at the following websites:

http://www.environment.gov.au/coasts/imps/index.html

http://www.daff.gov.au/animal-plant-health/pests-diseases-weeds/marine-pests/national-system

http://www.environment.gov.au/biodiversity/invasive/index.html.

The most comprehensive review of marine invasive species in Australia is by Hayes *et al.* (2005). They considered 53 non-native species present in Australia to be potential invasives. Table 1 (page 16) lists most of these species and includes several not covered in Hayes *et al.* (2005). Table 1 (page 16) also indicates whether any genetic probe development relevant to identification and detection has been done on these species. Unlike for other regions in the world where identification and detection of microalgae using genetic probes (Table 2, page 20) is well under way, few microalgae are listed in Table 1. Given this, and the fact that we are poised to commence development of genetic probes for GBR microalgae, based on cultured material now available and discussed in Heimann *et al.* 2009 (Part 1 of this Progress

[1] http://www.rrrc.org.au/mtsrf/theme_2/program_6.html

Report to the MTSRF), particular taxonomic groups of microalgae will be the main focus of this review.

2. Background to the development of genetic probes

2.1 Nature of the genetic material

DNA (sometimes RNA) is the vehicle for genetic instruction in all organisms. DNA consists of two long, antiparallel strands held together in a double helix by hydrogen bonds following the well-known base-pairing rules for the complementary bases (C≡G and A=T). The exact order of the bases A, C, G and T on these strands dictates the trajectory of an organism's development.

If a nucleic acid is in a single-stranded state (e.g. following heating or other manipulations), the bases along the single strand 'seek' to form hydrogen bonds with other bases, restoring a double-stranded condition. This process is termed *annealing* (in PCR) or *hybridisation*. Two regions of single-stranded nucleic acid along which numerous successive bases are complementary to one another are most likely to hybridise. This property of nucleic acids is exploited in many of the techniques discussed here.

2.2 The polymerase chain reaction (PCR)

The polymerase chain reaction is central to many aspects of genetic analytical techniques reported here. This technique, developed during the 1980s (see Saiki *et al.* 1988), permits the production of millions of copies of any particular, relatively short (< ~2,000 base pairs), tract of DNA. The actual DNA sequences flanking the region of interest must be known. This permits design of short (~20 bases) primers, one complementary to each flanking region but on opposite strands. Each primer forms a starting point for synthesis of the complementary strand. In addition to the template DNA and primers, the reaction mixture contains buffers, nucleotides (A, C, G and T) and a heat-stable DNA polymerase. When heated to 95°C, the two antiparallel strands of the template DNA separate. At about 55°C, the primers will anneal with their target regions, creating a short double-stranded region. Raised to about 72°C, the DNA polymerase will rapidly add bases, complementary to the bases on the single-stranded template DNA, to the 3' end of each primer. Thus each strand of the original template DNA is restored to a double-stranded condition. Each subsequent cycle of heating and cooling to these temperatures theoretically doubles the number of copies of the region of interest. Amplified in this way, the millions of copies of the template provide enough material for further manipulations such as sequencing, sizing on an agarose gel, or use as a probe. Other extensions of the basic PCR method are given in Section 3.

2.3 What are genetic probes?

In the context of techniques reported here, the term 'genetic probe' refers to short, synthetic DNA molecules (oligonucleotides) designed to anneal/ hybridise with regions of complementary sequence in the genome, or its RNA products. In the case of the PCR, two primers are oriented in opposite directions, permitting amplification of the region lying between them. In other methods, an oligonucleotide can hybridise with complementary regions of nucleic acid, thus permitting the extraction of this region from a complex mixture of molecules. Oligonucleotides can also be used as 'reporters', hybridising with captured nucleic acid molecules and bearing a fluorescent or other tag permitting visualisation of this.

2.4 The benefits and disadvantages of using genetic probes

Genetic techniques target the most fundamental level of an organism, the genetic instruction set. This remains unchanged throughout the life of the organism, no matter what environmental, developmental or physiological processes are influencing its morphology. Nucleic acids occur in virtually all living cells, so are plentiful as targets for probes. Amplification methods, such as the PCR, can provide satisfactory yields from remarkably small quantities of starting material. Thus, large sample volumes are not required. DNA molecules are tough and can withstand boiling, freezing and a range of other manipulations that destroy enzymes and other cellular components. Thus, technically difficult preservation techniques are not required. Of course, for optimal results, careful preservation is desirable.

There are some disadvantages to the use of genetic techniques. For PCRs, tiny quantities of starting material are usually used, rendering the process susceptible to contamination with DNA from extraneous sources, including laboratory staff themselves. Many genes are members of gene families that are more-or-less similar in sequence. Amplification of unsuspected paralogous genes can distort analyses and cause confusion. These problems can be circumvented or recognised by experienced workers.

2.5 Choice of gene region

Nuclear genomes of eukaryotes are large: the haploid human genome contains about 3.2×10^9 base-pairs (bp). The mean size of mollusc genomes is about sixty percent of that of the human genome (http://www.genomesize.com/index.php), and dinoflagellate genomes range from about the size of the human genome up to 2×10^{11} bp (Hackett *et al.* 2005; Lin *et al.* 2006). Thus, any given gene or region among the thousands represented in the genome is a small target. PCR can successfully amplify single-copy genes, but clearly repetitive sequences, occurring many times in a single nucleus, offer a better chance for amplification and characterisation. Most of the base-pairs in any genome do not encode genes. Genes constitute only about five percent of the human genome, and the value is probably lower for most dinoflagellates. There is much repetitive sequence in the non-coding regions, but such sequence tends to be highly taxon-specific, requiring considerable effort and cost to characterise, effort which must be repeated for each new taxon studied. In the near future, non-coding and highly specific repetitive DNA motifs will likely be exploited in species-specific probes. At present, ribosomal RNA genes are the favoured multi-copy targets in the nuclear genome. This is because (a) many copies are present, in tandem array, within a single nucleus; (b) the process of 'concerted evolution' results in identical copies throughout the array, thus avoiding the problem of paralogy mentioned earlier; (c) tracts conserved across great phylogenetic range, useful for the design of primers of broad specificity, alternate with highly variable regions that may differ even between 'strains' of the same species. Figure 1 shows the structure of the eukaryotic ribosomal RNA operon. As will be seen in the tables accompanying this report, ribosomal sequences are well represented among genetic probes. Ribosomal genes offer a repetitive target within the genome. In addition, ribosomal RNA, the product of these genes, occurs in large quantities within the numerous ribosomes. This can also be a target for genetic probes.

In contrast to nuclear genes, mitochondrial and chloroplast genomes are relatively small, circular, have limited amounts of non-coding sequence and generally do not contain paralogous genes. Animal mitochondrial genomes are typically in the range of 16 kbp-26 kbp: that of humans is at the lower end of the range. In most protists, mitochondrial genomes are around an order of magnitude larger than those of animals, and contain many genes. However, in the dinoflagellates, mitochondrial genomes are very complex – indeed they have been described as "chaotic" (Nash *et al.* 2008). Despite this, they encode only three genes. Eukaryotic cells may each contain hundreds or thousands of mitochondria,

making their genomes a tempting and abundant target for study. A few mitochondrial genes (cytochrome b, cytochrome oxidase subunit 1) have been used frequently in systematic studies, including on dinoflagellates (e.g. Lin *et al.* 2009), and shown to differ between species and strains. Chloroplast genomes have also been the targets for several systematic studies. In most species, chloroplasts contain over one hundred genes. However in dinoflagellates, only fourteen genes are present and these are arrayed on a series of minicircles (Nash *et al.* 2008).

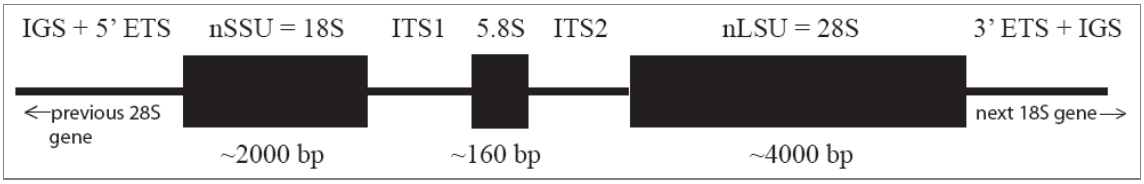

Figure 1: Structure of a eukaryotic ribosomal RNA gene operon within the long tandem array. Boxes indicate the three genes that encode ribosomal RNA used as the structural basis of ribosomes. These are separated by various spacer regions. Between the 18S and 5.8S genes lies the first internal transcribed spacer (ITS1) which is typically <250 bp in dinoflagellates. The ITS2 separates 5.8S and 28S genes and is variable in length, but typically around 200 bp. Because the sequences of the internal transcribed spacers are poorly conserved, usually differing between species, they are a common target for genetic probes, and can also be amplified by PCR using primers annealing with the highly conserved genes that flank them. The 3' end of the 28S gene is separated from the 5' end of the downstream 18S gene by the very variable and repeat-rich intergenic spacer. This region can exhibit intra-individual variation and is best not used in genetic probe work. The 28S gene (the 18S to a lesser extent) is a mosaic of highly conserved and very variable domains, providing many opportunities for design of probes to whatever level of specificity desired.

2.6 The role of genetic databases

Knowledge of particular gene sequences is required. These need to be species-specific or strain-specific if they are to be used successfully in genetic probe development. Fortunately, a number of major open-access genetic databases exist to which sequence data are routinely submitted by scientists. Indeed, many governments around the world, including those of the USA and the UK, require sequence data from publicly funded research to be made available in this way. The largest open-access database, GenBank (http://www.ncbi.nlm.nih.gov/), housed nearly forty-seven million sequences from eukaroytes when queried on 7 March 2009. Of particular value is the fact that gene regions regarded as useful for purposes such as probe design are highly represented in the databases, providing great volumes of data from an enormous range of taxa. The nucleotide database in GenBank houses 711,839 accessions containing the word 'ribosomal'. There are nearly 9,000 such accessions from dinoflagellates alone. Similarly, slightly over 700 accessions of dinoflagellate sequences contain the word "mitochondrial". We are now utilising database resources, and will add our own sequences to the database as our work proceeds.

