Port of Hastings National Demonstration Project – Verification of the Type II error rate of the Ballast Water Decision Support System (DSS)

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Final report prepared for:

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Port of Hastings national demonstration project :
verification of the Type II error rate of the ballast water
decision support system (DSS).

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ISBN 1 876996 67 6.

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Division of Marine Research.

363.728464

EXECUTIVE SUMMARY

In July 2001, Australia introduced a risk-based Decision Support System (DSS) to manage ballast water on international shipping. Up until now, however, the accuracy of the risk assessments made by the DSS have never been evaluated or tested. It was not until the DSS was considered as a mechanism to assist the management of domestically sourced ballast water, coupled with advances in genetic technologies, that the opportunity arose to evaluate the accuracy of the predictions of the DSS. The results of that evaluation are reported here.

Obtaining representative ballast water samples from ballast tanks is complicated by the physical geometry of the tanks and gradients of temperature, depth, light, etc. Ballast water sampling procedures have been the focus of several international workshops and were standardized in this study in an attempt to reduce this source of variability. We cannot garuntee that all of the ballast water in a tank is free of target species based on samples that test negative, however, this does not influence the Type II error rate (ballast water predicted to be free but samples prove to be positive). Identifying the species obtained in a ballast water sample is also 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.

All species have a unique genetic “signature”. Identifying DNA sequences that uniquely identify species is nowadays a routine process but one that typically operates on samples of individual species. Applying this existing technology to ballast water samples would require candidate individuals to be separated from the plankton sample thereby negating the advantages of genetic analysis over traditional morphological analysis. Here we report on a genetic technique – gene probes - that are capable of identifying the DNA of a single species, at low concentrations, in unsorted ballast water samples

Gene probes were developed for three species of concern – *Asterias amurensis*, *Crassostrea gigas*, *and Gymnodinium catenatum*. The gene probes target a unique DNA sequence in the mitochondrial CoI gene (*A. amurensis* and *C. gigas*) or large subunit (LSU) ribosomal DNA (*G. catenatum).* A nested polymerase chain reaction (PCR) technique is used to first amplify all DNA with similar sequences, and then to amplify the target DNA from this enriched sample. All three probes were tested against as many closely related species as could be obtained and the reaction conditions optimized for the maximum sensitivity providing 100 percent specificity.

The *Asterias amurensis* probe correctly differentiated this species (56 specimens, 8 locations, 3 countries) from 12 native Australian seastar species (including 5 from the family Asteriidae). The test did not distinguish *A. amurensis* from *Asterias rubens* (Belgium) nor *Asterias forbesi* (Atlantic Canada). The volume of ballast water arriving in Australia from the North Atlantic, however, is small so this is not seen as a serious limitation for use of the test in this country. The test was sensitive enough to routinely detect 10 or more larvae in a 200 mg plankton sample. Ballast water samples collected in this study contained between 53 and 1656 mg/m^3 plankton, suggesting a minimum density of between 3 and 80 *A. amurensis* larvae/ m^3 in order to be detected by the probe. This minimum sensitivity is well below typical *A. amurensis* densities in the Derwent River during the July-October spawning season.

G. catenatum (4 samples, 3 countries) was successfully distinguished from 14 dinoflagellate taxa (17 strains, 3 countries), including 2 identified *Gymnodinium* species and 5 unidentified *Gymnodinium* species. Testing the specificity of this probe was complicated by the confused taxonomy of the dinoflagellates. Despite this, the possibility of a false positive from other species not tested here is considered low because of the high interspecific variability of the primer binding site and the high specificity at low annealing temperatures. It would be prudent, as is the case for all probe results, to confirm positive results by sequencing or other techniques. The *G. catenatum* test was slightly less sensitive than the *A. amurensis* test: between 7 and >103 cysts/m^3 would be required for detection over the range of ballast water samples tested (53 – 1656 mg/m^3).

The *C. gigas*-specific probe successfully distinguished *C. gigas* (26 samples, Tasmania) from 8 bivalve species (9 samples, 5 countries), including 4 *Crassostrea* species. One *C. gigas* sample had a single base pair mismatch at the primed binding site, suggesting that there might be some intraspecific polymorphism at this site. Genetic characterization of the mt COI locus of *C. gigas* throughout its natural range in Asia as well as its *de novo* range, where it has been introduced, would be required to test the possibility of false negative results. The *C. gigas* probe detected 50 or more larvae at 6 hours post fertilisation and 5 or more larvae at 20 hours post fertilization when mixed with 150 mg plankton. This suggests that the probe would detect between 2 and 550 larvae/m^3 over the range of ballast water samples tested (53 – 1656 mg/m^3), depending on plankton biomass and larval size.

Overall the genetic probes developed in this study perform well and provide a new opportunity to test the efficacy of ballast water risk assessment and management, in addition to a variety of other environmental concerns. Research is starting to further develop the PCR technique so that abundance, as well as presence/absence, can be confirmed. The gene probes can be made more cost-effective by using micro-array techniques capable of testing several species at once.

Recommendation 1: A concerted effort be made to develop comparable genetic probes for all marine pests of concern to Australia – both those already here and those identified as likely to arrive.

In this study the genetic probes were used to test ballast water sampled from 63 vessels (80 vessel/tank/date combinations). The ballast water samples were chosen to assess the probability of Type II errors (the probability that "uninfected" ballast water actually contained detectable quantities of the target species at the end of the vessel's journey) arising from three error scenarios in the DSS:

- scenario 1 - the target species is recorded as absent from the donor port when in reality it is present;
- scenario 2 - the target species is recorded to be absent from the water column (and therefore unavailable to the vessel) when in reality it is present;
- scenario 3 - the target species is predicted to die prior to arrival in the recipient port when in reality it survives.

There are also four error scenarios in the sample collection and data reporting that could give rise to apparent Type II errors:

- scenario 4 - the sampling equipment is not thoroughly washed and dried between samples and is contaminated with water or sediment residues from a previous vessel;
- scenario 5 - cross-contamination occurs in the laboratory during DNA extraction and analysis;
- scenario 6 – the target species is present in the ballast water because it is completing (or attempting to complete) its life-cycle in the vessel's ballast tank; or,
- scenario 7 – information about the source of the ballast used in the risk assessment is incorrect.

In assessing the likelihood of each of these Type II error scenarios, we have used our best judgment, weighing the pros and cons for each scenario. In most cases, however, a dedicated scientific study, beyond the scope of this current study, is needed to confirm these assessments. While these assessments are subjective, full details of each ships history are provided in the text, so that they can be reviewed.

A high proportion (84%) of vessels predicted by the DSS to be free of *Asterias amurensis* were correctly identified as negative (*Asterias* was not detected at the end of the vessel's journey), suggesting that the DSS can provide effective risk mitigation for this species. Nine vessels (13%) predicted to be negative had detectable *A. amurensis*. Seven of these vessels are considered most likely to have tested positive because of carry-over of ballast water from infected ports. This indicates the importance of entering accurate information into the DSS. Additionally, for three of the vessels, the positive results suggest that *A. amurensis* may be able to survive in ballast water longer than has been previously estimated (and longer than specified in the DSS). Positive results for two of the vessels suggest that the timing and/or duration of the spawning period in the Northern Hemisphere may also be incorrectly recorded in the DSS.

Crassostrea gigas was identified in 43% of vessels predicted to be negative, indicating that the DSS is not adequately identifying positive vessels for this species. The majority (97%) of Type II errors in this instance can be attributed to *C. gigas* being present in ports where it is recorded as being absent. All of the ports in question – Sydney, Port Botany, Newcastle and Port Kembla – have been surveyed to the standards set down in the CRIMP port survey protocols. Alternative, but perhaps less likely, explanations are that *C. gigas* is present in the ballast tanks, piping or sea chests of a large number of vessels, or that *C. gigas* is present in the water column as larvae advected from adjacent coastal waters, but is not established in any of the ports. These results indicate that the CRIMP survey protocols may need revision, or at least an understanding that they may not have sufficient statistical power for risk management purposes. The cause of the Type II error is unclear in the remaining 3% of vessels, but again it is possible that *C. gigas* was completing its life cycle in the ballast tank or vessel piping and/or the samples were contaminated.

