

Techniques for Detection and Enumeration of Marine Biosecurity Risk Species and Compliance Testing

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EXECUTIVE SUMMARY

Identifying, detecting and enumerating species are integral components of border control, incursion response and pest management, but are typically inefficient in marine environments because of the constraints imposed by underwater sampling. New sampling tools for aquatic pest surveillance and monitoring are needed, as well as tools to detect, enumerate and assess the viability of marine species in different size categories as defined in the IMO Ballast Water Convention and New Zealand's Import Health Standard for ballast water.

Intermediate Outcome 4 (IO4) in the NIWA/Cawthron Outcome Based Investment programme, Effective Management of Marine Biodiversity & Biosecurity, (EMMBB), has been designed to provide the science for, and development of such tools. Recognising the need for a coordinated and innovative approach to the issue, a workshop hosted by Biosecurity NZ and attended by NIWA and Cawthron scientists, together with their national and international collaborators, was held as a first step in the development process.

The primary aims of the workshop were to identify the priority species/taxonomic groups for which rapid identification tools are available and/or needed, and the technologies needed for the detection and identification of species (in the pathway and at the border). Several recently-developed molecular techniques have potential for use in marine surveillance and monitoring, so much of the workshop comprised a review of molecular techniques and their applicability to New Zealand's needs. The amenability of techniques for translation to multi-species format for detection, enumeration and determining viability of organisms was also assessed as part of the review process. The workshop provided background information for formulation of a 10 year research plan for use by the EMMBB science providers to develop the most effective technology for detection and identification of new incursions.

These proceedings represent a summary of the workshop. They contain extended abstracts from the keynote speakers, and minutes from the meeting.

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1. INTRODUCTION

1.1. Workshop Purpose

Identifying, detecting and enumerating species are integral components of border control, incursion response and pest management, but effective and efficient tools are needed for application in the marine environments. Intermediate Outcome 4 (IO4) in the NIWA/Cawthron Outcome Based Investment programme, Effective Management of Marine Biodiversity & Biosecurity, EMMBB, has been designed to provide the science for, and development of such tools. Recognising the need for a coordinated and innovative approach to the issue, a workshop hosted by Biosecurity NZ and attended by NIWA and Cawthron scientists together with their national and international collaborators was held as a first step in the development process. The purpose of this workshop was to identify the priority marine species/taxonomic groups for which rapid identification tools are needed, and the technologies needed for the detection and identification of new incursions (in the pathway and at the border). In particular, the workshop sought to identify the most promising methods for the detection of marine pests and identify future avenues of research that are most likely to lead to the development of a detection platform that can be applied to detect high priority marine pests. The focus is on pests in ships' ballast water, to meet regulatory requirements under the IMO convention, and on tools for surveillance and monitoring.

The new IMO Convention for the management of ships' ballast water will require methods to enumerate and assess viability of organisms in different size categories so potential techniques for this purpose were also evaluated. The aim of the workshop was to:

- (i) appraise various molecular techniques which are (or likely to become) available for detection, enumeration, and assessment of viability of species and for organisms in different size categories as defined in the IMO Convention and by country and state specific legislation;**
- (ii) assess amenability of techniques for translation to multi-species format in regard to detection, enumeration and viability assessments;**
- (iii) provide background information to formulate a 10 year research plan that can be adopted by the science providers in EMMBB to deliver the most effective marine surveillance and monitoring technology for detection and identification of new incursions.**

For many years researchers have sought new means for discriminating among very closely related but genetically distinct species and novel techniques to increase the rate at which specific organisms are quantified, particularly in the context of field surveys. The biotechnology revolution has pointed to the use of molecular probes as being one way to accomplish these goals. As used here, the phrase "molecular probes" refers to a suite of

biological molecules that encompass lectins, antibodies and DNA. Each class of probe shares the ability to selectively adhere to molecules specifically associated with a particular species or group of species, thus serving as a basis for detecting specific organisms even when they occur in complex natural communities (*e.g.* Scholin & Anderson 1998).

Some of the first organisms for which molecular probes have been developed are the toxic phytoplankton. The use of molecular probes to aid the identification of harmful algal bloom (HAB) species was reviewed as early as 1995 by Anderson. Since that time, molecular methods using rRNA probes in both whole cell (*e.g.* fluorescence *in situ* hybridisation [FISH]) and sandwich hybridisation (SHA) formats (Scholin *et al.* 1996, 1997; Rhodes *et al.* 2001; Tyrell *et al.* 2002) have targeted organisms that span several classes of algae, diatoms, dinoflagellates and raphidophytes. The species studied are found in many coastal regions of the world where they pose substantial public health concerns and can have serious economic impacts (Hallegraeff 2003). Blooms of these organisms can also have deleterious impacts on wildlife (*e.g.* Scholin *et al.* 2000). Presently the Monterey Bay Aquarium Research Institute (MBARI) is focusing on the development of DNA probe arrays to simultaneously detect multiple targets in a single sample. The institute has demonstrated that species ranging from marine bacteria to phytoplankton and invertebrate larvae can be detected using tailored arrays and in some cases enumerated in near real-time using a common sample collection, preparation and processing protocol, known as the environmental sample processor (ESP) (Scholin *et al.* 2001; Goffredi *et al.* 2005).

Because a target species approach is central to regulations for biosecurity management in both New Zealand and Australia, the need to develop tools to assist in border inspections and surveillance (*e.g.* to establish that ships are compliant with regulations) has been seen by the respective regulatory agencies as being of high priority. Molecular methodologies have been successfully applied to monitor pest organisms in coastal situations but recognition that such methodologies may have potential in ballast water compliance monitoring has been relatively recent (Tyrrell *et al.* 1997; Evans *et al.* 1998; McGowen 1998). Researchers at CSIRO have published a PCR-based method for the detection of *Asterias* larvae in ships' ballast water (Deagle *et al.* 2003), and a whole cell hybridisation method for the detection of *Asterias amurensis* larvae has been described (Mountfort [In Press]). Some initiatives have also been taken to evaluate q-PCR for the detection of high priority pest species (reviewed in Bax *et al.* 2006) and application of the sandwich-hybridisation methods for detection of invertebrates (Goffredi *et al.* 2005) provides another potential technology for pest detection in ships' ballast.

These recent initiatives by various workers in the detection of marine pest species highlighted the need for a workshop to ensure that the most benefit for cost can be realised in developing a technique (or techniques that are likely to be of most value in compliance and regulatory authorities). Additionally, because relatively few groups are involved globally in this fast growing area, the need for a coordinated approach in evaluating methods (*e.g.* interlaboratory comparisons, validations with same sample *etc*) was recognised, so that advantages and

disadvantages of various candidate methods could be determined and appraised for their suitability as a compliance tools.

2. EXTENDED ABSTRACTS (MOLECULAR TECHNOLOGIES PROS AND CONS)

2.1. Fluorescence *in situ* hybridisation (FISH) assay (Doug Mountfort, Cawthron)

Whole cell assays, based on oligonucleotide probes targeted at nucleic acids, have been in use for the rapid and unequivocal identification of harmful algal bloom (HAB) species for well over a decade (Scholin *et al.* 2003; Miller & Scholin 1996). The assay is based on earlier versions aimed at the identification of prokaryotes and used extensively by microbiologists (for example, Amman 1995).

The key step in the technique involves hybridisation of a fluorescein tagged probe with ribosomal RNA of the target species followed by washing steps to remove unbound probe using the filtration rig shown in Figure 1. Ribosomal RNA is chosen as the target for FISH probes because of the large number of copies, and therefore large signal per cell. The LSU or 28S rRNA is usually targeted, but if there is enough genetic variability the SSU or 18S can be selected.



Figure 1. Filtration rig (left); epifluorescent microscope (middle) and view of positive assay using fluorescein-tagged oligonucleotide species-specific probe targeted at *Karenina mikimoto* (right).

The advantages of the FISH assay are its speed and ease of use. The assay takes approximately three hours and the first steps require minimal equipment (vacuum filtration system, custom-built manifold, water bath). Samples can be fixed and shipped or stored for several days before processing. Dried samples (on filters) can also be shipped as long as they are processed without delay (Scholin *et al.* 2003).

A disadvantage is the cost of an epifluorescent microscope, which is critical for the interpretation of the assay results, and therefore the successful application of the FISH assay. The objectives, filter set and light source have been shown to have an enormous bearing on the success of the assay (Scholin *et al.* 2003). The fluorescent moiety selected for tagging on to the oligonucleotide probe will depend on the target species. For example, fluorescein is usually favoured over Texas Red for HABs due to the red autofluorescence of chlorophyll.

Fixation of the target cell is critical and will need to be optimised for any particular organism. For example, alcohols are generally used as fixatives for HABs to ensure cell permeability and therefore access to the probe binding sites, but in some cases aldehydes may prove more suitable (Tyrrell *et al.* 1997).