3. Methods and their applications

3.1 Overview

The methods presented here all exploit the properties of nucleic acids described above. These methods have been used with different intents in different studies. The earliest studies focused on nucleic acid sequences as taxonomic aids and for construction of phylogenies (Section 3.3). Such studies continue to provide ever finer degrees of taxonomic resolution, especially in the dinoflagellates. The data accumulated in pursuit of these aims now provide the basis for detection of known strains and species through techniques such as real-time PCR (Section 3.4), fluorescent *in situ* hybridisation (Section 3.5), sandwich hybridisation (Section 3.6) and the use of "phylochips" (Section 3.7). These techniques and variants of them continue to be developed in search of ways to combine the greatest sensitivity and specificity with the lowest cost and repeatable, simple execution. All methods inevitably involve various levels of compromise.

3.2 Collection and storage of samples

Despite the toughness of DNA, and the fact that small quantities are adequate for PCR and other techniques, any laboratory worker will stress that reasonable quantities of good-quality starting material makes their life much easier, lowering cost and enhancing the likelihood of successful outcomes. Film and television crime shows verge on science fiction in their depiction of forensic molecular work.

Nucleic acids can be preserved in many ways. Freezing (especially to -80°C) and drying are both effective and long-term. However, these methods might not be available in field situations, and both can affect the structural integrity of whole organisms if downstream morphological work is also planned. Ethanol, in solutions of seventy percent or greater is also commonly used. However, its flammability leads to restrictions on shipping. Williams (2007) discussed the suitability of various alternatives to ethanol for preserving DNA in tissues of invertebrates, especially mollusks. She concluded that a commercial preparation (RNAlater™) and DMSO salt-saturated buffer (20% DMSO, 0.25 M EDTA, pH 7.5, NaCl saturated) are both suitable as media for shipping samples, but that ethanol is preferable and samples should be returned to ethanol after shipping. Sawada *et al.* (2008) also found ethanol to be the best preservative for DNA, in this case in bivalve larvae. Miller and Scholin (2000) demonstrated ethanol solutions to be the best preservative for diatoms and other microalgae prior to the use of fluorescent probes.

3.3 Basic PCR, DNA sequences, systematics and identification to any taxonomic level

Initial use of DNA sequence data was for molecular taxonomy/ phylogeny. Indeed, most of the data for marine invasive species and for dinoflagellates in public databases is derived from such studies. This has revolutionised our understanding of the taxonomy of many species, revealing previously unexpected relationships and many cryptic species. For example, many toxic and non-toxic microalgae from the genus *Alexandrium* are morphologically very similar (Balech 1995), and this, along with their small size can make morphological identification of field samples tentative at best. Scanning electron microscopy is required for confident morphological identification. Even so, it has been discovered that morphological traits previously thought to be 'stable' (e.g. cingulum displacement) are actually highly variable and influenced by environmental conditions. The degree of morphological plasticity in response to environmental factors (Lilly *et al.* 2005) is unknown and at present there is debate on whether *Alexandrium tamarense*, *A. catenella* and

A. fundyense (the '*tamarensis* complex') are distinct species or different strains of the same species. Molecular analyses of the large and small subunit rDNA found that the genetic variation did not coincide with morphotypically defined species, but did align with geographically distinct groups (Scholin and Anderson 1994, Scholin *et al.* 1994), showing evidence for microalgal phenotypic plasticity. Many *Alexandrium* cysts can not be identified to species level based on morphology (Kamikawa *et al.* 2005, Kamikawa *et al.* 2007), requiring use of DNA sequences. Similar histories exist for the genus *Gambierdiscus*, the main cause of ciguatera poisoning (Chinain *et al.* 1999; Richlen *et al.* 2008).

The application of PCR with species-specific primers remains one of the most straight-forward methods for detection of an organism. Once primers have been validated against related and unrelated species, to exclude the risk of false positives, amplification from environmental samples can be attempted. Reaction products can then be visualised by ethidium bromide staining and gel electrophoresis, with only samples positive for the target sequence producing reaction products. Godhe *et al.* (2001) used this method to detect two species of toxic dinoflagellates from within the plankton (*Gymnodinium mikimotoi* and *Alexandrium minutum*). However, levels of detection were not extremely high, and no product was amplified from a sample containing 115 cells of a target species. Patil *et al.* (2005a) performed a two-step nested PCR using universal primers to amplify the LSU rDNA primarily. Further amplification with species-specific primers went on to amplify a variable region within the template DNA. This boosted the level of detection from environmental samples, giving successful readings for samples with ≥5 cells. This method successfully detected ≥5 cysts from spiked ballast water samples. The same approach was used to detect the cysts of *Scrippsiella trochoidea*, *Protoceratium reticulatum* and *Lingulodinium polyedrum* from within sediments (Godhe *et al.* 2002). This is a good result as detection of microalgae from sediments often may not be as successful as from the water column due to sediments containing inhibitors of DNA extraction and PCR (Kamikawa *et al.* 2005).

The remaining methods discussed in this review have been developed in attempts to overcome problems associated with the basic PCR approach. These problems include lack of sensitivity, lack of quantifiability and inability to simultaneously screen for the presence of numerous strains or species.

3.4 Real-time PCR

Real-time PCR is a PCR-based technique aimed at simultaneously amplifying a target sequence and quantifying it in real time after each PCR cycle (Gibson *et al.* 1996, Heid *et al.* 1996). It was originally developed for gene expression studies, but in the last ten years it has been increasingly used for detection and quantification of micro-organisms from environmental samples (Bowers *et al.* 2000; Bowers *et al.* 2002; Dyhrman *et al.* 2006; Galluzzi *et al.* 2004; Godhe *et al.* 2007; Kamikawa *et al.* 2006; Moorthi *et al.* 2006; Ten Hove *et al.* 2008; Touzet and Raine 2007). In real-time PCR, quantification is carried out by either using fluorescent dyes which bind to double-stranded DNA (Galluzzi *et al.* 2004; Dyhrman *et al.* 2006), or by hybridisation of a sequence-specific (and hence strain- or species-specific) fluorescent probe which binds to a target sequence within the amplified fragment (Bowers *et al.* 2000; Bowers *et al.* 2002; Heid *et al.* 1996). In the latter case, a fluorescent indicator is present at the 5' end of the probe and a quencher dye at the 3' end (Lee *et al.* 1993). Fluorescence is only given off if the probe is degraded by the action of a polymerase synthesising a strand of DNA complementary to that on which the probe sits, and thus separating the indicator and the quencher. Fluorescence is detected and measured after each PCR cycle in a real-time PCR thermocycler. As the reaction proceeds, fluorescence increases above the baseline value (Gibson *et al.* 1996; Heid *et al.* 1996). An arbitrary threshold (C_t), based on the variability of the baseline data (usually 10 sd from the baseline fluorescence) is set and the number of cycles at which the threshold is reached is predictive of the starting quantity of the PCR target (Gibson *et al.* 1996; Heid *et al.* 1996).

Real-time PCR quantification of microalgae or other invasives involves constructing a calibration curve, where serial dilutions of cultures of target species are used to determine the relationship between cell concentration and C_t (Bowers *et al.* 2000; Bowers *et al.* 2002; Dyhrman *et al.* 2006; Galluzzi *et al.* 2004; Godhe *et al.* 2007; Kamikawa *et al.* 2006; Moorthi *et al.* 2006; Touzet *et al.* 2009). Once a reliable calibration curve has been constructed, real-time PCR can be used for monitoring concentration of harmful microalgae in the field (Figure 2). Real-time PCR is much quicker than cell counts from standard microscopy procedures, enabling the fastprocessing of a large number of samples (Bowers *et al.* 2002; Dyhrman *et al.* 2006; Galluzzi *et al.* 2004). If species-specific fluorescent probes are used for quantification, this enhances specificity, reducing the risk of cross-reactivity (Bowers *et al.* 2000; Bowers *et al.* 2002). The greatest advantage of real-time PCR is that it is an extremely sensitive technique, with which it is theoretically possible to detect environmental concentrations as low as one cell per litre of seawater, while traditional direct cell counting is normally limited to concentration of at least 1,000 cells/L (Touzet *et al.* 2009). Touzet and others (2009) reported a real-time PCR procedure to quantify the dinoflagellate *Alexandrium minutum* with a detection limit of 0.1 pg of DNA, which is equivalent to less than one cell. It would be possible to use real-time PCR to detect and quantify harmful algae before they reach critical concentrations, and real-time PCR would thus be a useful technique to monitor the dynamics of such populations in natural environments. However, intercalibration experiments clearly showed that real-time PCR has a low precision, showing standard deviations of ca. 25% and that real-time PCR often overestimates the number of target cells (Godhe *et al.* 2007). Quantification is usually in the same order of magnitude of estimates of cell concentration from standard techniques, however considerable discrepancies between real-time PCR estimate, direct cell counts and other quantification techniques such as FISH and SHA have been recorded (Godhe *et al.* 2007; Touzet *et al.* 2009).