All vessels sampled in this study were assessed by the DSS to be free of *Gymnodinium catenatum* because the distribution of this species is very limited in Australia. Almost half (40%) of these vessels, however, tested positive for *G. catenatum* - an unacceptably high Type II error rate. Carryover of cysts in ballast sediments could explain all of these positive results because of the highly resistant nature of dinoflagellate cysts. It is also possible, however, that ports in which native non-toxic *Gymnodinium* spp. have been identified – Botany Bay, Sydney, Port Kembla, Adelaide, Melbourne and Portland – do in fact contain the toxic non-native *G.*

catenatum. This is especially true for Melbourne because two vessels with positive identifications trade exclusively with between Melbourne and Burnie, and Burnie has recently been surveyed and found to be free of any *Gymnodinium* spp. Sixteen (52%) of the vessels that tested positive reported their ballast water source as ports thought to be free of any *Gymnodinium* spp. Eight of these vessels, however, had previously visited international ports where *G. catenatum* has been reported. The remainder had visited Australian ports with known or suspected populations emphasizing the importance of carryover. Two vessels testing positive reported their ballast water as sourced from Port Stanvac (not surveyed), suggesting that this port may contain *G. catenatum* as well.

Recommendation 2: routinely sample and test a proportion of low risk vessels for the presence/absence of marine pests to continually increase the sample size of Type II error results and assist in the on-going development of the DSS (all species);

Recommendation 3: encourage vessels' masters to maintain accurate ballast water logs, particularly where ballast water is mixed in the vessel's tanks. If ballast water is mixed within a tank it is imperative that all sources of the ballast water are recorded in the vessel log and reported to the DSS (all species);

Recommendation 4: review the port survey protocols, placing particular emphasis on the power of the resultant survey and the use of ancillary data and associated Quality Assurance issues, and where necessary re-design survey and/or monitoring methods, together with arrangements for their accreditation (all species);

Recommendation 5: sample ballast tanks for vessels ballasting in areas of known marine pest abundance, to determine whether they can complete their life cycle in ballast tanks (*A. amurensis* and *C. gigas);*

Recommendation 6: collect and analyse additional field samples (using the gene probe developed during this project) to provide a more accurate determination of the life-expectancy of larval in the ballast tanks of infected vessels, both with and without ballast exchange (*A. amurensis* and *C. gigas*).

Recommendation 7: confirm the presence or absence of *C. gigas* in Sydney, Port Botany, Newcastle, Port Kembla and Fremantle as soon as possible, and amend the DSS database accordingly;

Recommendation 8: confirm the presence or absence of *G. catenatum* in Botany Bay, Sydney, Port Kembla, Adelaide, Melbourne, Port Stanvac and Portland as soon as possible, either by applying the probes to the port survey material collected from these ports, or by collecting additional samples (and amend the DSS database accordingly);

Recommendation 9: review the efficacy of ballast water exchange as a risk management strategy for dinoflagellate, and other cyst producing, species, and collect additional ballast sediment samples to quantify the incidence of the "carry-over" of cysts between ports;

Recommendation 10: collect additional literature and field samples (if necessary) to verify the spawning and larval season of *A. amurensis* in Korea and Taiwan;

Recommendation 11: gather all available information on the life-cycle of *C. gigas* and amend the DSS database accordingly;

While this study has indicated the need for evaluation of the DSS system that is used to assist in the management of ballast water in Australia, the results should not be taken in isolation. The science of invasion biology is in its infancy, and nowhere more than in the marine realm. Australia leads the world in many aspects of responding to the growing threat of marine pests, through its scientific and management endeavors, but this should not be confused with concluding that the science is adequate to the task. Continued research, often based on new technologies as in this study, will be required in order to successfully reduce and/or mitigate the threat of marine pests to Australia. Such research is integral to an adaptive management approach, where new and existing management arrangements can be used to set up large-scale experiments that will improve our knowledge of the problem and provide for improved future management. Consistent with ISO14001 this requires a management structure that can adapt to maximize information return in addition to reducing the immediately perceived risks.

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1 INTRODUCTION

1.1 Background

The risks posed by ballast water to Australia's marine biodiversity and marine industries in the absence of effective management are well known. Introductions of exotic organisms into Australian marine waters threaten the biodiversity and ecological integrity of Australia's marine ecosystems, pose risks to human health, and threaten the social and economic benefits derived from the marine environment, including aquaculture, recreational and commercial fishing, tourism and domestic and international shipping.

In July 2001, the Australian Quarantine and Inspection Service introduced a mandatory ballast water management regime for international vessels, based on a risk-based Decision Support System (DSS), establishing Australia as the first country in the world to introduce risk-based management of ships' ballast water. At the same time it was recognised that effective management of ballast water would also include the management of ballast water on ships travelling between Australian ports, thus reducing the further spread of existing exotic organisms. The Hastings project was conceived to trial the operational and management arrangements of an integrated ballast water management model to assess its suitability for Australia wide application (Meyrick and Associates 2003). This included consideration of the environmental and administrative implications of the DSS.

The DSS, *inter alia,* provides an assessment of the probability that individual target species are present in individual ballast tanks. This assessment, however, is often hindered by insufficient or poor quality data. Lack of sufficient appropriate data can result in advice that characterises ballast water as infected with a target species when in fact it is not (Type I error), leading to unnecessary management intervention, or advice that characterises ballast water as uninfected with a target species when in fact it is (Type II error), leading to unexpected environmental risk.

In this project we aim to evaluate the likelihood of a target species being present in ballast water that has been deemed to be free of the target species (Type II error) to further aid in the development of the DSS. This evaluation includes the following components:

- a sampling programme targeting vessels (and ballast tanks) deemed free of the target species by the DSS;
- the development of genetic probes for three target species (*Crassostrea gigas, Asterias amurensis, Gymnodinium catenatum)* to evaluate the presence of these species in the ballast water; and,
- the evaluation of the Type II error rate.

Port of Hastings national demonstration project

It was considered that optimal benefits would be gained from the project by choosing a demonstration port in southeastern Australia. The reasons for this are: 1) there are high levels of domestic and international shipping traffic in this region; 2) there are large numbers of targeted ‘pest’ species already present but patchily distributed in the region; and 3) there is a strong species presence/absence knowledge base available due to completed port surveys in Victoria, New South Wales, and Tasmania.

The Port of Hastings was a practical port to implement the demonstration project because it has a moderate volume of international and domestic trade -180 ship visits in 1998/99 from 14 Australian and 15 international last ports of call - providing a tractable number of shipping movements for development and evaluation of the Ballast Water Management System. The Port of Hastings has also been surveyed for marine pests in accordance with standardised and vetted Port Survey Protocols.

Risk assessment framework

The risk assessment framework, developed by CSIRO Marine Research, Centre for Research on Introduced Marine Pests (CRIMP) was designed to meet the needs of the AQIS Decision Support System (DSS) for International Ballast Water Management. In this project it has been used to evaluate the risks associated with ballast water transport to the Port of Hastings from both International and Domestic vessels.

The risk assessment framework is subject to two types of errors, namely Type I and Type II errors where:

- Type I relates to errors where the risks are assessed (by the DSS) as high when in fact they are low. The implications of this are reversible with cost occurring on a vessel/voyage basis.

- Type II errors where the risks are assessed as low (by the DSS) when in fact they are high. The implications of this are potentially irreversible, passed onto future generations, in terms of environmental, social and economic consequences of a new species introduction.

1.2 Objective and reporting

The objective of this project was to provide an analysis of the Type II error associated with the risk-based DSS outcomes for a suite of vessels entering the Port of Hastings. This is the second and final project report. The first interim report (Patil *et al.* 2003) described the initial development of the DNA probes. This report describes: a) the methodology for rapid genetic diagnosis for three target species (*Crassostrea gigas, Asterias amurensis, Gymnodinium catenatum)*; b) an assessment of the Type II error rate outcomes for at least 70 ballast tank evaluations, from a range of origins appropriate for analysis, in the first year of operation of the DSS in Hastings; c) a discussion of the Type II error rate associated with the DSS and suggested methods of improvement; and, d) recommendations to improve the future accuracy of the DSS.