More recently a FISH assay has been developed for the detection of larvae of the invertebrate *Asterias amurensis* (the northern Pacific seastar), with the probe targeted at ribosomal RNA (Mountfort *et al.* In Press).

The FISH assay (Figure 1) is requested routinely in New Zealand by both the Ministry of Health and Marlborough Shellfish Quality Programme to refine risk assessments based on traditional counts for the amnesic shellfish poisoning group of HAB (Cawthron Micro-Algae Laboratory). The assay has been IANZ accredited against the ISO 17025 International Standards.

FISH may be an attractive option for use by port authorities ensuring that ships meet compliance standards because unlike many other molecular methods the assay is simple and easy to operate, is easily transportable (does not involve heavy equipment) and provides simultaneous visualisation and quantification at species level.

Table 1. Advantages and disadvantages of FISH

Disadvantages	Advantages
Does not provide viability assessment	Speed and ease of use
Requires the use of a fluorescence microscope which is expensive (<i>e.g.</i> \$60K NZ) (wavelength proved critical in interlaboratory trials)	Samples can be fixed and shipped or stored for several days before processing
Not readily amenable for translation to plate format	Has capability for detection and enumeration
Limited scope for multiplexing	N.B. clearing agents required for some fixatives

2.2. Sandwich hybridisation assay for the detection of marine pests (Lesley Rhodes, Cawthron)

The sandwich hybridisation assay (SHA) was originally developed for medical and dental clinical diagnostics, and is now available for a wide range of uses, including detection of nucleic acids from viruses and yeasts, tracking gene mutations, cell typing, monitoring of bioprocesses (mRNA) and even tracking bioremediation of contaminated sites. The assay was developed for harmful algal bloom species (HABs) by Chris Scholin, Monterey Bay Aquarium Research Institute (MBARI), California, United States of America, and has been available in New Zealand for a decade. The main purpose has been to provide a risk warning of HABs and their potential toxins for the aquaculture industries and public health. Recently the technology has been implemented for the detection of invertebrate larvae (Goffredi et al. 2005).

The oligonucleotide probes which are the basis of the assay include a species specific capture probe targeted at LSU rRNA and one or more signal probes (which attach to the captured rRNA, forming a “sandwich”) (Figure 2). The usual chemistry option is colorimetric, which uses streptavidin/HRP (1:1 covalent conjugate) with HRP substrate solution (4-methoxy-1 naphthol). However fluorescent and chemiluminescent options are also available. Once plates are loaded with the appropriate solutions and probes, the sample can be loaded and the assay run on an automated processor (Figure 3).

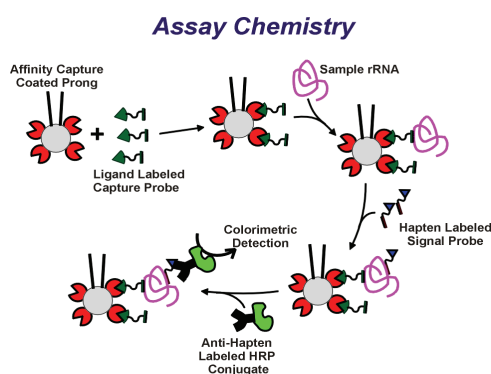


Figure 2. Sandwich hybridisation assay chemistry

Figure 3. Automated processor (Saigene) for sandwich hybridisation assay

The current main issues to address in terms of applicability of the SHA to biosecurity needs are viability, detection and enumeration of organisms and compliance with proposed IMO standards. Regarding viability, it is likely that any positive detects are from live cells/organisms, because rRNA has a short life once cells/organisms are lysed. This does mean that fresh samples are critical or assays need to be carried out on site. We are investigating “RNALater” as a suitable fixative but other fixatives are unsuitable (*e.g.* Lugols iodine, ethanol, methanol, formalin).

In the case of detection of biosecurity risk organisms, the main classes addressed so far are the Dinophyceae, Bacillariophyceae and Crustacea. The volume sampled will depend on the level of detection of the target organism; the greater the volume of seawater sampled, the greater the chances of detection but this will be limited by the amount of detritus and other non-target organisms in the sample. For the diatom *Pseudo-nitzschia australis* the Limit of Detection (LoD) is $>1,250$ cells L^{-1} , and for the barnacle *Balanus glandula* and the dinoflagellate complex *Alexandrium catanella/tamarense*, the LoD is 5,000 larvae or cells L^{-1} . Enumeration is based on a standard curve derived from known numbers of the cells or organisms and the identification validated with FISH probes. The SHA has been accredited for commercial use for *Pseudo-nitzschia* (Ayers *et al.* 2005; ISO 17 025).

The SHA is ideal for on site analyses, *e.g.* portside, and on board ships and aquaculture vessels (Tyrrell *et al.* 2002). MBARI are currently developing an Environmental Sample Processor (ESP) for *in situ* monitoring, using the SHA chemistry. Results are transmitted in real-time via a radio modem for on-shore analysis. The ESP is primarily for the detection of harmful algal blooms (HABs), but is also aimed at a range of organisms from heterotrophic and photosynthetic bacteria to small invertebrates found in the upper ocean. The capture probes are arrayed on membranes (contact Chris Scholin, MBARI, for more information).

Compliance with proposed IMO ballast water discharge standards will require enumeration of prokaryotes and eukaryotes in pre-determined size ranges. A general prokaryote assay is available, but the assay will only be amenable for general eukaryotes if size fractionation is carried out before analysis, due to the variable size ranges of target organisms, *i.e.* dinoflagellates ranging from <10 μm - >100 μm would all be positively detected by a general dinoflagellate probe in a sample.

The sandwich hybridisation assay differs from whole cell labelling in that it requires the preparation of a cell homogenate through collection of the cells on filters followed by lysis with a chaotropic solution. The probes and the nucleic acids (for HAB species rRNAs) target are exposed to each other during the assay process.

Pre-packaged test plates may be purchased or the solutions bought and the plates prepared at the time of the assay. The plates are processed automatically in a relatively simple bench-top system (Scholin *et al.* 1997, 2003). The two hybridisation reactions that occur allow for even greater specificity than can be obtained with the FISH assay. Total sample processing time from live sample, concentrated on a filter, to assay completion is one hour.

SHA kits are available for prokaryotic as well as eukaryotic micro-organisms, and several HAB species are being tested and refined. Kits are available from ORCA (contact Jason Ray for costs of assay plates and/or reagents; email: orcaresearch@yahoo.com). Robotic sample processors can be purchased or leased from Saigene Corp (view <http://www.saigene.com/>).

In summary, the SHA is a rapid method of detection, identification and enumeration. Field trials of the *Pseudo-nitzschia australis* SHA correlated well with FISH and traditional counts

and the SHA offers an excellent risk warning of HAB development for the aquaculture industry. Some anomalies can be expected, due to the varying physiological states of organisms and due to the SHA being an homogenate-based detection system. The costs are comparatively modest and the assay has been proven over time and internationally validated.

Table 2. Advantages and disadvantages of sandwich hybridisation assay

Disadvantages	Advantages
Requires fresh samples (may use RNALater)	Speed and ease of use
Requires relatively large (500 ml) samples if low cell/organism numbers	Can format 96-well plates to suit needs (<i>i.e.</i> 24 wells available to determine probe options suited to “on site” deployment)
Pre-prepared plates and prongs add to cost; can load “in house”	Equipment (<i>e.g.</i> processor, optical density reader) moderately priced; plates can be purchased pre-loaded
Limited scope for multiplexing	Has capability for detection and enumeration

Acknowledgements: Miguel de Salas, Gustaaf Hallegraef (Univ. Tasmania); Joe Jones, Chris Scholin (MBARI); Janet Adamson, Melissa Gladstone (Cawthron).

2.3. DNA Barcoding as a global universal tool for biosecurity (Shelley Ball, National Centre for Advanced Bio Protection Technologies, Lincoln University)

DNA barcoding on a large diversity of taxa has been recently developed as a global tool for species identification. It uses a small portion of the mitochondrial genome (~650 base pairs of the mitochondrial gene cytochrome c oxidase I (COI)) as a 'species-specific genetic identification tag'. Studies in a large diversity of taxa have shown that DNA barcodes are extremely effective for species identification, with success rates generally greater than 95%. This small portion of COI has several major advantages as a standardised region of the genome for species identification. Firstly, it is a protein coding gene and thus, extremely easy to manually align sequences due to the rarity of insertions and deletions. It is a ubiquitous gene and so a good target as a universal 'genetic identification tag'. There are universal primers for amplifying this portion of COI (Folmer primers: Folmer *et al.* 1994) that have been highly effective for metazoan animals. It is also relatively rapidly evolving and so possesses a good "taxonomic signal" for distinguishing species. For some taxa, it is also effective at distinguishing populations. In general, interspecific COI divergences are an order of magnitude greater than intraspecific divergences, meaning that species are easily distinguished. Limitations of COI-based identifications include distinguishing hybrids (nuclear genes will be needed for this) and sister species (which show little COI divergence).