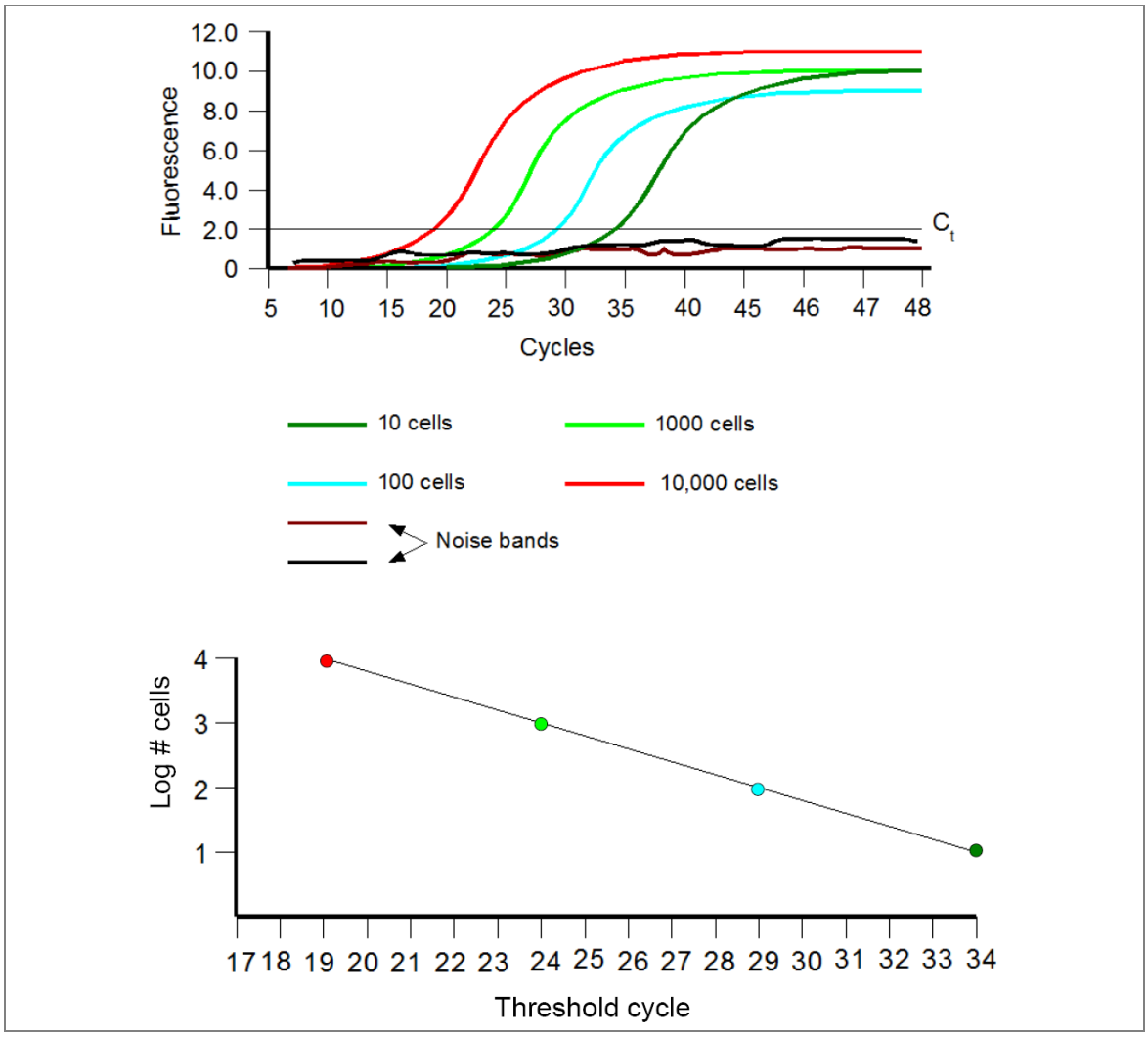

Figure 2: Real-time PCR assay for detection and quantification of target cells. The upper figure is a real-time PCR amplification plot. As the reaction proceeds, fluorescence increases above the baseline data (noise bands) and reaches the arbitrary threshold value (C_t). The number of cycles at which the threshold value is reached (the 'threshold cycle') is predictive of the starting quantity of the PCR template. Using serial dilutions of known concentrations of cultured cells, it is possible to construct a calibration curve (lower figure), which can be used to infer cell concentration in environmental samples from threshold cycle values.

3.5 Fluorescent *in situ* hybridisation

Fluorescent *in situ* hybridisation (FISH) is a technique for *in situ* detection of unicellular microbial organism which uses fluorescently (or radioactively) labelled rRNA probes (Congestri 2008; DeLong *et al.* 1989a; Miller and Scholin 1996). Probes are short (15-30 bases), synthetic, single-stranded DNA molecules which are complementary to taxon-specific sequences and carry at their 5' end fluorescent molecules (such as fluorescein) (Congestri 2008; DeLong *et al.* 1989a). Generally the oligonucleotide probes are designed to target rRNA as there are large numbers of copies of this within the cell giving a greater signal (Sako *et al.* 2004). However the fluorescent signal can be variable within a sample as the rRNA content of a cell can vary due to different physiological states and throughout the cell cycle (Adachi *et al.* 1996; DeLong *et al.* 1989b). Alternatively rDNA, the genomic DNA from which ribosomal RNA is transcribed, can be used as the target as the quantity of rDNA remains constant during the cell cycle. However, the signal from rDNA is often weak. In FISH, cells are firstly fixed and then permeabilised so that rRNA probes can penetrate the cell membrane while retaining cell structure. Cells are then mixed with the rRNA fluorescent probes in a hybridisation solution, and excess probes which did not hybridise are removed through a washing step (Congestri 2008; DeLong *et al.* 1989a). Target cells which successfully hybridise with the fluorescent probes can be visualised by epifluorescence microscopy. Labelled cells can be easily identified in environmental sample against non-labelled background cells without the need for morphological identification (Congestri 2008; Hosoi-Tanabe and Sako 2006; Scholin *et al.* 1997). Filter-based FISH allows collection of cells from known volumes of seawater on filter membranes, where the hybridisation and washing steps occur. Cells are visualised and counted directly on the filter membrane by epifluorescence microscopy permitting estimations of the original concentrations of target cells in the water sample (Miller and Scholin 1998; Godhe *et al.* 2007; Anderson *et al.* 2005).

One of the advantages of FISH is that it retains the intact cell structure of the target cells, which can be subsequently used for microscopy-based morphological studies and other analysis. Filter-based FISH has been largely used in the past decade to detect and quantify microalgae from algal culture and environmental samples (Touzet and Raine 2007; Touzet *et al.* 2009; Scholin *et al.* 1999; Scholin *et al.* 1997; Miller and Scholin 2000; Miller and Scholin 1996; Miller and Scholin 1998; Hosoi-Tanabe and Sako 2006; Hosoi-Tanabe and Sako 2005; Godhe *et al.* 2007; Congestri 2008; Anderson *et al.* 2005). Tests which used algal culture samples of known concentration show high correlations between expected densities and FISH estimates, showing that filter-based FISH recovers target cells quantitatively (Touzet and Raine 2007; Scholin *et al.* 1997; Miller and Scholin 1998; Godhe *et al.* 2007; Anderson *et al.* 2005). Despite this correlation, quantification by filter-based FISH was found to frequently underestimate the true abundance of target cells (Scholin *et al.* 1997; Godhe *et al.* 2007; Anderson *et al.* 2005). This is probably a result of cell damage during the filtration process and of cell loss in the post-hybridisation washing step. The assay measures only intact, fluorescently labelled cells, and thus it requires that cells survive sample collection and treatment (Anderson *et al.* 2005). FISH methods permits detection of concentrations as low as 100 cells/L, however the variance of results obtained from replicates of the same known concentration is usually high, and the deviation from the expected cell counts are often substantial (Godhe *et al.* 2007).

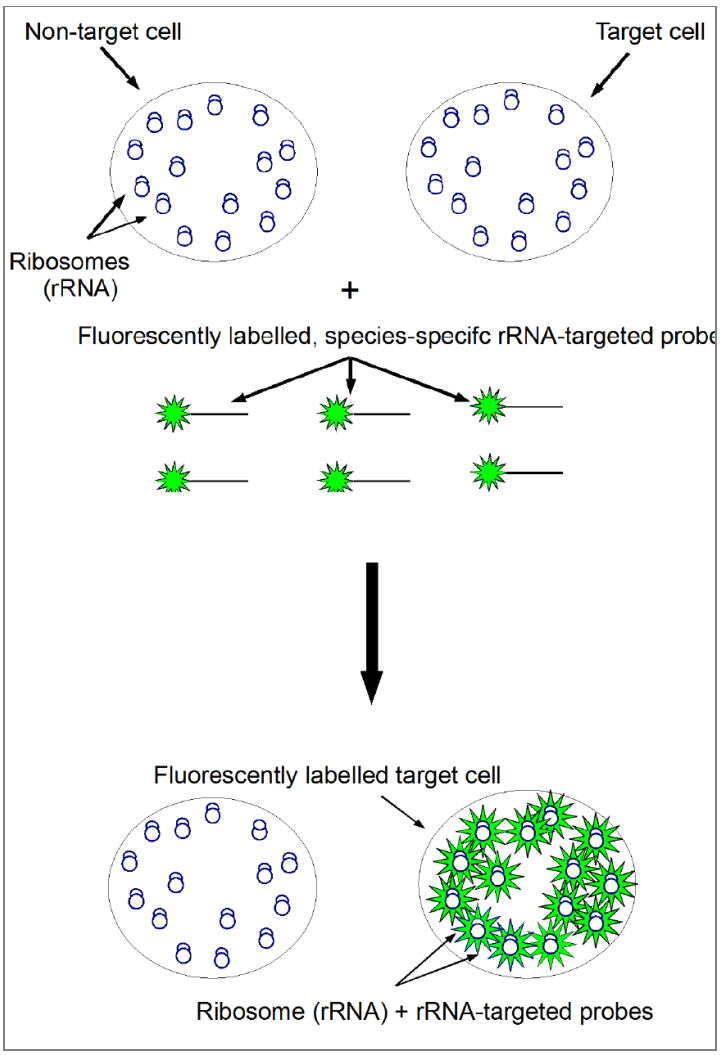

Figure 3: Fluorescent in situ hybridisation (FISH). See Section 3.5 for details.

3.6 Sandwich hybridisation

The Sandwich hybridisation assay (SHA) is a two-step hybridisation technique for the detection and quantification of extracted nucleic acids (usually rRNA released from ribosomes in crude biological sample lysates (Anderson *et al.* 2005; Diercks *et al.* 2008a; Scholin *et al.* 1997; Scholin *et al.* 1999; Tyrrell *et al.* 2002; Rautio *et al.* 2003). The target RNA is hybridised to two sets of probes, forming in this way a 'sandwich' hybrid complex. Hence detection is only achieved when the nucleic acids have complementary sequences to both probes (Tyrrell *et al.* 1997). In the first step, the target RNA is hybridised to a species-specific biotinylated capture probe which is attached to a streptadivin-coated solid support (Rautio *et al.* 2003; Scholin *et al.* 1997; Scholin *et al.* 1999). The capture probe immobilises the target nucleic acid sequence, after which a second probe, the reporter probe, is added. The latter carries a fluorescent, colourimetric or a chemiluminescent signal (Tyrrell *et al.* 1997). When it comes into contact with its complementary sequence in the sample, it forms a hybrid complex. This is then washed to remove any unbound reporter probe and fluorescence either measured directly (Ahn *et al.* 2006), or a further substrate is added causing a colourimetric or chemiluminescent signal when it comes into contact with the reporter probe (Tyrrell *et al.* 1997; Tyrrell *et al.* 2002; Jones *et al.* 2008; Diercks *et al.* 2008a). Quantification is achieved by constructing a calibration curve with cultured and field samples (Anderson *et al.* 2005; Diercks *et al.* 2008a; Scholin *et al.* 1997; Scholin *et al.* 1999; Tyrrell *et al.* 2002).