Chapters 2, 3 and 4 discuss the development of the genetic probes for *Asterias amurensis*, *Crassostrea gigas* and *Gymnodinium catenatum*, highlighting the techniques used to test the specificity of the probes. Chapter 5 provides presents the Type II errors for each of the species, including a detailed discussion of the patterns and probable causes in each case. Chapter 6 summarises the results of the project and makes a number of recommendations.

2. *ASTERIAS AMURENSIS*[1]

2.1 Introduction

The northern Pacific seastar, *Asterias amurensis*, is carried in ballast water and arrived in Australia from its native range (coasts of Japan, eastern Russia and Alaska) in the 1980s (Turner 1992, Ward and Andrew 1995). The first Australian record is from southern Tasmania in 1986 (Turner 1992) and the seastar has since become established along Tasmania's southeast coast as well as in Port Phillip Bay on mainland Australia (Garnham 1998). The species has a phenomenal rate of population growth – the Port Phillip Bay population increased from a small number of adults, first detected in routine scallop dredging in 1995, to over 100 million 5 years later (Talman *et al.* 1999). *A. amurensis'* wide environmental tolerances give it the potential to spread throughout Australia's temperate marine ecosystems (Hewitt *et al.* 2002). *A. amurensis* is difficult to identify from plankton or ballast water samples as its larval forms are indistinguishable from other closely related fauna.

A previous study of Tasmanian asteroid larvae provided a genetic method for identifying 14 Tasmanian seastar species, but the method requires isolation of individual larva and identifies only a subset of *Asterias amurensis* genotypes (Evans *et al.* 1998). Ensuring that genetic variation within a species is encompassed, while closely related species are excluded, is a common difficulty in developing species-specific DNA-based probes. Even when sequence data are available from widely dispersed populations of the target species and sister taxa, species specificity may be difficult to achieve (Bell and Grassle 1998). One way to deal with this difficulty is to develop a conservative group-specific probe which includes the target species and close relatives, then carry out secondary tests on positive results for species identification (Oldach *et al*. 2000).

In this chapter, we present a simple PCR amplification protocol that detects seastars in the genus *Asterias.* Since *Asterias amurensis* is the only Australian representative of the genus, a positive test would indicate its presence in Australian environmental samples. To boost the detection level in complex environmental/ballast water samples, a two-step nested PCR approach was used. The sensitivity of nested PCR was evaluated under realistic conditions by carrying out trials on ballast water samples that were spiked with known numbers of larvae. Ballast water arriving in Australian Ports from overseas could potentially contain other species closely related to *A. amurensis* and indistinguishable with our PCR test (e.g. additional non-Australian seastars species in the genus *Asterias*). Therefore to discriminate between positive results, we present a method using denaturing gradient gel electrophoresis (DGGE), a quick method for separating PCR products. Species level discrimination is expected not only to assist in assessment of potential invasion risk by other species of *Asterias* into Australian waters, but also in ecological studies in their native range of distribution.

[1] This chapter was published as a paper in Marine and Freshwater Research, **54** (6): 706-720, "Development and evaluation of a PCR based test for detection of *Asterias* (Echinodermata: Asteroidea) larvae in Australian plankton samples from ballast water" (Deagle *et al.* 2003).

2.2 Materials and methods

Sample collection

DNA samples from adults of 12 seastar species, broadly representative of Australian taxa, were obtained from a previous study in southern Tasmania (Evans *et al.* 1998). These samples encompass six families from three orders including two species (*Coscinasterias muricata* and *Uniophora granifera*) from the family Asteriidae.

Adult *Asterias amurensis* specimens from six Japanese populations and one Russian population were collected by Ward and Andrew (1995). Additional *A. amurensis* adults and samples of a native species, *Petricia vernicina* were collected during the present study near Hobart, Tasmania. Adult *A. amurensis* (Tasmanian) were bred in the laboratory to produce the larvae used in the study. Larvae were reared to the bipinnaria stage (13 days old) and then fixed in SET (0.75 M NaCl, 5mM EDTA, 80mM Tris HCl, pH 7.8) buffered 85% ethanol.

DNA extraction and sequencing

Genomic DNA isolated in this study was extracted from adult seastar tube feet (10-50 mg) using the Qiagen tissue extraction kit (Qiagen). For environmental and ballast water samples, collected material was concentrated by vacuum filtration through a 5µm pore-sized hydrophilic Durapore Filter (Millipore). The filtrate was allowed to air dry briefly, transferred to a 1.5ml tube and DNA was extracted using the DNeasy Plant Kit (Qiagen) following instructions of the supplier. DNA was retrieved in 200µl elution buffer and stored at 4°C.

Using primers previously employed on a wide range of seastar taxa (ECOLa and HCO; see Table 2.1 for sequence and references), a 735 bp segment of the mitochondrial cytochrome oxidase c subunit I (COI) gene was amplified. PCR products were purified using the Qiaquick PCR purification system (Qiagen). Sequencing reactions were carried out on both strands, using the original amplification primers, with the ABI Big Dye prism dideoxy sequencing dye terminator kit. Electrophoresis was carried out on an ABI-377 automated DNA sequencer and sequence data were edited with Sequence Navigator software (Applied Biosystems). Sequence data were aligned using CLUSTAL_X (Thompson *et al.* 1997). These sequences along with additional sequences from GenBank were used to assess the level of COI variation within *Asterias amurensis* and between this species and local seastars.

Asterias specific PCR

Several suitable primer pairs were identified in the COI sequence from *Asterias amurensis* using the software program OLIGO (Rychlik 1996). Multiple primer sets exhibiting significant interspecific variation were ordered. In preliminary trials, all pairs seemed to be genus specific. Primer pair CASF1 and CASR1 (Table 2.1) was used in all subsequent work as it consistently produced strongest PCR amplification.

Standard PCR reactions were done in a 25µl volume containing 0.4 µM of each primer, 0.125 mM dNTPs, 2.5 mM MgCl2, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions for the Asterias-specific primers (CASF1 and CASR1) were as follows: 94°C for 10 minutes then 35 cycles (94°C, 30s/61°C, 30s/72°C, 45s) followed by 72°C for 2 minutes. In single larva PCR, the ethanol fixed larva was isolated under a dissecting microscope and allowed to air dry. Using a pipette, 2µl of Milli-Q water was

used to rehydrate and transfer the larva directly into a PCR tube. The sample was snap frozen at -80°C, thawed to disrupt the cells and then the PCR cocktail (as above) was added directly to the tube.

For environmental samples, where the concentration of target DNA was low, nested PCR was carried out. Primary enrichment PCR was conducted using the COI primer pairs ECOLa and HCO (Table 2.1). Cycling conditions were: 94°C for 10 minutes then 15 cycles (94°C, 30s/56°C, 30s/72°C, 1 minute), followed by 72°C for 2 minutes. The secondary Asterias-specific PCR was carried out using the primer pairs CASF1 and CASR1 as described above with one tenth the volume of the primary reaction as template. A separate PCR reaction was carried out on all samples using universal ribosomal DNA primers (Table 2.1; NSF1179 and NSR 1642) to confirm suitability of each sample for PCR. Aerosol-resistant pipette tips where used with all PCR solutions and negative control reactions were performed with each PCR cocktail. The products of the Asterias-specific PCR and the 18S positive control PCR corresponding to each of the samples were mixed and separated together on either a 2.0% agarose or a 7.5% polyacrylamide gel. Gels were stained with ethidium bromide and visualized under UV light.