DNA barcoding offers many advantages over previously used molecular diagnostic tools such as PCR-restriction length polymorphisms (RFLPs), random application of polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs) and species-specific primers (Table 3). Using a single (or few) gene region(s), as proposed by DNA barcoding, means that the identification process is standardised. Rather than individual diagnostic laboratories independently developing their own, locally applicable diagnostic tools, the standardisation of barcoding means that diagnostic tools can be developed for taxa on a global scale. Such standardisation and universality means that efforts of individual diagnostic laboratories can be combined, leading to collaborative efforts which will result in the development of diagnostic tools for far more species than a single laboratory is capable of (*e.g.* new CBOL initiative to barcode the Fruitflies of the World). This makes barcoding operationally efficient and cost effective, given the rapid decrease in DNA sequencing costs and increased accessibility of sequencing technology in recent years. DNA barcoding is also anticipatory in that given the potential for global data collection efforts focused on target taxa, new exotic species can be identified far faster than traditionally possible. In other words, when a new invader appears on New Zealand's shores for the first time, if a DNA barcode for that species exists, then we can capitalise on these existing data and identify the specimen rapidly. Such an approach can be combined with invasive species risk analysis to ensure that DNA barcodes are collected for the most likely new invaders expected to reach New Zealand. This is a far more proactive approach compared to the traditional post hoc approach of designing diagnostic tools for species after they have invaded the country. Finally, DNA barcoding provides quantitative data, whereas approaches such as RFLPs do not. With RFLPs, for example, the restriction profile of an unknown will either match those of species in a reference data set or it will not.

In cases where there is no match, there is no way to identify the specimen. In contrast, DNA barcoding analysis tools such as neighbour-joining, results in two important pieces of quantitative data: i) % sequence divergence from the closest match; ii) bootstrap support for the placement of that sequence with its closest match. These data provide information to assess the accuracy of the identification. If an exact species match is not available, then DNA barcodes can often provide information at the next highest taxonomic level (*e.g.* identify the specimen to genus). Given the affordability, ease, and accessibility of DNA sequencing today, development of DNA barcoding as a regularly used species identification tool is feasible. Furthermore, establishment of the global Consortium for the Barcoding of Life has enabled rapid accumulation of DNA barcodes for a large number of taxa around the world. It has also facilitated collaborative efforts to collect barcodes for focal taxa (*e.g.* marine fishes of the world). The Barcoding of Life Database (BOLD; University of Guelph, Canada) and the barcode section of Genbank provides repositories for vouchered barcodes that are publicly accessible on-line. The International Network for Barcoding Invasive and Pest Species (INBIPS) is in place to support invasive and pest species barcoding around the world. Given these global initiatives and resources, DNA barcoding can provide an accessible and important tool for identification of exotic species in biosecurity. Furthermore, DNA barcoding has stimulated interest in developing new technologies for making DNA sequencing portable and feasible in the field as well as being capable of detecting multiple species in environmental samples.

Table 3. Advantages and disadvantages of DNA barcoding.

Disadvantages	Advantages
Universal primers not readily applied to detecting multiple species in a mixed sample	Universal (globally)
COI gene not appropriate for algae	Globally co-ordinated data coverage standardised
Recently diverged species may show little or no sequence divergence	Operationally efficient
	Anticipatory
	Quantitative (% seq. divergence and bootstrap support)

2.4. DNA barcoding: marine species (Peter Smith, NIWA)

The proposal to create a global DNA-based identification system for all animal species, based on sequence diversity in the COI gene region, has progressed rapidly over the last few years. Barcoding is generally recognised as an achievable and practical tool for specimen and product identification, and there are several Barcode of Life (BOL) global campaigns on fishes, birds, fruitflies, mosquitoes, and pest species, with regional campaigns emerging for sponges, bivalves, and squids. The barcoding concept has been fiercely debated in the scientific literature, with many of the arguments centred around the aims and potential for barcoding, in particular, using a portion of one gene to identify species from a wide taxonomic range. DNA barcoding aims to assemble a standardised DNA sequence reference library, based on voucher specimens with authoritative taxonomic identifications. The COI sequences will be freely available in electronic databases containing not only the barcodes, but also images of specimens, and geospatial coordinates of reference specimens. Once the Barcode of Life Database (BOLD) is established, any molecular laboratory will be able to sequence and identify suspect specimens, including parts of specimens, eggs and larvae.

The COI region was selected as the BOL marker because it is an easily recovered region of the genome that provides good taxonomic resolution. COI shows a high incidence of base substitutions at the third position nucleotides, allowing discrimination of closely related species, and the universal primers for this gene enable the recovery of COI sequences from most animal species. This simple “one method” for all animal species, coupled with the international reference database, are the key strengths of barcoding. The limitations to most PCR methods equally apply to DNA barcoding. The universal primers can be a disadvantage for mixed species samples, as they are non-selective, and species or genus specific primers need to be developed. Tissue samples can be processed in any laboratory set up for basic DNA extraction and amplification, and either sequenced on site, or DNA products mailed to a service provider, including overseas providers. Specimens are identified by matching their COI sequence against the BOLD public domain database. Because barcoding is based on the maternally inherited mtDNA, it cannot be used to detect hybrids. The COI gene is not appropriate for algae and alternative genes are being considered for barcoding plants.

Fishes are the most diverse group of vertebrates, with possibly as many as 30,000 species. As of July 2006, 2,177 (~7%) species of marine fishes had been barcoded, including 212 (~16%) NZ EEZ fishes. Preliminary results indicate low intra specific distances (<1%) and higher distances within genera (<9%). Occasional deep divergences found among specimens assigned to a single species may indicate the presence of cryptic species. Barcoding is being applied as a routine tool for specimen and fillet identification, replacing protein based methods.

COI data are available for most of the key potential marine invasives for New Zealand: the Mediterranean fanworm (*Sabella spallanzanii* and *S. crassicornis*); Northern Pacific seastar (*Asterias amurensis*); European shore crab (*Carcinus maenas*); Chinese mitten crab *Eriocheir sinensis*; and the swimming crabs *Charybdis hellerii* and *C. japonica*. Currently, there are no COI data available for the Asian clam *Potamocorbula amurensis*. Ironically there are few

barcodes available for native seastars and crustacea, although these sequences are being obtained and linked to vouchered specimens.

We have applied COI markers, in conjunction with morphological characters, to identify the invasive *C. japonica* in the Hauraki Gulf and more recently the invasive tunicate *Eudistoma elongatum* on oyster farms in Northland. The COI marker may be useful for population level studies in some species. In its native range the European shore crab *C. maenas* is found in the eastern Atlantic Ocean from Norway to Mauritania. A COI data set showed a major genetic break (11% sequence divergence) between Mediterranean and Atlantic samples, supporting their species-level status. Populations from the Faeroe Islands and Iceland were genetically distinct from populations around mainland Europe, and these showed further differentiation between populations in the central North Sea and those to the south (Roman & Palumbi 2004).

Molecular tools in general may be critical for addressing questions re the origins of some invasive species and the status of cryptogenic species. A recent invader in the Hauraki Gulf, the oyster blenny *Omobranchus anolius*, was identified with morphological and mtDNA sequences of the cytochrome b gene and the control region. The mtDNA base substitutions among populations of *O. anolius*, indicated that the New Zealand specimens were not derived from sites sampled in New South Wales, and that a more extensive sample collection throughout the range of *O. anolius* might allow identification of the source of the Hauraki Gulf population.

For some taxa, such as polychaetes, the soft tissues do not fix well, making traditional morphological comparisons with other species and holotypes difficult. The parchment tubeworm *Chaetopterus* has increased rapidly along the northeast coast of the North Island, since first reported by scallop fishers in 1997. The specific identity of this polychaete and its geographic origin are unknown. It might be a New Zealand endemic undergoing a population explosion or it might be a relatively recent invader.

The key strength of DNA barcoding is that it provides a simple universal method for identification of animal species, that can be applied to whole or parts of specimens. The universal one gene fits all approach has some limitations: recently diverged species may show little or no sequence divergence; and universal primers are not readily applied to detecting multiple species in a mixed sample. The rapid developments in molecular biology are leading to faster and automated low-cost sequencing, and when coupled with the growing international COI databases further strengthen the barcoding approach for specimen identification. In future barcoding might be undertaken in high throughput central processing laboratories or by field workers using low cost portable sequencers.

2.5. Microarrays: an emerging technology for biodetection (Neil Gemmell, Biological Sciences, University of Canterbury)

DNA microarray technology (Figure 4), with its ability to detect and measure thousands of distinct DNA sequences simultaneously, has been recognised as a potentially valuable tool for high throughput, quantitative and systematic studies for biodetection. Early applications of microarray technologies have included small-scale microarrays for overviews of the composition of microbial communities (El Fantroussi *et al.* 2003) and larger-scale microarrays that included both species and more inclusive taxonomic level probes (Wilson *et al.* 2002; DeSantis *et al.* 2005). Until recently there were few reports of successful quantification of individual species in the context of complex mixtures but recent work by Palmer and colleagues (Palmer *et al.* 2006) now show that it is possible, at least for microbial systems, to identify and quantitate 8,989 taxa simultaneously.