In the past decade SHAs have been successfully used for detecting and quantifying a number of harmful microalgae from environmental samples, including *Pseudo-nitzschia* spp. (Diercks *et al.* 2008b; Scholin *et al.* 1997; Scholin *et al.* 1999), *Alexandrium* spp. (Ahn *et al.* 2006; Anderson *et al.* 2005; Diercks *et al.* 2008a), *Prorocentrum* spp (Cai *et al.* 2006; Scorzetti *et al.* 2009), *Karenia brevis* (Haywood *et al.* 2007; Scorzetti *et al.* 2009), *Cochlodinium polykrikoides* (Mikulski *et al.* 2008) and *Heterosigma akashiwo* (Tyrrell *et al.* 2002). The detection limits, precision and accuracy of quantification by sandwich hybridisation are comparable to filter-based FISH (Anderson *et al.* 2005; Godhe *et al.* 2007; Scholin *et al.* 1997). However, in contrast with FISH, SHAs do not tend to underestimate the number of target cells (Godhe *et al.* 2007; Scholin *et al.* 1997). Furthermore, sample processing can be semi-automated by using robotic processors that enable the processing of large number of samples in 96 wells plates, with processing times as short as one hour (Anderson *et al.* 2005; Haywood *et al.* 2007; Jones *et al.* 2008). It is thus a technique very useful for processing large number of samples in short periods of time.

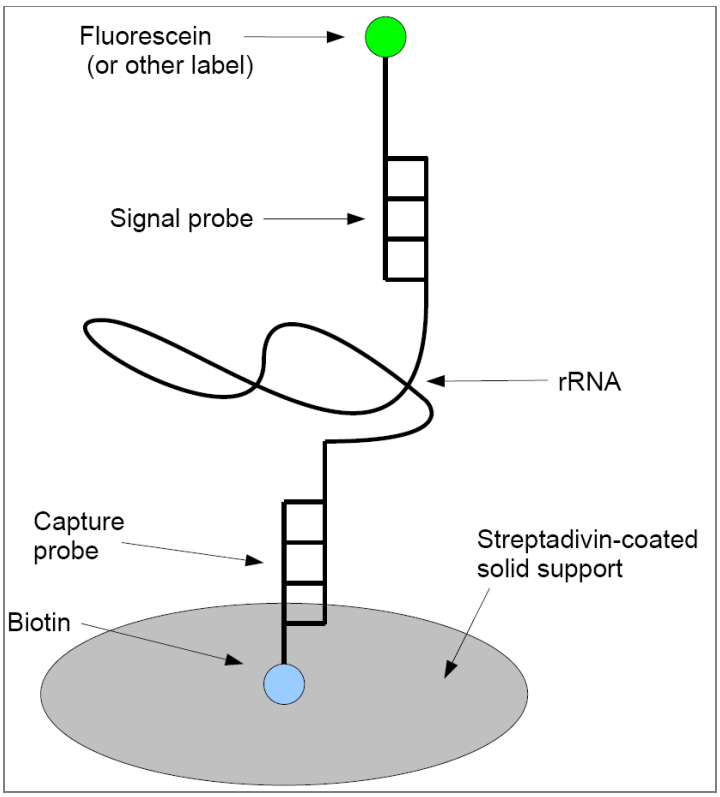

Figure 4: Sandwich hybridisation. See Section 3.6 for details.

3.7 Phylochips

All techniques previously described can be very specific in determining presence/absence of target species. However they are limited in that they permit the detection and quantification of only a single species at time. Microarray assays are hybridisation-based techniques for the parallel detection of multiple target sequences (Harrington *et al.* 2000; Hauser *et al.* 1999; Sherlock 2000; Xiang and Chen 2000). Microarrays were originally developed for gene expression studies, however in recent times they have been used in combination with taxon-specific rDNA probes for the identification of micro-organisms from environmental samples (phylochips) (Ahn *et al.* 2006; Gescher *et al.* 2008; Ki and Han 2006; Loy *et al.* 2002; Loy *et al.* 2005; Metfies *et al.* 2007; Metfies *et al.* 2008). Phylochips are created by immobilising taxon-specific probes on a streptavidin-coated glass slide (for low-density arrays) or by attaching the probes to chemical matrices on other solid surfaces (for high-density phylochips and microarrays) (Ahn *et al.* 2006; Gescher *et al.* 2008; Hauser *et al.* 1999; Ki and Han 2006; Loy *et al.* 2002; Loy *et al.* 2005; Metfies *et al.* 2007; Sherlock 2000; Xiang and Chen 2000). Templates of the DNA of interest are amplified from nucleic acids extracted from environmental samples with primers designed in regions highly conserved between species (Ahn *et al.* 2006; Gescher *et al.* 2008; Ki and Han 2006; Loy *et al.* 2002; Loy *et al.* 2005; Metfies *et al.* 2007). The purified PCR products are then fluorescently labelled and allowed to hybridise to the array. PCR products which successfully hybridised to the array can be visualised by fluorescence-based detection methods, determining presence/absence of target species from original samples (Ki and Han 2006; Loy *et al.* 2002; Loy *et al.* 2005). The hybridisation step follows PCR amplification, and since the final concentration of PCR products is not necessarily dependent on the initial concentration of templates (PCR is a qualitative, not a quantitative technique) quantification is not possible.

The multiplex nature of phylochip arrays and the high throughput of these approaches make arrays approaches an extremely useful technique for detecting presence/absence of multiple species at one time (Ahn *et al.* 2006; Gescher *et al.* 2008; Godhe *et al.* 2007; Ki and Han 2006; Loy *et al.* 2002; Loy *et al.* 2005). Gescher and others (2006) designed and tested a low-density oligonucleotide array which successfully detected and distinguished various species of the genus *Alexandrium*, which because of their morphological similarity are difficult to distinguish with standard microscopy. Another low density array was able to distinguish between ten species of harmful microalgae, including various *Alexandrium* spp., *Akashiwo sanguinea*, *Cochlodinium polykriroides*, *Chattonella marina* and various *Gymnodinium* species (Ki and Han 2006). One of the drawbacks of using phylochips is that, until recently, optimised protocols for using microarray technologies for detection of microalgae in the field were not available. Some optimised protocols for detection of harmful algae by phylochip approaches, however, are now available and such techniques are likely to play an important role in the future (Gescher *et al.* 2008; Ki and Han 2006; Metfies *et al.* 2007). Very interesting is the design of a protocol which combines sandwich-hybridisation with rRNA probes and a fiber-optic microarray approach for detection and quantification of *Alexandrium* spp and the diatom *Pseudo-nitzschia australis* (Ahn *et al.* 2006). The most obvious advantage of such a technique is that it combines multiplex detection from phylochip technology with the quantification techniques from sandwich hybridisation.

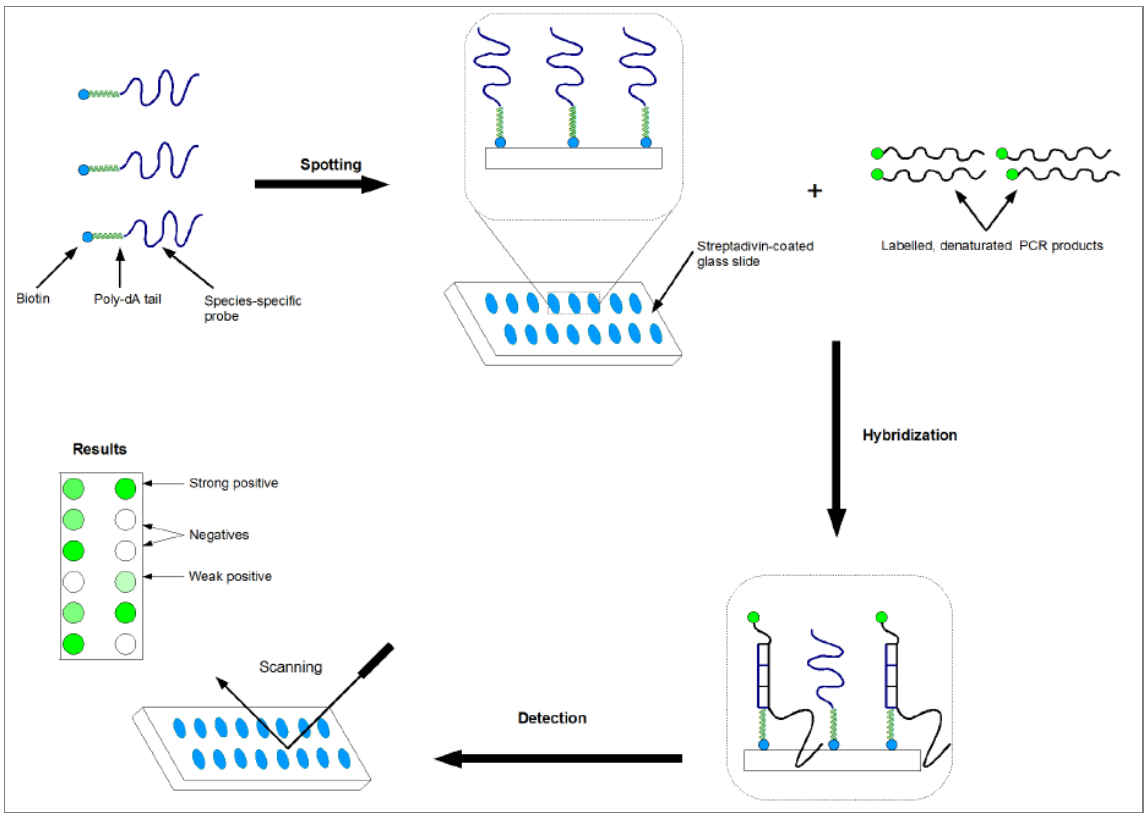

Figure 5: Phylochip method. See Section 3.7 for details.

4. Discussion and prospects

Outlined above are tools of exquisite sensitivity for detecting target organisms in natural samples or cultures. We will be evaluating these methods using microalgae cultured from the water of the GBR. Despite the promise these methods hold, they have not yet been applied to every potential or actual invasive species (Table 1). One of the principal reasons for this is the need to identify taxon-specific tracts of nucleic acid, which remains a time-consuming and potentially expensive process unless researchers resort to the well-known ribosomal RNA genes. However, next-generation sequencing techniques (Hudson 2008) will soon be able to produce huge volumes of sequence data very quickly and cheaply for any taxon. Researchers will be able to seek markers of appropriate specificity in the genomic data thus produced. Use of data from multiple genes will be particularly important in the case of dinoflagellates because single-gene studies may yield misleading results due to the extraordinary extent of horizontal gene transfer that has occurred in the history of this taxon, aided by various cycles of endosmymbiosis (Archibald 2009 *in press*).

Table 1: Non-native marine species considered to be potential invasives by Hayes *et al.* 2005. Several species have been added to update the list. Geographical locations are those recognised under the Integrated Marine and Coastal Regionalisation of Australia[2]. Geographical locations underlined are within the Great Barrier Reef Province. Also indicated in the table are any genetic probe studies carried out that might assist in identification and detection of these species.