Table 2.1 Sequences of primers used in this study

Name	Gene	Sequence (5'-3')	Application	Reference
(F)ECOLa	COI	ACCATGCAACTAAGAC GATGA	PCR + sequencing - Seastar	Knott and Wray 2000
(R) HCO	COI	TAAACTTCAGGGTGAC CAAAAATCA	PCR + sequencing - Seastar	Folmer *et al.* 1994
NSF 1179	18S	AATTTGACTCAACACG GG	PCR –Universal positive control	Wuyts *et al.* 2001
NSR 1642	18S	GCGACGGGCGGTGTG TAC	PCR –Universal positive control	Wuyts *et al.* 2001
CASF1 Forward	COI	GCACAACCGGGATCTT TACTTCAAG	PCR - Asterias specific	This study
CASR1 Reverse	COI	CATTTACCAAATCCTC CTAT	PCR - Asterias specific	This study
CASF1 Forward GC clamp	COI	CGCCCGCCGCGCCCCG CGCCCGTCCCGCCGCC CCCGCCCGCACAACCG GGATCTTTGCTTCAAG	Clamped PCR primer for DGGE	Sheffield *et al.* 1989 and this study

Simulated ballast water testing

To validate the PCR technique and determine its minimum detection level in field conditions, *in situ* ballast water conditions were simulated. Ballast water samples that were known not to contain *Asterias amurensis* were collected and spiked with known numbers of *A. amurensis* larvae. The "background" samples were collected from ballast tanks of the MV Iron Sturt, an Australian domestic bulk carrier that travels between ports in Tasmania, Victoria and South Australia (see Murphy *et al* . 2002). A total of six ballast water tanks were sampled in Hobart Tasmania in May 2002, four had been recently filled (<12 hours old) in the Port of Hobart and two tanks sampled were filled at Port Pirie in South Australia five days prior. Larval *A.*

amurensis are not expected to be present in Hobart water in May (Bruce *et al.* 1995) and *A. amurensis* has not been reported in Port Pirie.

The ballast water samples were taken through hatch coverings by vertical hauling of plankton net (100 µm mesh). From each of the six sampled tanks, three samples of 320 litres each were collected. Plankton were filtered from seawater through a 60 µm sieve, rinsed with 70 % ethanol and stored in 95% ethanol. The plankton obtained from all 12 Hobart water samples were pooled, in order to homogenize the background composition of each sample and then divided into 24 equal parts. Each of these 24 samples represents filtrate from 160 litres of ballast water; the settled volume of plankton in each sample was approximately 2 millilitres. Eighteen of the 24 samples were spiked with either 200 (n=2), 100 (n=2), 50 (n=2), 20 (n=2) 10 (n=3), 5 (n=3), or 1 (n=4) *Asterias amurensis* larvae, to simulate various seastar larval densities. Four samples were left un-spiked to serve as negative controls and two samples were reserved for reference purposes. Plankton samples from Port Pirie (n=6) water were not pooled. These samples were spiked with 10 (n=2) or 2 (n=2) *A. amurensis* larvae; the remaining two samples were left unspiked. Filtration, DNA extraction and nested PCR for all ballast water samples were performed as described above. Between 2 and 5 ng of DNA extract was used for each PCR. To confirm that the nested PCR method would detect *A. amurensis* larvae at densities naturally present in the environment, six plankton samples (410 litres each) were collected from the Derwent River, Hobart during the species' spawning season (September 2002).

Species discrimination

In order to encompass potential variation within *Asterias amurensis*, our PCR test was designed conservatively and therefore will detect other non-Australian species within the genus *Asterias*. We used DGGE to confirm that positive results were *A. amurensis*. DGGE separates PCR products that contain sequence differences (for a detailed description of DGGE see Nollau and Wagener (1997) and references within). To illustrate the utility of the approach, *Asterias* positive PCR products obtained using *A. amurensis*, *A. rubens* and *A. forbesi* genomic DNA were separated using the DCode™ system (Bio-Rad, Hercules CA). The forward primer was redesigned to incorporate a GC clamp for all samples separated by this method (Sheffield *et al.* 1989); all other PCR conditions were the same as in the standard *Asterias*-specific PCR. To determine a run-time that resulted in good separation of bands, time-series analysis on a 30-70% parallel gradient gel (6% acrylamide) was used. In some runs, heteroduplex molecules were formed by heat denaturation and reannealing of PCR products in the presence of amplified DNA from *A. amurensis*. This step results in the formation of heteroduplex DNA molecules (when two types of DNA are present), which form bands on a DGGE gel distinct from homoduplex DNA, increasing discriminating power of the method. To form heteroduplices, equal amounts of PCR products from the test sample and *A. amurensis* were mixed, denatured at 95°C for 2 minutes, incubated at 65°C for 1 hour and left for 2 hours at room temperature. All solutions for DGGE were prepared following the DCode™ system manual and gels were poured using a Model 475 Gradient Delivery System (Bio-Rad, Hercules CA). Gels were stained with ethidium bromide and viewed under UV light.

2.3 Results

Sequence analysis

Mitochondrial COI sequence data (619 nucleotides) were obtained from 25 individuals belonging to 11 seastar species. These sequences have been deposited in GenBank (accession numbers AY134989 - AY135013). Partial sequences of this region were also obtained for two samples of *Asterias rubens* (119 nucleotides). Alignment of the primer binding region (45 nucleotides) shows a minimum of 7 nucleotide differences between *Asterias amurensis* and the endemic Australian seastar taxa that we sampled (Table 2.2), with *Petricia vernicina* exhibiting closest similarity. Within *A. amurensis*, eight of the sequenced individuals matched the primer sequences identically and one individual (from Mutsu Bay, Japan) had a single nucleotide mismatch. *A. rubens* sequences show 2 or 3 mismatches over the primer region, while the *Asterias forbesi* sequences show 2 nucleotide differences.

Asterias specific PCR

Amplifications were carried out using the primers CASF1 and CASR1 on 48 samples from 13 endemic Australian seastar species to test the specificity of the *Asterias*-specific PCR (for example gel see Fig. 2.1). All samples produced negative results at an annealing temperature of 55°C, with the exception of *Petricia vernicina* (Table 2.3). This result is consistent with the sequence data since this species had the fewest nucleotide changes (seven) in primer sequence compared with *Asterias amurensis* (Table 2.2). When amplified with the *Asterias*-specific primers (CASF1 and CASR1) at higher stringency (annealing temperature of 61°C), no positive results were obtained with seven *P. vernicina* individuals. All further PCR tests were done with an annealing temperature of 61°C. The specificity of the nested PCR approach was confirmed with each of the species. The *Asterias*-specific PCR was carried out on 56 *A. amurensis* samples, including specimens from Tasmania, Russia and Japan, all of which produced positive results. Amplification also occurred using template DNA from *Asterias rubens* and *Asterias forbesi*. These results are summarised in Table 2.3.

To confirm that the test would work on individually isolated larva, 40 *Asterias amurensis* bipinnaria stage larvae were assayed. Every individual produced a strong band using the standard PCR method. Larvae from other seastar species were not available but individual ova (n=40) dissected from *Petricia vernicina* (the most similar Australian non-specific target tested) produced negative results. The *P. vernicina* single ova samples were the only templates which failed to amplify using the 18S ribosomal DNA positive control primers. Subsequent amplification of *P. vernicina* ova with the mtDNA primers ECOLa and HCO was successful. The failure using the 18S ribosomal primers was most likely due to the low copy number of the 18S rDNA in ova relative to mitochondrial copy number.

Table 2. *Asterias*-specific PCR primers aligned with corresponding sequence obtained from 12 species of seastar (a dot indicates the nucleotide is the same as in the top sequence). Numbers in parenthesis indicate number of individuals sequenced. The *A. amurensis* sequences include samples collected from Australia, Japan and Russia. The *A. rubens* and *A. forbesi* samples are from Belgium and Atlantic Canada respectively. The remaining seastars were collected in Tasmania.

Taxa	n		*Asterias* F →	←*Asterias* R	
Asterias amurensis	8	**5'**	GCACAACCGGGATCTTTACTTCAAG	ATAGGAGGATTTGGTAAATG	**3'**
*A. amurensis**	1		.................G.......	....	
A. rubens	1		........A..G.............	....	
A. rubens	1		........A..G.............	.....G..............	
A. forbesi	3		........A................	.....G..............	
Cocsinasterias muricata	2		..T.....A.....CC....A....	.....C........A..C..	
Uniophora granifera	2		........T..G...C.CT.A....	.....C...........T..	
Patiriella calcar	1		..C..G..A..T..AC.C..A....	.....G..T..C..A..C..	
Tosia magnifica	1		..C.....A.....CC..T.A....	..T...........A..C..	
T. australis	1		........A......C....C....	..T.....C..C..A..C..	
Nectria ocellata	1		........C......C....A....	..T..G..C.....A..T..	
Echinaster arcystasus	2		..C.....A.....C.....A....	.....T.....C..A..C..	
Plectaster decanus	1		........A..T...C.T...A...	..T...........G..T..	
Petricia vernicina	2		..T.....T..T..A..G.......	.....C........G.....	