Figure 4. Microarrays: small membranes or glass slides upon which large numbers of genes, potentially from multiple species, are physically bound and can be detected using hybridisation technologies.

Microarray technology was once the realm of a few very well equipped specialist laboratories but with custom-made microarrays now readily available, this field is rapidly changing. Microarray approaches are likely to be an important area of future research in the area of marine biodetection and surveillance, particularly as international ballast water standards are imposed. Microarray technologies provide the opportunity to simultaneously search for multiple organisms, altering the pervading paradigm from **whether** a specific invasive species is present, to **which** invasive species are present. There will be challenges associated with the uptake of this technology, but the potential benefits are large (Table 4).

Table 4. Key Advantages and disadvantages of Microarray Technologies

Disadvantages	Advantages
Requires <i>a priori</i> knowledge of organism gene sequences so initial development time could be quite long	Contain a very large number of DNA sequences (up to 500K)
Analyses statistically complicated, but good software now available	Only require small amount of sample (~250 ng of DNA) so small organisms are readily accommodated
Independent validation of positives may still be required	Can potentially survey for presence of a large number of species (1000s) simultaneously, quickly (<24 hrs) and reproducibly
	Low individual \$ dollar cost (potentially a few cents per species)
	Adaptable and future focused

2.6. PCR based genetic probes for detection and management of marine pests (Jawahar Patil, CSIRO, Hobart, Tasmania)

Background and Summary

Many marine invasive species are transported through the world's oceans at sizes that render them invisible to the naked eye and at developmental stages, indistinguishable from their native counterparts even under the microscope. To circumvent these limitations, over the last 5 years CSIRO Marine and Atmospheric Research (CMAR) has developed and deployed genetic probes for detection of five key marine pests of concern to Australia. Initially a standard nested PCR approach was adopted to differentiate target species from mixed biological samples (biofouling and ballast water) with the results feeding back to refine the Ballast Water Decision Support System used by Victoria to manage domestic ballast water. More recently we have developed quantitative PCR detection assays for 4 of the species and are currently applying these tools to acquire more accurate data on encounter rates, variability and uncertainties associated with sampling techniques as well as to obtain more accurate data on natural histories (spawning season, spatial and temporal heterogeneity *etc*). When more gene probes become available it would be prudent to amalgamate them on to a single platform incorporating the advantages of both signal amplification (PCR) and amenability for high throughput screening (e.g. microarray) afforded by the modern molecular techniques. However, the major bottleneck at the current time appears to be the lack of species specific probes for each of the target pest species of concern, and for marine species in general where the probes are being used to investigate recruitment dynamics and dispersal in plankton. In the light of this we suggest there should be a focus on further development of these genetic technologies (including developing more probes) and recommend that a collaborative international approach be initiated, if scientists and managers are to reap the rewards of these genetic technologies. Globally such collaborations will help the maritime states (both flag and port) to comply, monitor, and test the specific efficacy of ballast water management as required by the IMO Globallast convention (2004). The probes will also have application to testing management measures to restrict the spread of marine invasives by the many other vectors and thus conserve the identity of bioregions.

The Problem

To evaluate management and mitigation of the threats posed by translocations of marine pests, one must be able to first accurately and rapidly identify them. Identifying the species obtained in a ballast water or other plankton samples can be complicated and time consuming, because many of the species of interest are present in their early (planktonic) life stages and are often morphologically indistinguishable from other species in the same genus, family or higher taxonomic grouping. Moreover, the widely employed morphological tool, namely the light microscopy is cumbersome, time consuming and not easily amenable for automation.

A Molecular Solution

All species have a unique genetic signature. Gene probes are capable of identifying the DNA of a single species, at low concentrations, in unsorted ballast water samples. Gene probes that we developed target a unique DNA sequence in the mitochondrial COI gene (animal

applications) or large subunit (LSU) ribosomal DNA (plant applications). We employed a nested polymerase chain reaction (PCR) technique first to amplify (enrich) all DNA of closely related species, and then to amplify the target DNA from this enriched sample. In our experience it appears necessary to adopt a nested PCR approach to enhance sensitivity, when dealing with environmental samples. Typically probes were tested against as many closely related species as could be obtained and the reaction conditions optimised for the maximum sensitivity providing 100 percent specificity. The list of pest species of concern to Australia, for which we have developed gene probes are summarised in Table 5.

Table 5. Summary of gene probes developed by CSIRO Marine and Atmospheric Research (2005).

Species	Targeted gene	Number of taxa compared	Specificity	Sensitivity number *	Stage in ballast samples	Reference
<i>Asterias sp.</i>	COI	13	Genus	<5	bipinnaria	Deagle <i>et al.</i> (2003)
<i>Crassostrea gigas</i> **	COI	15	Species	5	D-hinge larvae	Patil <i>et al.</i> (2005a)
<i>Gymnodinium catenatum</i> **	LSU	8	Species	5	cysts	Patil <i>et al.</i> (2005b)
<i>Undaria pinnatifida</i>	ITS region	33	Genus	To be tested	zoospores	Hayes <i>et al.</i> (2005)
<i>Maoricolpus roseus</i> **	COI	7	Species	1 Egg capsule (~45 larvae)	??	Gunasekera <i>et al.</i> (2005)
<i>Asterias amurensis</i> **	COI	13	Species	<1	bipinnaria	Bax <i>et al.</i> (2006)

* sensitivity depends on the background plankton biomass.

** Real Time PCR probe

Ballast water Application

Australia has adopted a risk assessment based Decision Support System (DSS) for management of ballast water discharge domestically. Predictions from Australia's ballast water DSS were never empirically tested before. In this context, the gene probes developed at CMAR were used to test for presence/absence of target taxa in ballast water sampled from 63 vessels (80 vessel/tank/date combinations) in the Port of Hastings domestic ballast water management trial. These results are presented in Table 6 below and subsequently these findings have been incorporated to fine tune the DSS. A detailed report of the results in Table 2 has been published (Patil *et al.* 2004).

Table 6. Results of the Port of Hastings domestic ballast water trial. Tanks tested for each species, were those reported by DSS to be at low risk of carrying the species.

Species	Number tanks tested	Number positive (%)	Possible reasons for Type II error*
<i>Asterias sp.</i>	69	9 (13%)	Carryover (7), Northern hemisphere Plankton duration underestimated (2)
<i>Crassostrea gigas</i>	58	25 (43%)	Inadequate port sampling (22), unknown (3)
<i>Gymnodinium catenatum</i>	77	31 (40%)	Carryover in sediment (23), Inadequate port sampling (8)

* Numbers in brackets refer to the number of tanks

Environmental Applications.

We have deployed the probes for several environmental applications. For example, it was unknown whether, *Maoricolpus roseus*, present in Australia since the 1920s and now covering an area of the continental shelf the size of Tasmania, had a planktonic larval stage and could be spread in ship's ballast water. The *Maoricolpus roseus* gene probe found that this species was present in archived plankton samples from the Derwent estuary, indicating that it should be included in Australia's domestic ballast water management system. Results also indicated it to be present outside of the accepted reproductive season (for detail see Gunasekere *et al.* 2005).

In another environmental application, Real-time PCR was used as a quantitative tool to test plankton samples off Tasmania's east coast and Victoria's southern coast for the abundance of *Asterias amurensis*. Larvae were detected, confirming predictions of the model used for Management Strategy Evaluation (Details can be found in Bax *et al.* 2006).

2.7. Expanding the detection and monitoring capabilities for marine pests using real time PCR (Paul Rasmussen, Australian Water Quality Centre, Adelaide)

One way in which marine biosecurity may improve its capability to detect and monitor for marine pests is to move from measuring what we can physically see (cells, larvae, whole organisms) to other characteristics of the pests we are able to measure. This can be achieved by using the DNA from the pests of interest to identify them in a highly specific way (a less tedious version of the DNA profiling done in forensic science). DNA-based detection technologies fall into two broad groups, those that involve only DNA hybridisation (FISH, Sandwich Hybridisation, Microarrays) and those that involve DNA amplification and hybridisation (PCR, qPCR, NASBA, SDSA). The key areas in which a DNA-based amplification technology such as qPCR might improve the detection of marine pests by augmenting current approaches are: increased specificity, sensitivity, precision and throughput.

Many of the adult and larval forms of marine pests on the current register are ideal targets for qPCR detection as they have multi-copy target genes often present in cells that have multiple copies of the chromosome on which these genes are found. Since a well-designed qPCR assay can detect less than ten target copies in a reaction and amplify these 6-7 logs, this adds up to exceptional sensitivity. The linear relationship observed in a qPCR standard curve across target concentrations of substantial difference means that the quantitation can cope well with starting populations of widely different densities. Because a qPCR reaction has a three point control on specificity (two primers and a probe) and can be tested widely on closely related species before optimisation, the prospects for developing highly-specific assays are excellent.