Phylum	Class	Family	Species	Geographical location (IMCRA Bioregion)	First recorded in Australia	Molecular probe work?	Molecular probe publications
Dinoflagellata	Dinophyceae	Gonyaulaceae	*Alexandrium minutum*	HAW, LNE, SVG and VES	1980	Yes	Godhe *et al.* 2001; Guillou *et al.* 2002; Ki and Han 2006; Nagai *et al.* 2007; Diercks *et al.* 2008a
Dinoflagellata	Dinophyceae	Gymnodiniaceae	*Gymnodinium catenatum*	BAT, BRU, CVA, DAV, EYR, FRT, FLI, HAW, OTW, TWO and VES	1970	Yes	Patil *et al.* 2005a; Diercks *et al.* 2008a; Ki and Han 2006
Chlorophyta	Ulvophyceae	Cladophoraceae	*Cladophora prolifera*	COR, HAW, LMC, LNE, OTW, SVG and VES	1956	Yes (phylogeny)	Leliaert *et al.* 2007
Chlorophyta	Ulvophyceae	Codiaceae	*Codium fragile ssp. tomentosoides*	BRU and VES	1995	Yes	Lam *et al.* 2006, Provan *et al.* 2008
Chlorophyta	Ulvophyceae	Caulerpaceae	*Caulerpa* species	Beyond scope of this study: some native species		Yes	Beyond scope of this study. Booth *et al.* 2007 is a recent review.
Heterokontophyta	Phaeophyceae	Alariaceae	*Undaria pinnatifida*	BRU, FRT and VES	1988	Yes	Daguin *et al.* 2005; Muraoka and Saitoh 2005; Engel *et al.* 2008; Uwai *et al.* 2006
Rhodophyta	Florideophycae	Phyllophoraceae	*Schottera nicaeensis*	BRU, FRT, HAW, OTW, SVG, VES	1970	No	
Rhodophyta	Rhodophyceae		*Antithamnionella spirographidis*	SVG and VES.	1953	No	
Rhodophyta	Florideophycae	Rhodomelaceae	*Polysiphonia brodiaei*	BRU, CVA, FRT, OTW, SVG and VES	1977	No	
Rhodophyta	Florideophycae	Phyllophoraceae	*Gymnogongrus crenulatus*	COR, EYR, HAW, SVG and VES	1969	No	

[2] http://www.environment.gov.au/coasts/mbp/publications/imcra/pubs/map2-msb.pdf

Phylum	Class	Family	Species	Geographical location (IMCRA Bioregion)	First recorded in Australia	Molecular probe work?	Molecular probe publications
Porifera	Demospongiae	Halisarcidae	*Halisarca dujardini*	HAW and VES	1996	No (but mt genome completely sequenced)	
Cnidaria	Hydrozoa	Tubulariidae	*Ectopleura crocea*	HAW, LNE and VES	1885	No	
Cnidaria	Hydrozoa	Bougainvillidae	*Bougainvillia muscus*	BGS, BRU, HAW, LNE, VES and WSC	1918	Yes (but not as invasive species)	
Cnidaria	Hydrozoa	Clavidae	*Cordylophora caspia*	CVA, HAW, OTW and TMN	1931	Yes	Schable *et al.* 2008
Annelida	Polychaeta	Nereididae	*Alitta succinea*	HAW, LNE, SVG and VES	1930	No	
Annelida	Polychaeta	Spionidae	*Boccardia proboscidea*	CVA, EYR, LNE, OTW and VES	1975	No	
Annelida	Polychaeta	Sabellidae	*Euchone limnicola*	BGS, BRU, HAW, OTW and VES	1984	No	
Annelida	Polychaeta	Serpulidae	*Hydroides diramphus*	ANB, HAW and LMC	2000	No	
Annelida	Polychaeta	Serpulidae	*Hydroides ezoensis*	HAW and VES	1996	No	
Annelida	Polychaeta	Serpulidae	*Hydroides sanctaecrucis*	WTC	1999	No	
Annelida	Polychaeta	Spionidae	*Pseudopolydora paucibranchiata*	HAW, VES, BAT,EYR, SVG and TWO	1971	No	
Annelida	Polychaeta	Sabellidae	*Sabella spallanzanii*	BGS, LNE, SVG, TWO, VES and WSC	1965	No (some phylogenies)	
Annelida	Polychaeta	Spionidae	*Polydora cornuta*	SVG and VES	1975	No (only one pop-gen study)	
Annelida	Polychaeta	Spionidae	*Polydora websteri*	HAW	1885	No	
Mollusca	Ploylacophora	Chitonidae	*Chiton glaucus*	BRU	1910	No	
Mollusca	Gastropoda		*Moaricolpus roseus*	BRU		Yes	Gunasekera *et al.* 2005
Mollusca	Bivalvia	Ostreidae	*Crassostrea gigas*	BGS, BRU, COR, CVA, EYR, FRT, HAW, SVG, TWO, WSC and VES	1947	Yes	Li *et al.* 2003; Hosoi *et al.* 2004; Pie *et al.* 2006; Le Goff-Vutry 2007 Wang *et al.* 2008 Patil *et al.* 2005b

Phylum	Class	Family	Species	Geographical location (IMCRA Bioregion)	First recorded in Australia	Molecular probe work?	Molecular probe publications
Mollusca	Bivalvia	Mytilidae	*Mytilopsis sallei*	VDG	1999	Yes	Unpublished
Mollusca	Bivalvia	Mytilidae	*Musculista senhousia*	BGS, LNE, SVG, VES and OTW	1982	Yes (phylogeny only)	
Mollusca	Bivalvia	Mytilidae	*Perna viridis*	WTC	2001	Yes	Blair *et al.* 2006
Mollusca	Bivalvia	Corbulidae	*Varicorbula gibba*	BGS, BRU, OTW and VES	1987	No	
Mollusca	Bivalvia	Terredinidae	*Teredo navalis*	BAT, COR, CVA, CWC, EUC, EYR, HAW, LNE, MAN, MUR, NSG, OTW, SGF, SVG, MN, TWO, VES and WSC	?	No	
Mollusca	Bivalvia	Semelidae	*Theora lubrica*	HAW, LNE, NSG, OTW and VES	1958	No	
Bryozoa (or ectoprocta)	Gymnolaemata		*Bugula flabellata*	BAT, EYR, HAW, LNE, MAN, OTW, SCT, SVG, TWO, VES and WSC	1933	No	
Bryozoa (or ectoprocta)	Gymnolaemata		*Bugula neritina*	ANB, BAT, CVA, EYR, FLI, HAW, LMC, LNE, OTW, PIN, SC, SGF, SVG, TWO, VES and WSC	1880	No (only phylogenies)	
Bryozoa (or ectoprocta)	Gymnolaemata	Cryptosulidae	*Cryptosula pallasiana*	BGS, EYR, HAW, LNE, SC, SVG, TWO, VES and WSC	1880	No	
Bryozoa (or ectoprocta)	Gymnolaemata	Schizoporellidae	*Schizoporella errata*		1982	No	
Bryozoa (or ectoprocta)	Gymnolaemata	Schizoporellidae	*Schizoporella unicornis*	BAT, BGS, EYR, HAW,LMC, LNE, SC, SVG, VES and WSC	1982	No	
Bryozoa (or ectoprocta)	Gymnolaemata	Scrupocellaridae	*Scrupocellaria bertholetti*	BRU and VES	1970	No	
Bryozoa (or ectoprocta)	Gymnolaemata	Curculionoidea	*Tricellaria occidentalis*	BAT, BRU, EYR, HAW, LNE, SVG, VES and WSC	1880	No	
Bryozoa (or ectoprocta)	Gymnolaemata	Watersiporidae	*Watersipora arcuata*	BAT, CVA, EYR, HAW, LMC, VNE, SVG, TMN, TWO, VES, WSC	1940	Yes	Mackie *et al.* 2006
Bryozoa (or ectoprocta)	Gymnolaemata	Watersiporidae	*Watersipora subtorquata*	OTW, VES, SC, HAW, BGS, LMC	1970	Yes	Mackie *et al.* 2006

Phylum	Class	Family	Species	Geographical location (IMCRA Bioregion)	First recorded in Australia	Molecular probe work?	Molecular probe publications
Bryozoa (or ectoprocta)	Gymnolaemata	Vesciculariidae	*Zoobotryon verticillatum*	CWC, HAW, LMC LNE, PIN, SC, SGF, SVG and WSC	1970	No	
Kamptozoa	Not divided	Barentsiidae	*Barentsia benedeni*	HAW and SVG	1952	No	
Crustacea	Malacostraca	Portunidae	*Carcinus maenas*	BGS, BRU, COR, CVA, FLI, FRT, LNE, SVG, TWO and VES	1890	Yes	Geller *et al.* 1997; Tepolt *et al.* 2006; Darling *et al.* 2008; Harvey *et al.* 2009
Crustacea	Malacostraca		*Apocorophium acutum*	HAW	1937	No	
Crustacea	Malacostraca	Corophiidae	*Monocorophium acherusicum*	BGS, BRU, FRT, LNE, TWO and VES	1921	No	
Crustacea	Malacostraca	Corophiidae	*Monocorophium insidiosum*	BRU, CVA, HAW, LNE, OTW and VES	1973	No	
Crustacea	Malacostraca	Sphaeromatidae	*Sphaeroma walkeri*	HAW, LMC and WTC	1924	No	
Chordata	Ascidiacea	Styelidae	*Styela clava*	VES	1972	Yes	Dupont *et al.* 2006
Crustacea	Cirripedia	Balanidae	*Balanus amphitrite*	TS		No	
Crustacea	Cirripedia	Balanidae	*Notomegabalanus algicola*	BAT, HAW and MAN	1943	No	
Crustacea	Cirripedia	Balanidae	*Megabalanus rosa*	BAT, CAN, EMB, HAW, MAN, NIN, PIN, PIO, SBY and ZUY	1981	No	
Crustacea	Cirripedia	Balanidae	*Megabalanus tintinnabulum*	ANB, AWS, BAT, BON, CAB, CAN, COB, CVA, CWC, ECY, EMB, FLI, HAW, KIM, KSD, LMC, LNE, MAN, NIN, OTW, PIN, PIO, SC, TWO, VDG, VES, WTC and ZUY	1949	No (only one study phylogeny)	
Crustacea	Cirripedia		*Balanus reticulatus*	CWC, PIN and WTC	1981	No	
Echinodermata	Asteroidea	Asteriidae	*Asterias amurensis*	BRU, FRT, VES and CVA	1985	Yes	Deagle *et al.* 2003; Ward *et al.* 2008
Chordata	Ascidiacea	Cionidae	*Ciona intestinalis*	BRU, HAW, LNE, OTW, SC, SVG, VES and WSC	1899	Yes (but not as an invasive species)	
Chordata	Osteicthyes	Gobiidae	*Tridentiger trigonocephalus*	BAT, LNE, HAW and VES	1973	Yes (but not as invasive species)	Mukai *et al.* 1997

Table 2: Studies relevant to genetic-probe detection of microalgal species. Method abbreviations: FISH = fluorescent *in*-situ hybridisation; NASBA; NPA-SH = nuclease protection assay – sandwich hybridisation; SH = sandwich hybridisation. Note: (1) Detection and/or quantification from environmental samples; (2) Detection and/or quantification from algal cultures; (3) Work done as part of optimisation of sample fixation; (4) Simultaneous detection of multiple species; (5) Detection from ballast water; (6) Work done for comparison of classical and molecular techniques for detection and quantification.