* specimen collected from Mutsu Bay, Japan

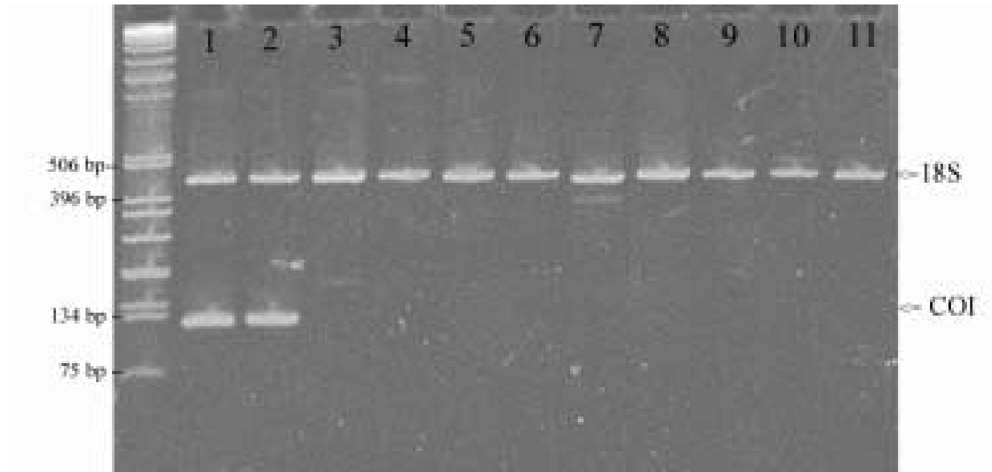


Fig. 2.1 A representative gel photograph showing PCR products separated on a 7.5% polyacrylamide gel. The upper band is the positive control reaction (18S) and the lower band the *Asterias*-specific (COI) PCR product. The left lane contains standard size markers (1Kb DNA ladder, Invitrogen). Templates for samples 1 and 2 were *A. amurensis* genomic DNA from Tasmania (1) and Japan (2). Samples 3-11 used genomic DNA from Australian seastars as templates: *Patiriella calcar* (3), *P. regularis* (4), *Tosia magnifica* (5), *Nectria ocellata* (6), *Echinaster arcystasus* (7), *Plectaster decanus* (8), *T. australis* (9), *Coscinasterias muricata* (10) and *Uniophora granifera* (11).

Table 2.3 List of seastar species included in this study and results of specificity trials of the *Asterias*-specific primer pair using PCR amplification (+ indicates clearly visible band on a 1.8% agarose gel stained with ethidium bromide, - indicates no visible band). Template for PCR was genomic DNA extracted from adult seastars, all samples gave PCR products when amplified using 18S rDNA positive control primers

Family	Species	Collection Location	n	*Asterias* PCR Result (Annealing temp.)
Asteriidae	*Asterias amurensis*	Australia-		
		Hobart	16	
		Japan-		
		Yochi	2	
		Nemuro Bay	3	
		Suruga Bay	7	
		Ariake Sea	4	
		Mutsu Bay	2	
		Tokyo Bay	2	
		Russia -		
		Vladivostok	20	
			Total=56	All + (61°C)
Asteriidae	*A. rubens*	Belgium	3	+ (61°C)
Asteriidae	*A. forbesi*	Atlantic Canada	3	+ (61°C)
Asteriidae	*Coscinasterias muricata*	Tasmania	9	- (55°C)
Asteriidae	*Uniophora granifera*	Tasmania	8	- (55°C)
Asterinidae	*Patiriella calcar*	Tasmania	5	- (55°C)
Asterinidae	*P. regularis*	Tasmania	5	- (55°C)
Asterinidae	*P. brevispina*	Tasmania	1	- (55°C)
Goniasteridae	*Tosia magnifica*	Tasmania	4	- (55°C)
Goniasteridae	*T. australis*	Tasmania	2	- (55°C)
Oreasteridae	*Nectria ocellata*	Tasmania	2	- (55°C)
Echinasteridae	*Echinaster arcystasus*	Tasmania	1	- (55°C)
Echinasteridae	*Plectaster decanus*	Tasmania	2	- (55 °C)
Asteropseidae	*Petricia vernicina*	Tasmania	7	+ (55°C)
				- (61°C)
Goniasteridae	*Pentagonaster dubeni*	Tasmania	1	- (55 °C)

Simulated ballast water testing

The average weight of plankton in 22 Hobart ballast water samples was 230 mg (SD 19 mg) after vacuum filtration. DNA extraction efficiency with these samples was relatively low when compared with fresh plankton samples, with the amount of DNA recovered ranging between 2.0 and 5.0 μg per sample. Results of the *Asterias*-specific nested PCR carried out on these samples are summarized in Figure 2.2. The unspiked control ballast water plankton samples were consistently negative (Fig. 2.2, lanes 2-5). Among the spiked samples, all samples that had >10 larvae successfully amplified the expected 119 bp band (Fig 2.2, COI arrows). We did not reach a lower sensitivity threshold with this test, however, at the low concentrations of spiked *A. amurensis* larva (1 and 5 individuals) some of the PCR tests produced false negative results (Fig 2.2, lanes 7 and 12).

The amount of plankton recovered from the 5-day old Port Pirie ballast water was considerably less than the recently loaded Hobart ballast water, ranging between 17-122 mg. The amount of DNA recovered was between 4.4-22.8 μg, consistent with our expectations based on preliminary DNA extractions from plankton samples. The *Asterias*-specific PCR produced strong bands in the samples spiked with 2 larvae and 10 larvae; no amplification occurred in the unspiked negative control reactions.

The lack of a PCR product in the unspiked control reactions indicate non-specific amplification products were not obtained from a diverse group of estuarine planktonic organisms commonly found at these locations at this time of year. We processed six unspiked environmental plankton samples (~ 150 mg total plankton in each) collected during the *Asterias amurensis* spawning season (September) and all samples produced positive *Asterias* specific PCR result.

Species discrimination

Separation of the PCR products obtained from three species of *Asterias* was accomplished using DGGE. The best separation of bands was achieved using a 30-70% parallel gradient, 6% acrylamide gel with running conditions of 60 volts for 5 hours 15 minutes at 56°C. Under these conditions, the DGGE detected two allelic variations of *Asterias amurensis* and *Asterias forbesi* and usually separated the three species of *Asterias*. It was occasionally difficult to separate one of the alleles of *A. amurensis* from the band produced by *Asterias rubens*. Incorporation of heteroduplex analysis into the assay increased the resolving power of the DGGE. This analysis generated signature patterns that discriminated between species and detected the alleles within *A. amurensis* (Fig 2.3a; lanes 1-5) and *A. forbesi* (Fig 2.3a; lanes 8-10). Sequence data from these samples indicate that the DGGE-heteroduplex method identified all of the point mutations present (Fig 2.3b). The intra-specific differences in both species were single base substitutions (Figure 2.3b; third position T⇔C transitions).