The common target for qPCR is DNA, a much more tractable molecule than RNA, but one which may not necessarily accurately reflect the viability of the target species detected. For border control-related testing this is not likely to be an issue because whether live or dead the identification of the potential vector for introduction is the key. For more routine monitoring such as ballast water testing or control and management of introduced pests this may be an issue. The issue can be handled with qPCR using assay variants such as EMA-qPCR, where the addition of EMA renders the DNA of compromised cells unamplifiable or by performing qRTPCR and targeting the RNA (the latter more difficult and likely to be much less precise in quantitation). Studies which have examined the half-life of DNA in seawater matrices also observe that free DNA (outside cells) does not persist very long (minutes/hours), suggesting that a qPCR estimate might only include a fraction of signal from non-viable cells. The converse is true of sediment samples where free DNA can persist for months or years.

The quality of information from qPCR (or any other DNA detection technology) will depend upon the sampling and preparation techniques. These issues are inseparable and deserve as much consideration as the choice of detection technology itself. A poor sampling regime or ineffective sample preparation will ensure a highly appropriate detection technology returns an inappropriate answer. Opting for a generic sampling and sample preparation technique would be the sensible option and given how difficult sampling may be (ballast water), making the

most out of each sample by having a tiered filtration protocol would again be sensible. The statisticians can argue about the right model to assume or the number of samples needed for confidence limits but the important feature of qPCR is the speed, throughput, multiplexing and cost; which ultimately should allow more samples to be taken and more targets analysed.

Some devices capable of qPCR are also portable and are becoming available at moderate cost. In the future, qPCR devices will be available with “dumbed-down” easy-to-use software at increasingly reduced size and cost. This provides additional flexibility but usually limits throughput, so whilst it may be ideal for decentralised border control, it may not be appropriate for high-throughput routine sampling. Collecting and returning samples to a centralised facility will always be the easiest option but should be considered against the cost/logistics of sample collection operations.

Comparing DNA-detection technologies available at the present time, qPCR has a number of attributes that suggest that it is well-equipped for the detection of marine pests.

In simple terms, qPCR is the ideal technology for asking a specific question such as “is this marine pest in the sample or not; and if it is, how much is there?” Technologies such as microarrays are better at asking questions such as “of this set of many marine pests, which might be present in the sample”; however, at this stage the sensitivity, precision and quantification are not comparable to qPCR. This stems from restrictions of the microarray platform, the increased complexity of information and the data processing requirements. In the future the lines between DNA-detection technologies that use hybridisation, amplification, or both, may blur as advances are made in miniaturisation and materials science; however, the trajectory of these developments really is uncertain.

Alternative technology platforms not considered are biosensors, either portable or in-line. Handheld biosensors might have some potential as either antibody, aptamer or oligonucleotide-mediated detection devices but often suffer from an inability to sample appropriate volumes for sensible detection limits to be achieved. In-line sensors (as in ballast tanks) do not at this stage detect marine pests specifically and are always going to suffer from costly high maintenance requirements.

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5. APPENDICES

5.1. Workshop Minutes

15 AUGUST 2006

PRESENT: Naomi Parker, Dan Kluza, Brendan Gould, Susy Keeling, Susie Wood, Andrew Bell, (Biosecurity NZ), Graeme Inglis, Wendy Nelson, Peter Smith, Roberta D'Archino, Barb Hayden (NIWA), Mike Taylor, Lesley Rhodes, Doug Mountfort (Cawthron Institute), Judy Bloom (University of Otago), Neil Gemmell, Sharyn Goldstein (University of Canterbury), Miguel de Salas (University of Tasmania), Jawahar Patil (CSIRO Hobart), Paul Rasmussen (Australian Water Quality Centre), Shelley Ball (Lincoln's terrestrial insect Bioprotection Centre).

Mike Taylor welcomed all and outlined the aim of the workshop; to develop a coordinated research strategy to guide development of molecular and biochemical technologies for rapid detection and enumeration of unwanted species in transport pathways and at ports of entry.

Naomi Parker (Senior Science Advisor) outlined Biosecurity NZ's needs for rapid detection tools and Graeme Inglis discussed the aims of the workshop in the context of the Marine Biodiversity & Biosecurity OBI, EMMBB, of which he is joint Science Leader. Invited speakers then outlined the strengths and weaknesses of various molecular techniques.

5.1.1. Summary of talks

Naomi Parker-BNZ Perspective

Naomi outlined the structure of BNZ, its governance and legislative responsibilities where molecular tools could be of value. These include:

- (i) Managing the Invasion Process
 - Border controls
 - Surveillance & Incursion Response
 - Pest Management
- (ii) Supporting Arrangements
 - Diagnostics & Taxonomy

Border control needs:

- (i) to test compliance with Regulation D-2 of the Ballast Water Convention,
- (ii) to provide rapid ID of taxa that arrive,
- (iii) Tools to provide rapid identification of aquarium trade taxa of concern

Surveillance and Incursion Response needs:

- (i) Rapid diagnostics for field surveillance
- (ii) ID of cryptic species for incursion response (*e.g.* differentiating from a native species)

Pest management needs:

- (i) to identify when a particular pest is present in the water column

General needs:

- (i) Species prioritisation is an ongoing BNZ concern – need to work together on this
- (ii) Fast, cheap and multi-species tools required – need to target methods that will deliver these
- (iii) Tools must be easy for quarantine officers, regional councils *etc.* to use
- (iv) Portable “labs” for incursion investigators and surveillance teams
- (v) Important to combine molecular tools with whole organism taxonomy
- (vi) “Downstream” use further along invasion pathway (beyond border), even though developed initially for border controls.

Graeme Inglis commented: Also need a test for compliance with Australian standards.

Graeme Inglis- Marine Biodiversity & Biosecurity OBI, EMMBB

Aims of the workshop:

- Summarise the strengths and weaknesses of techniques by purpose
- Identify species for which tools are available or needed
- Identify gaps/opportunities for research

EMMBB milestones:

In collaboration with key end-users and national and international collaborators, a plan has been formulated that identifies the priority species/taxonomic groups for which rapid identification tools are needed, and the technologies needed for the detection and identification of new incursions (in the pathway and at the border). The plan will draw on species risk profiling being undertaken by Biosecurity NZ, and will complement tool development contracted by it under Vote Biosecurity.

Identification tools:

Tools to identify priority unwanted species, genera or families and their source regions have been developed to allow stakeholders to rapidly distinguish them from native New Zealand analogues. These tools will range from simple morphological and taxonomic keys to a hierarchy of molecular identification tools.

Surveillance tools for high risk species

A portable format for molecular biodetection assay(s) that can be applied for the detection and quantification of particular high-priority marine pests has been developed.

Surveillance tools for the IMO ballast water standard

Molecular biodetection assays for the enumeration of eukaryotic marine organisms in the two size categories (*i.e.* 10–50 μm and $>50 \mu\text{m}$) specified in the IMO ballast water standard have

been evaluated for performance against less efficient conventional methods, and a rapid format for enumeration has been developed and trialled.

Doug Mountfort-*In situ* Fluorescence hybridisation assay (FISH)

Background

- (i) Based on early versions for identification of prokaryotes
- (ii) Chris Scholin took technique to use for eukaryotes with HAB probes
- (iii) Early versions also used to detect and identify bacteria.

Can have universal probes and species-specific probes.

FISH probes target Ribosomal RNA. Method is simple.

Technology already used for Ministry of Health and MSQP for counts on ASP group of HABs, *e.g.* can differentiate between toxic and non-toxic species of *Pseudonitzschia*.

Blooms of *Pseudonitzschia* can be as high >100,000 cells/ml.

Can discriminate between nine species of potentially toxic and non-toxic *Pseudonitzschia*.

Detection

Detection limit is 5,000 cells/L.

FISH can be used to detect all size categories specified in the IMO convention; bacteria (<10 µm); phytoplankton (10-50 µm), zooplankton (>50 µm), larvae (all species specific probes).

Could be used as a universal probe by filtering samples into these three size ranges.

Enumeration

- By direct counts of organisms by fluorescence microscopy, or
- Non-specific probes can be used to enumerate different size classes of organisms from filtering using different mesh sizes

But cannot be used to indicate viability (rRNA exists in cell after death); may need a viability marker, *e.g.* (CTC) used in tandem with the probe.

Compliance

Could be used by port authorities to ensure that ships discharging ballast water comply with New Zealand IHS.

- Quick and simple takes ~3 hours for results
- Relatively easily transportable.
- Simultaneous visualisation and enumeration at species level.