TAXA	Geographic Location	Method	Gene Target	Reference	Notes
Alexandrium spp.	Gulf of Maine	FISH, SH	LSU rRNA	Anderson *et al.* 2005	1
Alexandrium spp.	Japan	FISH	LSU rRNA	Hosoi-Tanabe and Sako 2005	1
Alexandrium spp.	Japan	FISH	LSU rRNA	Hosoi-Tanabe and Sako 2006	1
Pseudo-Nitzschia spp. *(Bacillariophyceae)*	Algal cultures (Japan)	FISH	LSU rRNA	Miller and Scholin 1998	2
Pseudo-Nitzschia spp. *(Bacillariophyceae)*	Japan	FISH	LSU rRNA	Miller and Scholin 2000	3
Pseudo-Nitzschia australis	California	FISH, SH	LSU rRNA	Scolin *et al.* 1997	1,2
Pseudo-Nitzschia australis	Algal cultures (California)	FISH, SH	LSU rRNA	Scholin *et al.* 1996	2
Alexandrium minutum, *Alexandrium andersoni*	Ireland	FISH	LSU rRNA	Touzet *et al.* 2007	1,2
Alexandrium spp.	Southern Ireland	FISH, Real-time PCR	LSU rRNA, LSU rDNA,	Touzet *et al.* 2009	1,2
Prorocentrum spp.	Algal cultures, various origins	NPS-SH	LSU rRNA	Cai *et al.* 2006	2
Alexandrium spp.	Algal cultures	SH	SSU rRNA	Diercks *et al.* 2008	1,2
Gymnodinium; *Proceratium*; *Lingulodinium*; *Pyrmesium*; *Chrysochromulina*; *Pseudo-nitzschia*	Algal cultures	SH	SSU rRNA	Diercks *et al.* 2008b	2
Karenia brevis; *Gymnodinium*	Florida, California, New Zealand, Mexico	SH	LSU rRNA	Haywood *et al.* 2007	1,2
Cochlodinium	Korea	FISH, SH	LSU rRNA	Mikulski *et al.* 2008	1,2
Pseudo-Nitzschia spp. *(Bacillariophyceae)*	California	FISH, SH	LSU rRNA, SSU rRNA	Scholin *et al.* 1999	1,2
Karenia; *Alexandrium*; *Amphidium*; *Heteocapsa*; *Katodinium*; *Prorocentrum*; *Proceratium*; *Crippsiella*; *Lingulodinium*	Florida	Phylochip	LSU rDNA	Scorzetti *et al.* 2009	4
Heterosigma	Isolates from Australia, Japan, Korea, Spain, USA	SH	rRNA (not specified)	Tyrrell *et al.* 2002	1
Alexandrium spp.	Mediterranean	Qualitative PCR	5.8S, ITS1	Galluzzi *et al.* 2005	1,2
Alexandrium minutum; *Gymnodium mikimotoi*	Sout-West India	Qualitative PCR	SSU rDNA	Godhe *et al.* 2001	1,2
Lingulodinium polyedrum; *Protoceratium reticulatum*; *Scrippsiella trochoidea*	Arabian Sea (India), Sweden	Qualitative PCR	LSU rDNA	Godhe *et al.* 2002	1,2

TAXA	Geographic Location	Method	Gene Target	Reference	Notes
Alexandrium spp.; *Dinophysis* spp.; *Karenia* spp.	France (Atlantic coast and British channel)	Qualitative PCR	LSU rDNA	Guillou *et al.* 2002	1,2
Gymnodinium catenatum	New South Wales, Tasmania	Qualitative PCR	LSU rDNA	Patil *et al.* 2005a	1,5
Pfiesteria spp.	North-East Pacific (algal cultures)	Real-time PCR	SSU rDNA	Bowers *et al.* 2000	1,2
Karenia brevis	Florida	Real-time PCR	rbcL mRNA	Casper *et al.* 2004	1,2
Alexandrium fundyense	Gulf of Maine	Real-time PCR	LSU rDNA	Dyhrman *et al.* 2006	1,2
Lingulodinium polyedrum	California	Real-time PCR	SSU rDNA	Moorthi *et al.* 2006	1,2
Cochlodinium polykrikoides; *Karenia mikimotoi*; *Heterocapsa circularisquama*; *Chattonella antique*; *C. marina*; *C. Ovata*; *Heterosigma akashiwo*	Japan	Real-time PCR	LSU rDNA	Kamikawa *et al.* 2006	1,2
Alexandrium spp.	Mediterranean	Real-time PCR	LSU rDNA	Galluzzi *et al.* 2004	1,2
Karenia brevis	Gulf of Maine	Real-time PCR	rbcL mRNA	Casper *et al.* 2007	2
Alexandrium spp.	Algal cultures, various origins	Phylochip	LSU rDNA, SSU rDNA	Gescher *et al.* 2008	1,2,4
Alexandrium spp.; *Pseudo-Nitzschia austalis*	Algal cultures, various origins	Phylochip	LSU rRNA	Ahn *et al.* 2006	1,2,4
Alexandrium spp., *Akashiwo sanguinea*; *Cochlodinium polykrikoides*; *Chattonella marina*; *Gymnodinium* spp.; *Heterosigma akashiwo*	Algal cultures, various origins	Phylochip	LSU rDNA	Ki and Han 2006	2,4
Alexandrium fundyense	Algal cultures	FISH, SH, Real-time PCR	rRNA/rDNA	Godhe *et al.* 2007	6

5. References

Adachi, M., Sako, Y. and Ishida, Y. (1996). Identification of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* (Dinophyceae) using DNA probes and whole-cell hybridisation. *Journal of Phycology,* 32: 1049-1052.

Ahn, S., Kulis, D.M., Erdner, D.L., Anderson, D.M. and Walt, D.R. (2006). Fiber-optic microarray for simultaneous detection of multiple harmful algal bloom species. *Applied and Environmental Microbiology,* 72: 5742-5749.

Anderson, D.M., Kulis, D.M., Keafer, B.A., Gribble, K.E., Marin, R. and Scholin, C.A. (2005). Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. *Deep Sea Research Part II: Topical Studies in Oceanography,* 52: 2467-2490.

Archibald, J.M. (2009 in press). The origin and spread of eukaryotic photosynthesis: evolving views in light of genomics. *Botanica Marina* 51.

Balech, E. (1995) The Genus *Alexandrium* Halim (Dinoflagellata), Sherkin Island Marine Station, Cork, Ireland, 151 pp.

Booth, D., Provan, J. and Maggs, C.A. (2007). Molecular approaches to the study of invasive seaweeds. *Botanica Marina,* 50: 385-396.

Bowers, H.A., Tengs, T., Glasgow, H.B., Jr., Burkholder, J.M., Rublee, P.A. and Oldach, D.W. (2000). Development of Real-Time PCR Assays for Rapid Detection of *Pfiesteria piscicida* and Related Dinoflagellates. *Appl Environ Microbiol,* 66: 4641-4648.

Bowers, H.A., Tengs, T., Goto, S., Tomas, C., Ono, C., Yoshimatsu, S. and Oldach, D.W. (2002). Development of Real-Time PCR assays for the detection of *Chattonella* species in culture and environmental samples. IN Steidinger, K.A., Landsberg, J.H., Tomas, C. and Vargo, G.A. (Eds.) *Harmful Algae 2002*

Cai, Q., Li, R., Zhen, Y., Mi, T. and Yu, Z. (2006). Detection of two *Prorocentrum* species using sandwich hybridisation integrated with nuclease protection assay. *Harmful Algae,* 5: 300 - 309.

Casper, E.T., Paul, J.H., Smith, M.C. and Gray, M. (2004). Detection and Quantification of the Red Tide Dinoflagellate *Karenia brevis* by Real-Time Nucleic Acid Sequence-Based Amplification. *Applied and Environmental Microbiology,* Aug 2004: 4727-4732.

Casper, E.T., Patterson, S.S., Bhanushali, P., Farmer, A., Smith, M., Fries, D.P. and Paul, J.H. (2007). A handheld NASBA analyzer for the field detection and quantification of *Karenia brevis*. *Harmful Algae,* 6: 112-118.

Chinain, M., Faust, M. and Pauillac, S. (1999). Morphology and molecular analysis of three toxic species of *Gambierdiscus* (Dinophyceae): *G. pacificus*, sp. nov., *G. australes*, sp. nov and *G. polynesiensis*, sp. nov. *Journal of Phycology,* 35: 1282-1296.

Congestri, R. (2008). FISH methods in phycology: Phototrophic biofilm and phytoplankton applications. *Plant Biosyst,* 142: 337-342.

Daguin, C., Voisin, M., Engel, C. and Viard, F. (2005). Microsatellites isolation and polymorphism in introduced populations of the cultivated seaweed *Undaria pinnatifida* (Phaeophyceae, Laminariales). *Conservation Genetics,* 6: 647-650.

Darling, J.A., Bagley, M.J., Roman, J., Tepolt, C.K. and Geller, J.B. (2008). Genetic patterns across multiple introductions of the globally invasive crab genus *Carcinus*. *Molecular Ecology,* 17: 4992-5007.

Deagle, B.E., Bax, N. and Patil, J.G. (2003). Development and evaluation of a PCR-based test for detection of *Asterias* (Echinodermata : Asteroidea) larvae in Australian plankton samples from ballast water. *Marine and Freshwater Research,* 54: 709-719.

Delong, E., Wickham, G. and Pace, N. (1989a). Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science,* 243: 1360-1363.