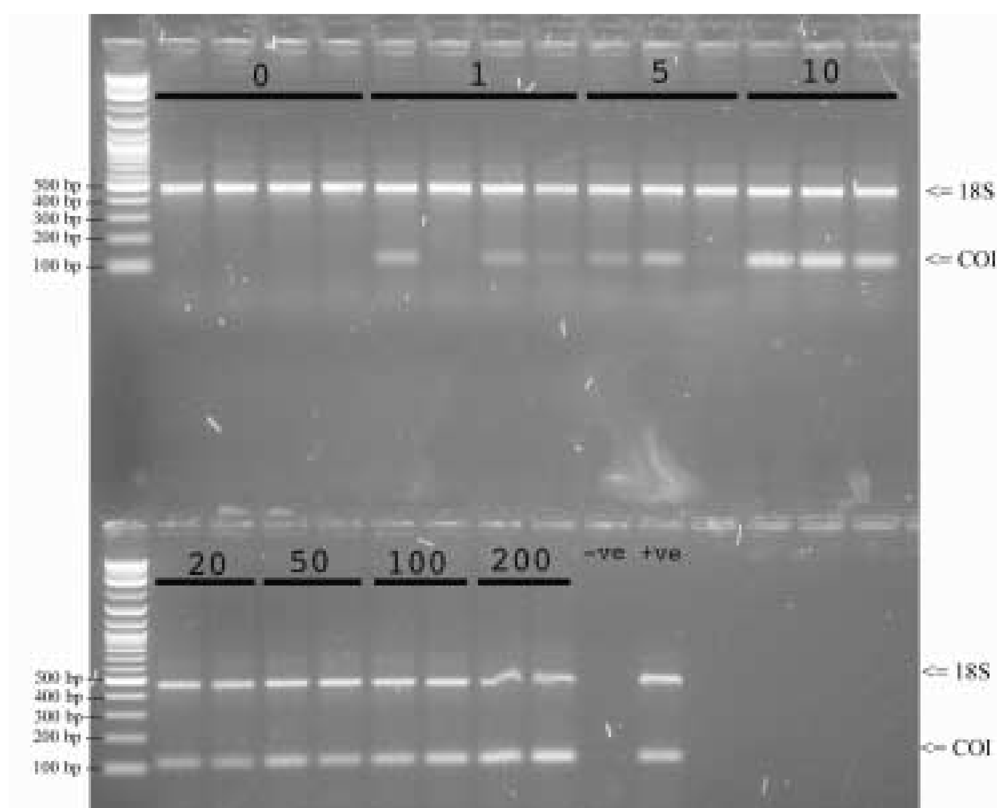
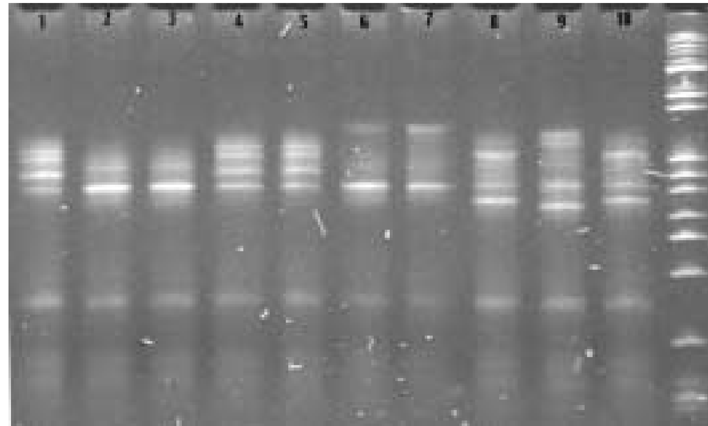


Fig. 2.2 PCR test results from the sensitivity trial using Hobart ballast water separated on a 1.8% agarose gel. The left-hand lanes on both the top and bottom contain standard size markers (2-log ladder, New England Biolabs). In the remaining lanes are PCR products from mixed plankton samples spiked with known numbers of *A. amurensis* larvae. Numbers above the lanes represent the number of larvae spiked in the sample. For each lane, the upper band is the positive control reaction (18S) and the lower band the *Asterias*-specific PCR product (COI).

(a)



(b)

(1) *A. amurensis* 5'

ATGATCAAATTTATAAAGTTATAGTAACTGCTCATGCTCTTGTAATGATATTTTTTATGGTGATGCCTATTATG 3'

(2) *A. amurensis*

.............C..

(3) *A. amurensis*

.............C..

(4) *A. amurensis*

....

(5) *A. amurensis*

....

(6) *A. rubens*

.C...................................C..C....................A............

(7) *A. rubens*

.C...................................C..C....................A............

(8) *A. forbesi*

.............C..........................C..G.................A............

(9) *A. forbesi*

.C...........C..........................C..G.................A............

(10) *A. forbesi*

.............C..........................C..G.................A............

Fig. 2.3 (a) Seastar COI gene fragments separated using a parallel denaturing gradient gel with heteroduplexes formed using DNA from sample #2 (*A. amurensis* from Ariake Sea, Japan). Samples are *A. amurensis* (lanes 1-5), *A. rubens* (lanes 6 and 7) and *A. forbesi* (lanes 8-10). This technique allows for separation of mtDNA from the three species of *Asterias* and identifies variation within *A. amurensis* and *A. forbesi*. In the right hand lane is a DNA ladder (2-log ladder, New England Biolabs)

Fig. 2.3 (b) Sequence data (74 bp) between the specific primers obtained from the samples run on the gel. A dot indicates the nucleotide is the same as in the first *A. amurensis* sequence.

2.4 Discussion

Previous work on identification of Australian asteroid larval employed a PCR-based restriction fragment length polymorphism (RFLP) procedure targeting a 1200 bp region of mtDNA (Evans *et al.* 1998). Although this is an efficient tool to identify individual adults or individually isolated larva, the non-specificity of the primers to the genus *Asterias*, preclude its use for species identification in mixed species samples. Using this method to detect *Asterias amurensis* specimens in ballast water would also be complicated as RFLP analysis of Japanese samples revealed nine different haplotypes (Evans *et al.* 1998). The simple *Asterias*-specific PCR test we have developed in the present study overcomes these limitations, making it possible to identify not only isolated *A. amurensis* larvae, but also to detect them in mixed-species environmental samples. As demonstrated, our test is robust enough to incorporate allelic variation within *A. amurensis*.

As in other studies that have used PCR-based techniques to discriminate between closely related specimens, our method relies on identification of group specific DNA fragments. To ensure that native Australian seastars would not produce false positive results we obtained sequence information and tested several Australian taxa. Since it is not feasible to obtain DNA sequence information or test all related taxa, the specificity of a test cannot be known with complete certainty. Australian representatives of the family Asteriidae are the most likely to be genetically similar to *Asterias amurensis*, but stochastic variables rather than relatedness alone can affect small DNA regions critical for primer binding. This is highlighted in the present study by the positive result of *Petricia vernicina* (Asteropseidae) in preliminary low stringency tests and negative results of other species more closely related to *A. amurensis*. Ideally, specimens of all members of Asteriidae recorded in Australian waters (18 species in 11 genera, Rowe and Gates 1995) would have been tested during the present study however several were unavailable. Even without these data we feel the possibility of a false positive from an Australian seastar species is highly unlikely for a number of reasons. First, the Australian genera *Astrostole* and *Allostichaster* have sequences as distinct from the Northern hemisphere *Asterias* as the Australian genera that we did test (sequences available in Genebank). Second, several of the untested Australian Asteriidae are deep-water species (i.e. species in the genera *Coronaster*, *Cosmasterias*, *Perissasterias* and *Stylasterias*). Even in the improbable event that they would be detected, they are unlikely to be present in waters being tested for *A. amurensis* larvae. Lastly, even when we lowered the stringency of the test (by lowering the PCR annealing temperature by 6º), negative results were obtained in the tested Australian Asteriidae - indicating a significant buffer zone. Of course it would be prudent to confirm the identity of a subset of positive results using DGGE as we have described or by sequencing of the PCR product.

Detection of microscopic organisms in mixed species environmental samples has previously been achieved (Morgan and Rogers 2001, Rublee *et al.* 2001), but is considerably more difficult than genetic identification of pure samples (see Godne *et al.* 2001). One of the problems with environmental samples is the low concentration of target DNA. In this regard, the use of mitochondrial genes should have an advantage compared with nuclear genes due to the large number of copies of mtDNA in each cell providing a higher concentration of template for detection. This feature is illustrated in the present study by the failure of 18S rDNA (nuclear) to amplify from single ova whereas mtDNA amplification was successful. Another way to deal with a low concentration of target DNA is the use of nested PCR. Our results

indicate that the use of nested PCR increases sensitivity by at least 100 times (unpub. data). Other studies have demonstrated 10 000 times higher sensitivity of nested compared with standard PCR (Miserez *et al.* 1997).