To detect a pest in ballast water, need to filter 10L through 5µm filter

Disadvantages

- Can't assess viability
- Requires expensive fluorescence microscope (*e.g.* NZ\$60K); wavelength proved critical in inter-lab trials
- Not amenable to plate format so won't be easy to automate
- Limited scope for multiplexing

Advantages

- Speed and ease of use; assay takes approximately three hours
- Samples can be fixed and shipped or stored for several days before processing
- Has capability for detection, enumeration
- N.B. clearing agents required for some fixatives

Paul Rasmussen commented (in response to question from floor): Flow cytometry not good for water as anything that is sticky clogs it.

Lesley Rhodes- Sandwich hybridisation assay (SHA)

For detection/enumeration of marine pests –

- Developed for medical and dental diagnostics
- Detects nucleic acids from viruses and yeasts, gene mutations, cell typing
- Used for monitoring of bioprocesses (mRNA); bioremediation of contaminated sites
- Developed for HABs with aim of dipstick probes
- Applicable for marine pests, *e.g.* invertebrate larvae

Targets:

- rDNA stable, low abundance, double stranded
- rRNA degrades, abundant, single strand
- Eukaryotes LSU or 18S; SSU or 28S; ITS1 or 2
- Where finer taxonomic resolution needed can explore other regions of DNA, *e.g.* mtCO1 gene

Small processing unit, simple in practice

Chemistry options:

- Colorimetric
- Fluorescence
- Chemiluminescence

Viability

- rRNA has short life once cells/organisms are lysed
- Fresh samples are critical or assay on site

- Liquid nitrogen is a storage option or RNALater but NOT Lugols iodine, ethanol, methanol, formalin *etc*
- RNase pretreatment of recently killed *Cryptosporidium* oocysts destroys residual rRNA (FISH assay); incorporate vanadyl ribonucleoside complex (VRC) before permeabilisation steps to neutralize RNase activity

Detection

Good for

- dinoflagellates
- diatoms
- raphidophytes – fish killers
- invertebrates – *Asterias*, *Balanus*

Cross reactivity testing is critical (DNA sequence data is a guide only); for micro-algae Cawthron uses the Cawthron Culture Collection of ≈ 100 species

Detection level

Detection increased by increasing sample size but limited by amount of detritus and number of other organisms that clog the filter.

LoD $> 1,250$ cells/L *Pseudonitzschia australis*, but compromised by chain formation.

Balanus glandula, *Alexandrium catanella/tamarense* complex LoD 5000 larvae or cells L⁻¹

May have released rDNA before assay done – so may get slightly higher results than other methods, *e.g.* microscopy

Enumeration

Gives good estimate – not accurate enumerator.

Applications

- Ideal for on site analyses, *e.g.* portside, onboard ship
- Chris Scholin at MBARI uses sandwich hybridisation chemistry in an *in situ* environmental sample processor (ESP) that transmits results in real-time via a radio modem for on-shore analysis. Aimed at invertebrate larvae BUT won't be cheap.

Melissa Gladstone has written report on international validation of technique for enumerating HABs.

Compliance

- General prokaryote assay available.
- Not suitable for eukaryotes unless size fractionation before analysis.
- Variable size ranges of target organisms, *e.g.* dinoflagellates $< 10 \mu\text{m}$ - $> 100 \mu\text{m}$ and invertebrate larval stages similar problem
- Rapid for detection, identification and enumeration.
- Good for early warning of HABs.

- Costs – modest – could reformat to dipstick relatively easily.

FISH will indicate unknowns.

SHA will not indicate unknowns.

Shelley Ball- DNA Barcoding

Species specific, unique identifications.

Using rRNA - use a fragment of 650 base pairs, primarily used for animals.

Problems when using for plants.

In past used PCR-RFLP primarily but problems when new species found; hard to add in new ones. So moved to DNA bar-coding.

Benefits of DNA bar-coding

Universal (globally)

Standardised

Globally co-ordinated data coverage – great if get a new arrival, get very quick identification based on existing codes

Operationally efficient

Quantitative (% sequence divergence and bootstrap support) - how different is it from similar species and how reliable is the identification

Other methods e.g. RFLP

Lack of interoperability

Little co-ordinated data coverage

Lack of inter-agency standardisation

Operationally inefficient (limited taxa)

Not anticipatory

Detection

- Success rate extremely high
- “False positives” – not an issue
- Will get lack of identification if no existing code on database (can also get mis-identification (sister taxa).

Viability

- Easy and cheap to amplify and sequence DNA
- High throughput facilities on the increase
- DNA extraction and PCK can be done on site, need access to sequencer, e.g. at Lincoln, Massey.

Flexibility

- Currently being done for most animals groups.
- CO1 (the gene used) does not work for plants (other genes will need to be studied for these)
- Rapid/portable? – 24 hours or less.

CBOL

- CBOL consortium for the barcode of life (www.barcoding.si.edu) – global bar-coding of all life. High technology companies are being involved to think about future high tech coding/ID tools.

Limitations

- How do we define species limits? (*i.e.* how different do they have to be to decide they are different spp.)?
- No single gene will work for all taxa.
- May not discriminate young species pairs.

INBIPS (within CBOL) – International Network for Barcoding Invasive and Pest Species (Shelley co-chairs).

Databases

- BOLD = Barcoding of Life Database, based in University of Guelph, is accessible through the web. www.barcodinglife.com. Currently 20,000 species in database.
- Fish -Bol – marine fishes
- Freshwater invertebrates for biomonitoring (within CBOL). Bioprotection Centre thinking about working on these.

Developing new technologies – future identifications

- Identification of many taxa at once
- Mixed species samples *e.g.* environmental samples
- Nanotechnology

Brendon asked: How good is barcoding at distinguishing same spp. from different regions. Shelley replied: Depends on taxon.

Jawahar asked: How can it be used in ballast water (for instance). Shelley replied: Can't at present – that is why there is interest in how can develop probes for detecting several taxa at once.

Peter Smith- DNA Barcoding of Marine Species

CO1 (cytochrome oxidase subunit 1) region is the mitochondrial DNA gene unit of choice. Cytochrome b region ended up with ~10 pairs of primers for fishes whereas CO1 region only need one pair of primers to differentiate between all fish species.

As a tool for assigning specimens to known species, it can be used on fragments/damaged specimens, eggs and larvae, gut contents, fillets, archived material, faecal pellets BUT can't be used on formalin preserved material.

Databases

- Global library – aiming for standardised reference sequence library for all species and voucher specimens with authoritative taxonomic identifications
- CBOL's FISH-BOL global fish database development is a long term campaign (5+ years), as is the bird one (ABBI). Peter has been involved with various taxa including fish, crustaceans, mussels and squids. Short term campaigns (two years) for fruit-flies, mosquitoes, databases.
- Local/special initiatives include International Network for Barcoding Invasive and Pest Species (INBIPS), bivalve and squids, Canadian Biota

Advantages

- universal method
- rapidly growing international database
- Good spp. differentiation tool, e.g. *Carcinus*, *Eudistoma*
- Need only small amounts of tissue
- Bar-coding can be applied to whole animal or small parts
- COI data available for Med. fanworm *Sabella spallanzanii*, European shore crab *Carcinus maenas*, Northern Pacific seastar *Asterias amurensis*, Chinese mitten crab *Eriocheir sinensis*, Swimming crab *Charybdis hellerii* BUT NOT Asian clam *Potamocorbula amurensis* (and algae such as *Caulerpa taxifolia*).

Current Limitations

- Technique is PCR based so it has all the same limitations as PCR
- Only works for single species e.g. specimens
- Often don't have bar-codes of our own species e.g. crabs and seastars
- Doesn't identify closely related/recently diverged species with little or no divergence (<20,000 years)
- Cannot detect hybrids
- Relatively quick but not yet mobile
- Different markers required for plants
- requires molecular lab/sequencer

N.B. Barcoding is not DNA taxonomy

NIWA used it for *Charybdis*. There are 47 sp. of *Charybdis* in Indo-Pacific but only two (*C. hellerii* and *C. japonica*) were in the database. Interestingly, these two were the only two intertidal and sub littoral species.

Graeme Inglis asked: Is there collection information in database? Peter replied: Depends on who entered data but collection data should be there.

Miguel asked: Can marine species <2 mm in size be barcoded?

Peter replied: Not good for small stuff e.g. have to culture phytoplankton to identify them.

Barcoding good for bigger stuff only, i.e. organisms than can be individually picked out of a sample.

Paul Rasmussen commented: No good for small and mixed samples. Pre-empted by actual taxonomic knowledge.

Neil Gemmell commented: Metagenomics will allow identification based on DNA sequence in future and won't require prior taxonomic knowledge.

Neil Gemmell- Microarrays: an emerging Technology for Biodetection

Microarrays

Consist of a small membrane or glass slide containing samples of many genes/species arranged in a fixed, regular pattern.