Delong, E.F., Wickham, G.S. and Pace, N.R. (1989b). Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science,* 243: 1360-1363.

Diercks, S., Medlin, L.K. and Metfies, K. (2008a). Colorimetric detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridisation in a microtiter plate assay. *Harmful Algae,* 7: 137 - 145.

Diercks, S., Metfies, K. and Medlin, L.K. (2008b). Molecular probe sets for the detection of toxic algae for use in sandwich hybridisation formats. *Journal of Plankton Research,* 30: 439-448.

Dupont, L., Viard, F. and Bishop, J.D.D. (2006). Isolation and characterization of twelve polymorphic microsatellite markers for the invasive ascidian *Styela clava* (Tunicata). *Molecular Ecology Notes,* 6: 101-103.

Dyhrman, S.T., Erdner, D., Du, J.L., Galac, M. and Anderson, D.M. (2006). Molecular quantification of toxic *Alexandrium fundyense* in the Gulf of Maine using real-time PCR. *Harmful Algae,* 5: 242-250.

Engel, C., Billard Marie Voisin, E. and Viard, F. (2008). Conservation and polymorphism of mitochondrial intergenic sequences in brown algae (Phaeophyceae). *European Journal of Phycology,* 43: 195-205.

Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garces, E. and Magnani, M. (2004). Development of a Real-Time PCR Assay for Rapid Detection and Quantification of *Alexandrium minutum* (a Dinoflagellate). *Applied and Environmental Microbiology,* 70: 1199-1206.

Galluzzi, L., Penna, A., Bertozzini, E., Giacobbe, M.G., Vila, M., Garcés, E., Prioli, S. and Magnani, M. (2005). Development of a qualitative PCR method for the *Alexandrium* spp. (Dinophyceae) detection in contaminated mussels (*Mytilus galloprovincialis*). *Harmful Algae,* 4: 973-983.

Geller, J.B., Walton, E.D., Grosholz, E.D. and Ruiz, G.M. (1997). Cryptic invasions of the crab *Carcinus* detected by molecular phylogeography. *Molecular Ecology,* 6: 901-906.

Gescher, C., Metfies, K. and Medlin, L.K. (2008). The ALEX CHIP - Development of a DNA chip for identification and monitoring of *Alexandrium*. *Harmful Algae,* 7: 485-494.

Gibson, U.E., Heid, C.A. and Williams, P.M. (1996). A novel method for real time quantitative RT-PCR. *Genome Research,* 6: 995-1001.

Godhe, A., Cusack, C., Pedersen, J., Andersen, P., Anderson, D.M., Bresnan, E., Cembella, A., Dahl, E., Diercks, S., Elbrächter, M., Edler, L., Galluzzi, L., Gescher, C., Gladstone, M., Karlson, B., Kulis, D., Legresley, M., Lindahl, O., Marin, R., Mcdermott, G., Medlin, L.K., Naustvoll, L.-J., Penna, A. and Töbe, K. (2007). Intercalibration of classical and molecular techniques for identification of *Alexandrium fundyense* (Dinophyceae) and estimation of cell densities. *Harmful Algae,* 6: 56-72.

Godhe, A., Otta, S.K., Rehnstam-Holm, A.-S., Karunasagar, I. and Karunasagar, I. (2001). Polymerase Chain Reaction in Detection of *Gymnodinium mikimotoi* and *Alexandrium minutum* in Field Samples from Southwest India. *Marine Biotechnology,* 3: 152-162.

Godhe, A., Rehnstam-Holm, A.-S., Karunasagar, I. and Karunasagar, I. (2002). PCR detection of dinoflagellate cysts in field sediment samples from tropic and temperate environments. *Harmful Algae,* 1: 361-373.

Guillou, L., Nezan, E., Cueff, V., Denn, E.E.L., Cambon-Bonavita, M.A., Gentien, P. and Barbier, G. (2002). Genetic diversity and molecular detection of three toxic dinoflagellate genera (*Alexandrium*, *Dinophysis*, and *Karenia*) from French coasts. *Protist,* 153: 223-238.

Gunasekera, R.M., Patil, J.G., Mcennulty, F.R. and Bax, N.J. (2005). Specific amplification of mt-COI gene of the invasive gastropod *Maoricolpus roseus* in planktonic samples reveals a free-living larval life-history stage. *Marine and Freshwater Research,* 56: 901-912.

Hackett, J.D., Scheetz, T.E., Yoon, H.S., Soares, M.B., Bonaldo, M.F., Casavant, T.L. and Bhattacharya, D. (2005). Insights into a dinoflagellate genome through expressed sequence tag analysis. *BMC Genomics,* 6.

Harrington, C.A., Rosenow, C. and Retief, J. (2000). Monitoring gene expression using DNA microarrays. *Current Opinion in Microbiology,* 3: 285-291.

Harvey, J.B.J., Hoy, M.S. and Rodriguez, R.J. (2009). Molecular detection of native and invasive marine invertebrate larvae present in ballast and open water environmental samples collected in Puget Sound. *Journal of Experimental Marine Biology and Ecology,* 369: 93-99.

Hauser, N.C., Scheideler, M., Matysiak, S., Vingron, M. and Hoheisel, J.D. (1999). DNA arrays for transcriptional profiling. *Methods in Microbiology, Vol 28.*

Hayes, K. Sliwa, C., Migus, S., McEnnulty, F., Dunstan, P. (2005), National priority pests: Part II Ranking of Australian marine pests, Independent report for Department of Environment and Heritage, Australian Government Department of Environment and Heritage, Canberra, Australia 94 pp.

Haywood, A.J., Scholin, C.A., Marin Iii, R., Steidinger, K.A., Heil, C. and Ray, J. (2007). Molecular detection of the brevetoxin-producing dinoflagellate *Karenia brevis* and closely related species using rRNA-targeted probes and a semiautomated sandwich hybridisation assay. *Journal of Phycology,* 43: 1271-1286.

Heid, C.A., Stevens, J., Livak, K. and Williams, P.M. (1996). Real Time quantitative PCR. *Genome Research,* 6: 986-994.

Heimann, K., Sparrow, L. and Blair, D. (2009) Interim Report on laboratory culture of marine microalgae of the Great Barrier Reef toxin dinaflagellate cultures established at the North Queensland Aglal Identification/Culturing Facility (NQAIF). March Interim Report (Part 1) to the Marine and Tropical Sciences Research Facility. Reef and Rainforest Research Centre Limited, Cairns (8pp.).

Hosoi, M., Hosoii-Tanabe, S., Sawada, H., Ueno, M., Toyohara, H. and Hayashi, I. (2004). Sequence and polymerase chain reaction restriction fragment length polymorphism analysis of the large subunit rRNA gene of bivalve: Simple and widely applicable technique for multiple species identification of bivalve larva. *Fisheries Science,* 70: 629-637.

Hosoi-Tanabe, S. and Sako, Y. (2005). Rapid detection of natural cells of *Alexandrium tamarense* and *A. catenella* (Dinophyceae) by fluorescence in situ hybridisation. *Harmful Algae,* 4: 319-328.

Hosoi-Tanabe, S. and Sako, Y. (2006). Development and application of fluorescence *in situ* hybridisation (FISH) method for simple and rapid identification of the toxic dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella* in cultured and natural seawater. *Fisheries Science,* 72: 77-82.

Hudson, M.E. (2007). Sequencing breakthroughs for genomic ecology and evolutionary biology. *Molecular Ecology Resources,* 8: 3-17.

Jones, W.J., Preston, C.M., Marin Iii, R., Scholin, C.A. and Vrijenhoek, R.C. (2008). A robotic molecular method for *in situ* detection of marine invertebrate larvae. *Molecular Ecology Resources,* 8: 540-550.

Kamikawa, R., Hosoi-Tanabe, S., Nagai, S., Itakura, S. and Sako Y (2005). Development of a quantification assay for the cysts of the toxic dinoflagellate *Alexandrium tamarense* using real-time polymerase chain reaction. *Fisheries Science* 71:987-991

Kamikawa, R., Asai, J., Miyahara, T., Murata, K., Oyama, K., Yoshimatsu, S., Yoshida, T. and Sako, Y. (2006). Application of a Real-time PCR Assay to a Comprehensive Method of Monitoring Harmful Algae. *Microbes and Environments,* 21: 163-173.

Kamikawa, R., Nagai, S., Hosoi-Tanabe, S., Itakura, S., Yamaguchi, M., Uchida, Y., Baba, T. and Sako Y (2007). Application of real-time PCR assay for detection and quantification of *Alexandrium tamarense* and *Alexandrium catenella* cysts from marine sediments. *Harmful Algae* 6:413-420.

Ki, J.S. and Han, M.S. (2006). A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridisation of consensus PCR products of LSU rDNA D2 domain. *Biosensors and Bioelectronics,* 21: 1812-1821.

Lam, D.W. and Zechman, F.W. (2006). Phylogenetic analyses of the Bryopsidales (Ulvophyceae, Chlorophyta) based on rubisco large subunit gene sequences. *Journal of Phycology,* 42: 669-678.

Le Goff-Vitry, M.C., Chipman, A.D. and Comtet, T. (2007). In situ hybridisation on whole larvae: a novel method for monitoring bivalve larvae. *Marine Ecology-Progress Series,* 343: 161-172.

Lee, L.G., Connell, C.R. and Bloch, W. (1993). Allelic discrimination by nick-translation PCR with fluorgenic probes. *Nucl Acids Res,* 21: 3761-3766.

Leliaert, F., De Clerck, O., Verbruggen, H., Boedeker, C. and Coppejans, E. (2007). Molecular phylogeny of the Siphonocladales (Chlorophyta: Cladophorophyceae). *Molecular Phylogenetics and Evolution,* 44: 1237-1256.

Li, G., Hubert, S., Bucklin, K., Ribes, V. and Hedgecock, D. (2003). Characterization of 79 microsatellite DNA markers in the Pacific oyster *Crassostrea gigas*. *Molecular Ecology Notes,* 3: 228-232.

Lilly, E.L., Halanych, K.M. and Anderson, D.M. (2005) Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group. *Harmful Algae* 4:1004-1020.

Lin, S.J., Zhang, H., Hou, Y., Miranda, L. and Bhattacharya, D. (2006). Development of a dinoflagellate-oriented PCR primer set leads to detection of picoplanktonic dinoflagellates from long island sound. *Applied and Environmental Microbiology,* 72: 5626-5630.

Lin, S., Zhang, H., Hou, Y., Zhuang, Y. and Miranda, L. (2009). High-level diversity of dinoflagellates in the natural environment, revealed by assessment of mitochondrial cox1 and cob genes for dinoflagellate DNA barcoding. *Applied and Environmental Microbiology,* 75: 1279-1290.