Experiments with dilutions of pure DNA can provide an absolute lower limit of detection but these levels are unlikely to be reached in reality and are of little practical importance. Our approach to determine the lower level of detection measured the number of *A. amurensis* larvae that consistently produced a positive PCR result when mixed with approximately 200 mg of plankton. With a nested PCR approach, our results gave a value of ten or more larvae. In order to convert this value to the detectable density of *A. amurensis* larvae in the water column, the amount of non-specific organisms present must be considered. Of the ballast water samples we collected, the highest density of total plankton (1656 mg/m^3) would require a density of >80 *A. amurensis* larvae per cubic meter for a consistent positive PCR result from a 200 mg plankton sample. At the lowest background plankton level we encountered (53 mg/ m^3) it would be possible to detect *A. amurensis* at any density greater than three larvae per cubic meter. Typical *A. amurensis* densities in the Derwent River near Hobart during the July-October spawning season were above these thresholds; peak densities were (400-1100/ m^3) (Bruce *et al* . 1995).These calculations indicate the importance of background plankton levels in determining sensitivity relative to volume of water sampled. By increasing the number of samples tested, we could expect to increase sensitivity considerably since positive results were obtained in many of the ballast water samples spiked with less than ten larvae.

The probes reported here were used to analyse fortnightly triplicate samples from three sampling stations in Port Philip Bay between May and December 2002. The first positive results for *Asterias amurensis* was in early May; by the end of May all samples were positive. Samples were all positive until one negative sample in late October; two weeks later all samples were negative and remained that way until the study ended in December (J. Patil unpublished data). Previous morphological studies (e.g. Parry and Cohen 2000), have indicated a shorter larval duration (June to September), perhaps indicating an increased sensitivity of the probe technique over manual sorting at low larval densities, although interannual variability cannot be ruled out.

Sensitivities lower than achieved in this study could be found in environmental samples where chemicals and detritus in the water affected extraction efficiency and the performance of the PCR test. Since the composition and quality of environmental/ballast water samples are expected to vary a great deal, it is difficult to arrive at a standard minimum level of detection. The appropriate use of positive control reactions (i.e. spiking and testing of sub-samples) will be critical for accurate detection of *Asterias* larvae in environmental and ballast water samples.

Since we have shown at least three non-Australian species of *Asterias* will be detected by our PCR test, further analysis is needed to confirm the identity of positive results obtained from ballast water originating outside Australia. The ability to detect species of *Asterias* other than *Asterias amurensis* is beneficial, since the presence of any *Asterias* in Australian waters would be of concern. A common method to differentiate between PCR amplification products is RFLP analysis (e.g. Evans *et al.* 1998). However, in the present study we amplify a small PCR fragment lacking any informative restriction enzyme sites, so we investigated the use of DGGE. This mutation detection technique is commonly used in microbial ecology to separate PCR bands produced from mixed templates (Muyzer 1999) and it can be used to quickly and cheaply screen large numbers of samples (e.g. Miller *et al* . 1999). Standard DGGE detects most sequence differences and when used in conjunction with heteroduplex analysis, can detect close

to 100% of single base pair changes (Nollau and Wagener 1997). All of the sequence variation that we observed within the target fragment for the three *Asterias* species used in this study was identified using combined heteroduplex and DGGE analysis. This resolution indicates that any new species could be distinguished and further characterized through sequencing. The presence of many alleles within a species is a potential weakness of this method, since new alleles cannot be distinguished from a new species. To assess the level of sequence variation in this region we looked at a survey of COI sequence data that is available for *Asterias rubens* from samples collected in eastern North America, Iceland and Europe (Wares and Cunningham 2001). These data (n=48) show no variation within the DNA region used in our DGGE species identification test. Some additional COI sequences are also available in GenBank for *A. amurensis* (n=3) and *Asterias forbesi* (n=8) over the 119bp fragment of interest; these sequences are also identical to the ones identified in the present study. Given this information, our DGGE protocol will reliably identify most *A. amurensis*, *A. forbesi* and *A. rubens* samples and excessive intra-specific variation is unlikely to be a problem.

In situ, rapid and unequivocal detection of alien species in ballast water provides an early warning signal for their potential introduction, and can be used to establish their transportation routes, frequency and where management intervention is most needed. These genetic probes were developed to detect *Asterias* in ballast water samples collected as part of an Australian ballast water demonstration project at the Port of Hastings, where *Asterias amurensis* has yet to arrive. The aim of the larger study is to determine the accuracy of predictions of the biological risk assessment (Hayes and Hewitt 2000), used by the Australian Quarantine Inspection Service (AQIS) to manage ballast water. Of particular interest are cases where *A. amurensis* is found in ballast tanks predicted to be low risk. Results from this study will contribute to measuring the reliability of Australia’s compulsory ballast water management program. We are currently extending this approach to other high profile alien marine species either in Australia or likely to arrive in the future. Our long-term aim is to provide a rapid, high throughput and cost-effective method for routinely screening domestic and foreign ballast water for alien species that have the potential to cause environmental and economic damage to Australia’s marine environment.

3. *GYMNODINIUM CATENATUM* [2]

3.1 Introduction

Marine species are being transported around the world at a rate that is increasing exponentially (Cohen and Carlton 1998). Many species are – 10,000 at any one time (Carlton 1996) are transported in the ballast water of ships, oil rigs and even racing yachts. Others are transported on the hulls, in the sea chests of commercial and recreational vessels or on their gear and sometimes in their cargos of marine produce (e.g. Hewitt *et al.* 2004). Most of these species will not survive the vessel voyage and the discharge into a foreign environment. Even fewer will establish a self sustaining population in the species new environment, and of those that establish still fewer will become invasive, threatening the marine environment, industries and amenities. But some will, and it is these species we need to concentrate and understand and manage. The first step in reducing the risk from this relatively small number of likely invasive species is to identify them (e.g. Hayes and Sliwa 2003). Then one will need to establish which of the myriad and possible vectors from which of the world's ports transport them around the oceans. Lastly if we are to eradicate or respond to those that escape preventative measures in place, we will need to identify them rapidly and take corrective action (Bax *et al.* 2002).

Many marine species are transported in their early larval stages in the plankton or as biofouling when they are indistinguishable from one another using conventional taxonomic means. What is needed is a technique that can distinguish the most threatening species rapidly and efficiently, preferably with the minimum of cost and time-consuming sample sorting. Studies on genetic introgression (e.g. Echelle and Echelle 1997), identification of source population of introduced species (e.g. Scheffelke *et al.* 2002) and understanding of invasion dynamics (e.g. Geller *et al.* 1994) have all benefited by molecular genetic tools. Further, a variety of molecular methods have been used for the identification of planktonic organisms from environmental samples (see Olson *et al.* 1991; Scholin *et al.* 1999; Hill *et al.* 2001). In particular, a PCR approach in conjunction with morphological sorting has been used to study the distribution and abundance of microreticulate dinoflagellates (including *G. catenatum*) from several locations around the world (Bolch and Reynolds 2002). However, the application of molecular techniques to the detection of exotic species in ballast water has not been possible to species level (Drake *et al.* 2002) or has required isolation and culture of larvae prior to molecular verification (Geller *et al.* 1994). More recently it has been shown that a two-step nested PCR targeted at the mitochondrial (mt) COI locus can specifically and reliably detect the seastar *Asterias amurensis* larvae from ballast water samples (Deagle *et al*. 2003).

The aim of the present study was to develop a simple genetic method to detect cells and cysts of the toxic dinoflagellate *G. catenatum* in ballast water and environmental samples. This dinoflagellate was first described from the Gulf of California in 1943 (Graham 1943) and has been subsequently linked with paralytic shellfish poisoning (PSP) in humans (e.g. Estrada *et al.* 1984; Mee *et al.* 1986). The known global distribution of the species has increased rapidly in the last decade, occurring in estuarine and coastal waters of both tropical and temperate regions (Hallegraeff and Fraga 1998). In Australia, *G. catenatum* was first reported in southern

[2] This chapter is in review (Biological Invasions); "Development and evaluation of a PCR based assay for detection of the toxic dinoflagellate, *Gymnodinium catenatum* in ballast water samples" (Patil *et al.*).