Advantages

- Contain very large number of DNA sequences (up to 500,000)
- Only require small amount of sample (~250 ng of DNA) so small organisms readily accommodated, i.e. very sensitive – single small organisms
- Can potentially reproducibly survey for presence/quantity of a large number of species (1000s) simultaneously in <24 hours
- Low individual dollar cost (potentially a few cents per sp.) although not yet – still in development stage
- Adaptable and future focused
- May need a bunch of these at one time
- Moving from 'Is a specific invasive present?' to 'Which bioinvasives are present?'

Probably will use DNA for presence/identification and RNA for viability.

The microarray could have sequences for different sections of the gene on the same array, not just CO1.

Get fluorescent spots which are measured quantitatively at two wave lengths.

Microarrays being used for pathogen detection especially in medical field and Neil believes they could be adapted to our needs. For example, has been used in a Multiple Pathogen Identification array for 18 different pathogens, including 11 bacteria, 5 RNA viruses, 1 fungi, 1 dinoflagellate. Limit of detection was 10 fg of genomic DNA (*Bacillus anthracis*) so very sensitive.

Phylochip- Rapid quantitative profiling of complex microbial populations

- Can identify 8,989 taxa simultaneously (prokaryotic and eukaryotic organisms)
- It detected 73 of 81 sub-families detected by clone library - also detected an additional 97 sub-families

Disadvantages

- Requires *a priori* knowledge of organism gene sequences so initial development time could be quite long
- Analyses are statistically complicated but good software now available
- Cost per microassay still quite high but dropping (\$100s off the shelf; \$1000s if has to be developed). Currently gene chips cost ~0.1c(US)/gene.

Shelley Ball commented: Fish and Chips - Limit to how many taxa can go on.

Jawahar Patil- PCR based Probes & qPCR (Quantitative PCR)

- Started on PCR because that was all that was available at the time but now working on qPCR.
- Have PCR probes for *G. catenatum*, *A. amurensis*, *C. gigas*, *Undaria pinnatifida*, *Maoricolpus roseus* (NZ screw shell).
- Next pest list in Australia now includes 33 more species than original ABWMAC 1994 list of 14species.

Molecular tools:

- Require a minimum amount of sample material
- Molecular very sensitive because can detect a small portion of the target organism
- Specific
- Can be automated; hence cost and time effective

Approaches

Protein based.

- Antibody binding assays

Nucleic acid based.

- Target Amplification (PCR and variants) - sensitive
- Hybridisation - good for high throughput but not so sensitive
- Combination of the two (best option).

PCR tools

Very sensitive in the laboratory but not so in the field so have gone to nested PCR, uses 2-stage targets – primary enrichment PCR and secondary specific.

Agreement on CO1 for animals great; hopefully plant guys will soon settle on a zone also.

Sensitivity

- Tried 3-80 *A. amurensis* larvae/m³ - picked up >10 larvae/m³. [95% sensitivity means a species detected in 95% of samples]
- Sensitivity depends on life stage and target copy number
- Tried 0-200 *A. amurensis* larvae in 200 mg of plankton (background)

Viability

- Most methods are indirect methods
- Current tools unable to detect single larvae consistently in ballast water
- DNA based tools most sensitive and cost effective
- 2 stage amplification is the best

Application of gene probes

- They deployed gene probes for *Asterias amurensis*, *G. catenatum* and *C. gigas* on 63 vessels (= 80 vessel/tank/dates) and took >500 samples, all in Port of Hastings.
- *A. amurensis*-9 vessels (13%) type II error of which 7 most likely due to carry-over from a previous positive port.
- *C. gigas*-33 vessels (43%), Type II errors of which 97% most likely due to inadequate port surveys.
- *G. catenatum*-29 vessels (40%) type II error - Most likely due to carryover
- *M. roseus* - thought not to have planktonic larval phase but does have a free-living larval form therefore has ballast water implications (not on original ABWMAC list). Detected *M. roseus* DNA in plankton samples.

PCR most cost effective tool in survey comparing Plankton trawl (PCR Probe), Plankton trawl (Visual), Diver deployed quadrat, Snorkel transect (good visibility), Snorkel transect (bad visibility) – see Hayes, Cannon, Neil and Inglis.

qPCR – allows evaluation of sensitivity; PCR is just presence/absence.

A. amurensis has spread from Derwent north to Henderson's Lagoon and St Helens – adults in both places. Think natural dispersion of larval. Used nested PCR.

Sampling technique is very critical

- Ballast or port
- Zooplankton, phytoplankton, bacteria virus, *i.e.* target group
- Pump, net haul, bottles, flow through
- Preservation

- Sub sampling
- DNA extraction

Technical issues with sampling

- Error
- Variability
- Uncertainty
- Encounter rate
- Replicates
- Contaminants

Environmental issues

- Heterogeneity – temporal; spatial, *e.g.* diurnal migration

Single Sampling Platform

A.J. Richardson *et al.* Progress in Oceanographic (2006) 37-74.

- Great but mesh size is 270 μm although Lesley Rhodes commented that 11 μm now available.
- Will trap phytoplankton but may under-sample these taxa.

Automation and high through-put

Thinks nestled PCR on glass slide best but not commercialised, do first amplification in liquid phase, then second in a solid phase on a glass slide.

Other novel technologies,

- Microarrays – hybridisation
- Quantum dots
- Bioconjugated nanoparticles
- Micro and nano fluidics
- Massive parallel sequences

Bottleneck for most of these things is doing the sequencing of species.

Future work

- Develop probes for all critical species (Next pest list Trigger list?)
- Deploy available probes for variety of environmental studies
 - o Threatened species
 - o Life history
 - o Ballast water management (National system)
- Develop semi-quantitative high-throughput screening as a tool for certification of ports/self-certification of vessels
- Single platform sampling
- Automation of remote sensing and low cost sample processing

- Coastal: Oceanic ratios

Paul Rasmussen - Real time PCR

Real-time PCR amplifies the DNA 220 times, *i.e.* very sensitive. Can detect 10 or less starting target sequences. Requires PCR primers, probe and target sequence matching up.

DNA or RNA can be targeted

DNA or RNA?

DNA quite tractable:

- Heat labile
- Resistant to mild acid and moderate alkali
- Resistant to many detergents and surfactants

RNA difficult to prepare high quality

- Unstable
- Variable expression
- Susceptible to acids and alkali

Detection versus quantitation

qPCR can detect at very low cell densities but can also generate a measure of gene copies, *i.e.* enumerate.

Most end-user stakeholders want this but the key questions are:

- What does it mean compared to conventional measure (cells/mL)
- How can it be co-opted for guideline development

Hardware for qPCR

- Range from ~\$70K - \$100K.
- 25-45 minute turn-around time.
- A couple of simpler versions being developed for easy detection of *e.g.* anthrax in Army, getting down to ~\$17K.
- Can do these assays out on a boat.

Sampling

- Pre-filter 20L to remove macroinvertebrates
- Then plankton net 20-100 μ
- Ultra filter 20 μ m and smaller makes PCR easier because gets rid of the amount of crud in the samples.

Inhibition

Is an issue, *e.g.* in ballast water, have humic substances, carbohydrate goo *etc.*, these may increase Ct or may destroy amplification altogether, can be overcome with PCR additives.

Controls

- False positives (Type II error) – eliminated by good assay design and validation using a wide range of closely-related species (need for comprehensive culture collection)
- False negatives (Type I error) – can be handled by external heterologous control (separate PCR reaction on split sample) or internal heterologous control (separate target in the same PCR reaction)
- Greatest advantage in removing human error.

Multiplexing

Limited only by number of channels of the machine although is still tricky (6 channels = 6 gene targets per reaction).

Viability

Free DNA in seawater gone in 10 hours but in sediments it takes 93 days so cannot be used on sediments (will have DNA from ages ago). RNA only expressed from viable organisms

EMA-PCR for quantification of viable and dead cells in complex samples. EMA goes through damaged (dead) membranes – but probably excessive except for sediments.

Sample preparation

Need general rapid, robust method for getting DNA out – sonication and bead-beating best.

Portability

Yes, he brought his to workshop but better to do at a central place because highly likely that there will be a need for peripheral equipment for sample concentration and preparation.

Table 7 Comparison of qPCR with other technologies

	Sensitivity	Specificity	Throughput	Precision	Multi target	Quantification
Microscopy	X	X	X	X	√√	√√
ELISA	X	X	√√	√	X	X
Antibody rapid tests	X	X	√√	√	X	X
DNA hybridisation	√	UD	X	UD	X	UD
Conventional PCR	√	√	X	√	X	X
Real time PCR	√√	√√	√	√√	√	√
Microarray	UD	√	√√	√	√	√

Does not think online probes are feasible, better to go with rapid assays.

5.1.2. Discussion

Graeme Inglis: Clarified difference between EMMBB and operational research.

Ballast water standard: 10-50 μm eukaryotes; >50 μm eukaryotes

Jawahar: Need to involve Biosecurity managers from other countries.

Naomi: Have developed joint DAFF/MAF technical group for research directions, policy *etc.*

Border compliance needs include quantitative monitoring of ballast water for IHS (exchange standard) and IMO (treatment standard), and proposed biofouling IHS.