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.-H. and Wagner, M. (2002). Oligonucleotide Microarray for 16S rRNA Gene-Based Detection of All Recognized Lineages of Sulfate-Reducing Prokaryotes in the Environment. *Applied and Environmental Microbiology,* 68: 5064-5081.

Loy, A., Schulz, C., Lucker, S., Schopfer-Wendels, A., Stoecker, K., Baranyi, C., Lehner, A. and Wagner, M. (2005). 16S rRNA Gene-Based Oligonucleotide Microarray for Environmental Monitoring of the Betaproteobacterial Order "Rhodocyclales". *Appl Environ Microbiol,* 71: 1373-1386.

Mackie, J., Keough, M. and Christidis, L. (2006). Invasion patterns inferred from cytochrome oxidase I sequences in three bryozoans, *Bugula neritina*, *Watersipora subtorquata*, and *Watersipora arcuata*. *Marine Biology,* 149: 285-295.

Metfies, K., Berzano, M., Mayer, C., Roosken, P., Gualerzi, C., Medlin, L. and Muyzer, G. (2007). An optimized protocol for the identification of diatoms, flagellated algae and pathogenic protozoa with phylochips. *Molecular Ecology Notes,* 7: 925-936.

Metfies, K., Borsutzki, P., Gescher, C., Medlin, L.K. and Frickenhaus, S. (2008). PHYLOCHIPANALYZER- a program for analysing hierarchical probe sets. *Molecular Ecology Resources,* 8: 99-102.

Mikulski, C.M., Park, Y.T., Jones, K.L., Lee, C.K., Lim, W.A., Lee, Y., Scholin, C.A. and Doucette, G.J. (2008). Development and field application of rRNA-targeted probes for the detection of *Cochlodinium polykrikoides* Margalef in Korean coastal waters using whole cell and sandwich hybridisation formats. *Harmful Algae,* 7: 347-359.

Miller, P. and Scholin, E.C.A. (1996). Identification of cultured and *Pseudo-nitzchia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes. *Journal of Phycology,* 32: 646-655.

Miller, P. and Scholin, C. (1998). Identification and enumeration of cultured and wild *Pseudo-nitzchia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridisation. *Journal of Phycology,* 34: 371-382.

Miller, P.E. and Scholin, C.A. (2000). On detection of *Pseudo-nitzschia* (Bacillariophyceae) species using whole cell hybridisation: sample fixation and stability. *Journal of Phycology,* 36: 238-250.

Moorthi, S.D., Countway, P.D., Stauffer, B.A. and Caron, D.A. (2006). Use of quantitative real-time PCR to investigate the dynamics of the red tide dinoflagellate *Lingulodinium polyedrum*. *Microbial Ecology,* 52: 136-150.

Mukai, T., Naruse, K., Sato, T., Shima, A. and Morisawa, M. (1997). Multiregional introgressions inferred from the mitochondrial DNA phylogeny of a hybridizing species complex of gobiid fishes, genus *Tridentiger*. *Molecular Biology and Evolution,* 14: 1258-1265.

Muraoka, D.A. and Saitoh, K.E. (2005). Identification of *Undaria pinnatifida* and *Undaria undarioides* Laminariales, Phaeophyceae using mitochondrial 23S ribosomal DNA sequences. *Fisheries Science,* 71: 1365-1369.

Nagai, S., Nishitani, G., Yamaguchi, S., Yasuda, N., Lian, C.L., Itakura, S. and Yamaguchi, M. (2007). Development of microsatellite markers in the noxious red tide-causing dinoflagellate *Heterocapsa circularisquama* (Dinophyceae). *Molecular Ecology Notes*.

Nash, E.A., Nisbet, R.E.R., Barbrook, A.C. and Howe, C.J. (2008). Dinoflagellates: a mitochondrial genome all at sea. *Trends in Genetics,* 24: 328-335.

Patil, J.G., Gunasekera, R.M., Deagle, B.E., Bax, N.J. and Blackburn, S.I. (2005a). Development and evaluation of a PCR based assay for detection of the toxic dinoflagellate, *Gymnodinium catenatum* (Graham) in ballast water and environmental samples. *Biological Invasions,* 7: 983-994.

Patil, J.G., Gunasekera, R.M., Deagle, B.E. and Bax, N.J. (2005b). Specific detection of Pacific oyster (*Crassostrea gigas*) larvae in plankton samples using nested polymerase chain reaction. *Marine Biotechnology,* 7: 11-20.

Pie, M.R., Ribeiro, R.O., Boeger, W.A., Ostrensky, A., Falleiros, R.M. and Angelo, L. (2006). A simple PCR-RFLP method for the discrimination of native and introduced oyster species (*Crassostrea brasiliana, C. rhizophorae* and *C. gigas*; Bivalvia: Ostreidae) cultured in Southern Brazil. *Aquaculture Research,* 37: 1598-1600.

Provan, J., Booth, D., Todd, N.P., Beatty, G.E. and Maggs, C.A. (2008). Tracking biological invasions in space and time: elucidating the invasive history of the green alga *Codium fragile* using old DNA. *Diversity and Distributions,* 14: 343-354.

Rautio, J., Barken, K., Lahdenpera, J., Breitenstein, A., Molin, S. and Neubauer, P. (2003). Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates. *Microbial Cell Factories,* 2: 4.

Richlen, M.L., Morton, S.L., Barber, P.H. and Lobel, P.S. (2008). Phylogeography, morphological variation and taxonomy of the toxic dinoflagellate *Gambierdiscus toxicus* (Dinophyceae). *Harmful Algae,* 7: 614-629.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487-494.

Sako, Y., Hosoi-Tanabe, S. and Uchida, A. (2004). Fluorescence in situ hybridisation using rRNA-targeted probes for simple and rapid identification of the toxic dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella*. *Journal of Phycology,* 40: 598-605.

Sawada, H., Saito, H., Hosoi, M. and Toyohara, H. (2008). Evaluation of PCR methods for fixed bivalve larvae. *Journal of the Marine Biological Association of the United Kingdom,* 88: 1441-1449.

Schable, N.A., Kuenzi, A.M., Drake, C.A., Folino-Rorem, N.C. and Darling, J.A. (2008). Microsatellite loci for the invasive colonial hydrozoan *Cordylophora caspia*. *Molecular Ecology Resources,* 8: 968-970.

Scholin, C.A. and Anderson, D.M. (1994). Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae) I. RFLP analysis of SSU rRNA genes I. *Journal of Phycology* 30:744-754

Scholin, C.A., Herzog, M., Sogin, M. and Anderson, D.M. (1994). Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. sequence analysis of a fragment of the LSU rRNA gene. *Journal of Phycology* 30:999-1011.

Scholin, C., Miller, P., Buck, K., Chavez, F., Harris, P., Haydock, P., Howard, J. and Cangelosi, G. (1997). Detection and Quantification of *Pseudonitzschia australis* in Cultured and Natural Populations Using LSU rRNA-Targeted Probes. *Limnology and Oceanography,* 42: 1265-1272.

Scholin, C.A., Marin Iii, R. and Miller, P.E. (1999). DNA probes and a receptor-binding assay for detection of *Pseudo-nitzchia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. *Journal of Phycology,* 35: 1356-1367.

Scorzetti, G., Brand, L.E., Hitchcock, G.L., Rein, K.S., Sinigalliano, C.D. and Fell, J.W. (2009). Multiple simultaneous detection of Harmful Algal Blooms (HABs) through a high throughput bead array technology, with potential use in phytoplankton community analysis. *Harmful Algae,* 8: 196-211.

Sherlock, G. (2000). Analysis of large-scale gene expression data. *Current Opinion in Immunology,* 12: 201-205.

Stafford, H., Willan, R.C., Neil, K.M., 2007. The invasive Asian Green Mussel, *Perna viridis* (Linnaeus, 1758) (Bivalvia: Mytilidae), breeds in Trinity Inlet, tropical northern Australia. *Molluscan Research* 27, 105-109.

Ten Hove, R.J., Verweij, J.J., Vereecken, K., Polman, K., Dieye, L. and Van Lieshout, L. (2008). Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102: 179-185.

Tepolt, C.K., Bagley, M.J., Geller, J.B. and Blum, M.J. (2006). Characterization of microsatellite loci in the European green crab (*Carcinus maenas*). *Molecular Ecology Notes,* 6: 343-345.

Touzet, N., Keady, E., Raine, R. and Maher, M. (2009). Evaluation of taxa-specific real-time PCR, whole-cell FISH and morphotaxonomy analyses for the detection and quantification of the toxic microalgae *Alexandrium minutum* (Dinophyceae), Global Clade ribotype. *Fems Microbiology Ecology,* 67: 329-341.

Touzet, N. and Raine, R. (2007). Discrimination of *Alexandrium andersoni* and *A. minutum* (Dinophyceae) using LSU rRNA-targeted oligonucleotide probes and fluorescent whole-cell hybridisation. *Phycologia,* 46: 168-177.

Tyrrell JV, Bergquist PR, Saul DJ, MacKenzie L, Bergquist PL (1997) Oligonucleotide probe technology as applied to the study of harmful algal blooms. New Zealand Journal of Marine and Freshwater Research 31:551-560

Tyrrell, J.V., Connell, L.B. and Scholin, C.A. (2002). Monitoring for *Heterosigma akashiwo* using a sandwich hybridisation assay. *Harmful Algae,* 1: 205-214.

Uwai, S.Y., Nelson, W., Neill, K., Wang, W.D., Aguilar-Rosas, L.E., Boo, S.M., Kitayama, T. and Kawa, H. (2006). Genetic diversity in *Undaria pinnatifida* (Laminariales, Phaeophyceae) deduced from mitochondria genes origins and succession of introduced populations. *Phycologia,* 45: 687-695.

Wang, Y., Ren, R. and Yu, Z. (2008). Bioinformatic mining of EST-SSR loci in the Pacific oyster, *Crassostrea gigas*. *Animal Genetics,* 39: 287-289.

Ward, R.D., Holmes, B.H. and O'Hara, T.D. (2008). DNA barcoding discriminates echinoderm species. *Molecular Ecology Resources,* 8: 1202-1211.

Williams, S.T. (2007). Safe and legal shipment of tissue samples: does it affect DNA quality? *Journal of Molluscan Studies,* 73: 416-418.

Xiang, C.C. and Chen, Y.D. (2000). cDNA microarray technology and its applications. *Biotechnology Advances,* 18: 35-46.