Paul: 10-50 μm range may be challenging for molecular technologies.

When organisms are very small, are enumerating genes rather than organisms. Will need to assess relationship between number of genes and number of organisms and variability around this. Will also need to assess variability around sampling methods.

Doug: All techniques need to be validated before utilisation/application.

Neil: Likely that will have to use non-molecular tools for some needs *e.g.* spectroscopy, dye uptake to include viability.

Graeme: MITS getting so many species, including juveniles barnacles, scuzzy algae, mytilids – may be another source of groups to develop quick molecular tools in conjunction with operational work.

Need to review species on existing list, *e.g.* Lesley noted all of the three *Alexandrium* sp. are all around New Zealand

Naomi: Thinks Australian list will be out in ~6 months. New Zealand list? – depends on Dan Kluza's timetable. List will likely be similar to the list that Graeme Inglis used in this workshop.

5.1.3. Summary- Identification of gaps and opportunities for research

1. Need to compare techniques and validate against traditional methods.
2. Review applications and most appropriate avenues for pursuit.
3. Sample preparation is a big issue for use and applications. Concentrations and DNA extraction, especially as increasingly doing environmental sampling. Will depend on detection thresholds.
4. Sampling for using molecular tools (statistician plus molecular biologists).
5. Future proofing and future casting. Where do we want to be in 12 years time *e.g.*
6. Understanding relation between cells/ml and genes/ml.

Neil:

- Future molecular
- Homeland security.
- Environmental vote – Green Genes
- Medical applications
- Need to link with Ministry of Health, ESR *etc.* because the gene chips are expensive.

CSIRO strategy – focusing on PCR and qPCR with enough spots, with view to taking them to further applications later, *e.g.* microarrays. Need high density of spots (number of species) for the technology to be advanced, *e.g.* to microassays. Costs same to have 10 spots as ~1,000 spots so developers sticking with 10 spots but this is inadequate.

A preliminary evaluation of the methodologies in terms of compliance, pathway spread analysis, species identification and targeted surveillance derived from the workshop is shown in Table 8. A list of known molecular markers that are known to have been developed for marine pests is shown in Table 9.

Detection and Enumeration Techniques for Marine Biosecurity

Table 8. Preliminary evaluation of molecular methods with respect to their suitability in biosecurity

Biosecurity function	Focus	Use	Technique				Comment		
			FISH	SHA	PCR	Bar coding		qPCR	Other
Border compliance with IMO std. for ballast water	Multiple species	Needs to enumerate and test viability in 2 size fractions			No	No	Yes but too much hassle. Will be good if single species, e.g. <i>Vibrio</i> is targeted in standards	Dye exclusion or dye uptake and then fluorescence spectroscopy. Could cook half sample to kill all and subtract	Need to trial various techniques (e.g. PCR and FISH) – may not be molecular techniques
Pathway spread analysis (where has species come from and relation to others, e.g. population analyses)	Unidentified species, target spp., multiple spp.	Detection Enumeration						Micro satellites	Marker depends on organism and question, e.g. haplotype work on <i>Undaria</i> . None of these useful for population genetics. Study used simple PCR for differentiate introduced and non-introduced <i>Caulerpa</i> spp.
Species identification	Unidentified species, target spp., multiple spp.	Detection Enumeration				Bar coding CO1			All methods potentially useful but depends on whether you want speed, cost likely to be done first, probably go for a couple of different genes
Targeted surveillance	Unidentified species, target spp., multiple spp. May be single species and multi-species arrays	Detection Enumeration	Labour intensive, not as sensitive as PCR.	Rapid, cheap, potentially can screen for 24 species at once.	Will pick up <u>any</u> DNA even if not in an organism		Not over-sensitive, wide range of enumeration, good specificity, less labour intensive than microscopy and quicker, cheap, high throughput so good for getting lots of samples, e.g. when trying to detect rare species.		

Detection and Enumeration Techniques for Marine Biosecurity

Table 9. Known molecular markers developed for marine pests

Species	Common name	Significance	Marker type	Research group	Comment
<i>Asterias amurensis</i>	Northern Pacific seastar	1,2,4	PCR, qPCR, Barcode	CSIRO/Cawthron/Phillip Weir	
<i>Carcinus maenas</i>	European green crab	1,2,4	PCR, qPCR/ barcoded	CSIRO/EPA USA/Stanford/ NIWA	
<i>Charybdis japonica</i>	Lady crab, Ishigani Chinese mitten crab	1,2,3	barcoded		
<i>Eriocheir sinensis</i>	crab	1,2	barcoded, mt-DNA 586 bp COI	? Gen bank (Hanfling <i>et al.</i> 2002. MEPS 238: 307-310)	
<i>Hemigrapsus sanguinensis</i>	Japanese shore crab	2			
<i>Sabella spallanzanii</i>	Mediterranean fan worm	1,2,4	qPCR / barcoded / FISH	SARDI / gen bank / Cawthron	not complete
<i>Hydroides ezoensis</i>	Serpulid tubeworm	2,3,4			
<i>Chaetopterus sp. A</i>	Hauraki	3	16S for genus	gen bank	
<i>Maoriculpus rosea</i>	NZ screwshell	4	PCR, qPCR, gen bank	CSIRO	
<i>Crassostrea gigas</i>	Pacific oyster	2,3,4	PCR, qPCR, gen bank/ microsats, COI, 16S, snips	CSIRO/multiple including delaware	
<i>Potamocorbula amurensis</i>	Brackish-water Corbula clam	1,2	?	Japan	
<i>Corbula gibba</i>	Marine Corbula	2,4	qPCR	SARDI	not complete
<i>Musculista senhousia</i>	Asian nesting mussel	1,2,3,4	qPCR / gen bank	SARDI	not complete
<i>Perna viridis</i>	Asian green mussel	2,4	PCR / lot of genetic data for genus	JCU	
<i>Mytilopsis sallei</i>	Black-striped mussel	2			
<i>Limnoperna fortunei</i>	Golden mussel	2			
<i>Undaria pinnatifida</i>	Asian kelp	1,2,3,4	gen bank, microsats, cox3, PCR	French, Japanese, CSIRO (PCR)	
<i>Caulerpa taxifolia</i>	Aquarium weed	1,2,4	lots internationally, ITS, PCR	CSIRO, U. Otago/NIWA	
<i>Polysiphonia brodiaei</i>	red macroalga	2,3,4	PCR	Korean, NIWA (Queens Uni Ireland)	native sub-species may be an issue for this species
<i>Codium fragile ssp. tomentosoides</i>	Green macroalga	2,3,4	lots internationally,	States (A state)	

Detection and Enumeration Techniques for Marine Biosecurity

<i>Styela clava</i>	Clubbed tunicate	1,2,3,4	PCR, microsatellites	NIWA/ Plymouth	U. Canterbury/U.	work in progress
<i>Didemnum vexillum</i>	Colonial ascidian	3?	16S / gen bank (not this species),	NIWA. G. Lambert UC		
<i>Eudistoma elongatum</i>	Colonial ascidian	3?	COI,	NIWA		
<i>Ciona intestinalis</i>	Sea vase	2,3,4	gen bank,			
<i>Gymnodinium catenatum</i>	Toxic	2,3,4	PCR, qPCR, FISH, Sandwich, lots	CSIRO, UTAS		
	dinoflagellate		internationally			
<i>Alexandrium minutum</i>	Toxic	2,3,4	FISH, Sandwich, qPCR	UTAS, AWQC, Cawthron, MBARI		
<i>Alexandrium catenella</i>	Toxic	2,3,4	FISH, Sandwich, qPCR	UTAS, AWQC, Cawthron, MBARI		
<i>Alexandrium tamarense</i>	Toxic	2,3,4	FISH, Sandwich, qPCR	UTAS, AWQC, Cawthron, MBARI		
<i>Bugula neritina</i>	dinoflagellate	2,3,4				
<i>Schizoporella errata</i>	Bushy bryozoan	2,3,4				
<i>Pseudodiaptomus marinus</i>	Sheet bryozoan	2,3,4				ask museums
<i>Balanus eburneus</i>	Calanoid copepod	2				ask museums
<i>Tridentiger bifasciatus</i>	Barnacle	2	lot on genus level on gen bank	MBARI (not sure this species)		
<i>Neogobius melanostomus</i>	Japanese goby	2	mitochondrial	Japanese		
<i>Omobranchus anolius</i>	Round goby	2	mitochondrial	Japanese		
IMO indicator organisms	Oyster blenny	3	COI, mitochondrial genes, cytochrome B, control re.g.ion	NIWA		
Aquarium species of concern						
Molecular markers for baseline information libraries, for specific taxa - barnacles, juvenile bivalves/mustellids, juvenile Ulva, anemones - taxonomic issues worldwide						
		1=	NZ Unwanted organism,			
		2=	Australian pest or next pest list			
		3=	NZ incursion			
		4=	Australian incursion			