Tasmania, in association with PSP leading to closure of commercial shellfish farms (Hallegraeff and Sumner 1986). Absence of the *G. catenatum* cells in historical plankton samples prior to 1980 and the absence of its distinctive resting cysts in ^{230}P- or ^{137}Cs-dated sediment cores prior to 1972 led to the hypothesis that it was introduced into Australian waters. (McMinn *et al.* 1997). Whilst it is plausible that the natural dispersal of the dinoflagellate by oceanic and coastal currents might have contributed to its introduction into Australia, the known coastal distribution with specific micronutrient requirements (Doblin *et al.* 2000) suggests that this is unlikely (Blackburn *et al*. 2001) and does not explain its absence prior to 1972. Introductions might have occurred via the ballast water discharge of ships visiting southern Tasmania from Japan/Korea since 1972 (wood-chip carriers) or from Spain/Portugal during the 1960s (fruit carriers), or associated with the introduction of Pacific oyster from Japan into southern Tasmania in 1943 (McMinn *et al.* 1997). Ballast water has been implicated as a vector for its spread within Australia (Hallegraeff and Bolch 1992; Bolch *et al.* 1999a).

In lieu of limited sequence data on the mitochondrial genome of dinoflagellates, we targeted both small subunit (SSU) and large subunit (LSU) ribosomal DNA (rDNA) loci for developing *G. catenatum* specific probes. The SSU (Saunders *et al.* 1997) and LSU (Daugbjerg *et al.* 2000) rDNA loci have been extensively used to address phylogeny and evolutionary history of dinoflagellates. The existence of hundreds of copies of these ribosomal genes in nuclear genome of eukaryotes makes them nearly as abundant as mtDNA and therefore expected it to cause little or no compromise on detection levels. Presented here is a method that detects *G. catenatum* DNA, based on specific amplification of the LSU rDNA locus. In addition, a two-step nested PCR approach was developed to boost the detection level in environmental/ballast water samples. The sensitivity of nested PCR was evaluated by carrying out trials on ballast water samples that were spiked with a known number of cysts.

3.2 Materials and Methods

Strains and cultures

Cultures of most species and strains of dinoflagellates used in this study were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Collection of Living Microalage (http://www.marine.csiro.au/microalgae/collection.html). The origin and isolation details of the species/strains are presented in Table 3.1. The 21 different strains used in this study represent fifteen different species of dinoflagellates. Of the four different strains of *G. catenatum*, two originated from Tasmanian waters and one each from Japan and Portugal. Cellular DNA of *G. nolleri*, *G. microreticulatum, Gyrodinium uncatenum, Karlodinium micrum* and *Karenia umbella* was a gift from Dr. C. Bolch (University of Tasmania, Launceston, Tasmania). *G. catenatum* cysts used in this study were generated by mating between compatible strains (GCDE06 X GCHU11) isolated from Tasmanian waters as described previously (Blackburn *et al*. 2001) and the resulting cysts were isolated and fixed in 90 % ethanol, until required.

DNA extraction and sequencing

Extraction of genomic DNA directly from the dinoflagellate cultures, environmental and ballast water samples were carried out in a similar manner. Briefly, the biomass was concentrated by vacuum filtration through a 5µm pore-sized hydrophilic Durapore Filter (Millipore). The filtrate was briefly air dried, and the weight measured. Filtrate was then transferred to a 1.5ml

tube and DNA was extracted using the DNeasy Plant Kit (QIAGEN) following supplier's instructions. DNA was retrieved in 200µl elution buffer and stored at 4°C.

Published sequences of LSU-rDNA loci from different species of *Gymnodinium* and other dinoflagellates were aligned using CLUSTAL_X (Thompson *et al.* 1997) and a highly variable region flanked by conserved regions was identified. This region corresponds to sequence number 201 to 836 bp of a published *G. catenatum* (Accession: AF375855) LSU-rDNA sequence. Accordingly, primers (DinoLSU201F and DinoLSU836R; see Table 3.2 for sequences) were designed to amplify the 636 bp region from a wide range of dinoflagellates.

Standard PCR reactions were done in a 25µl volume containing 0.2 µM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions were: 94°C for 9 minutes then 35 cycles (94°C, 30s / 54°C, 30s / 72°C, 1 minute) followed by 72°C for 5 minutes.

The amplified PCR products were purified using the QIAquick PCR purification kit (QIAGEN). Sequencing reactions were carried out on both strands, using the original amplification primers, with the ABI Big Dye dideoxy terminator cycle sequencing kit (Applied Biosystems). Electrophoresis was carried out on an ABI-377 automated DNA sequencer and sequence data were edited with Sequence Navigator software (Applied Biosystems). Sequence data were aligned with other known sequences from GenBank and used to assess the level of variation within *G. catenatum* and between this species and other dinoflagellates.

Gymnodinium catenatum-specific PCR

Several suitable primer pairs were identified in the 636 bp LSU-rDNA sequence of *G. catenatum* using the software program OLIGO (Rychlik 1996). Multiple primer sets exhibiting significant interspecific variation were designed and synthesised. In preliminary trials, two pairs (CGCS511F; CGCS721R and CGCS484F; CGCS721R) seemed promising. However the latter pair of CGCS484F and CGCS721R (Table 3.2) turned out to be the more specific of the two and was used in all subsequent work. *G. catenatum*-specific PCR reactions were done in a 25µl volume containing 0.4 µM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions for the *G. catenatum*-specific primers (CGCS484F and CGCS721R) were as follows: 94°C for 9 minutes then 40 cycles (94°C, 30s / 61°C, 30s / 72°C, 15s) followed by 72°C for 5 minutes.

Table 3.1 Dinoflagellate species/strains that were tested with *G. catenatum* specific LSU-rDNA primers and universal SSU-rDNA internal control primers.

Species	Strain	Collection Location	SSU internal control PCR	"*G. catenatum* specific" PCR Result
Gymnodinium catenatum	GCDE08	Derwent estuary, Tasmania	+	+
	GCHA01	Hastings Bay, Tasmania	+	+
	GCJP01	Harimanada, Japan	+	+
	GCPT03	Portugal	+	+
G. nolleri	GDKB03	Kiel Blight, Baltic sea	+	-
G. microreticulatum	GMUR02	Uruguay	+	-
	NC01-2	Newcastle, NSW	+	-
Gymnodinium sp.	CS380-2	Devonport, Tasmania	+	-
	CS381	Huon River, Tasmania	+	-
	CS409	Port Arthur, Tasmania	+	-
Gyrodinium uncatenum	CS289	Bathurst Harbour, Tasmania	+	-
Karlodinium micrum	LIGG03/	Lake Illawara, NSW	+	-
=*G. galatheanum*	CS310			
Karenia umbella	GY2DE	Derwent estuary, Tasmania	+	-
Alexandrium affine	AABB01	Bell Bay, Tasmania	+	-
A. catenella	ACPP01	Port Philip Bay, Victoria	+	-
A. margalefi	AMaDE01	Derwent Estuary, Tasmania	+	-
A. tamarense	ATBB01	Bell Bay, Tasmania	+	-
Heterocaspa niei	CS89	Port Hacking, NSW	+	-
Kryptoperidinium foliaceum	CS291	Port Philip Bay, Victoria	+	-
Scrippsiella sp.	CS297	Port Arthur, Tasmania	+	-
Woloszynskia sp.	CS341	Port Arthur, Tasmania	+	-

Table 3.2 Sequences of primers used in this study

Name	Gene	Sequence (5'-3')	Application	Reference
DinoLSU201F	LSU-rDNA	CGAGACCGATAGCAAACAAGTA	PCR & sequencing	This study
DinoLSU836R	LSU-rDNA	GTCAGTATCGCTACGAGCCTCC	PCR & sequencing	This study
NSF 1179	SSU-rDNA	AATTTGACTCAACACGGG	PCR – Universal positive control	Wuyts *et al* . 2001
NSR 1642	SSU-rDNA	GCGACGGGCGGTGTGTAC	PCR – Universal positive control	Wuyts *et al.* 2001
CGCS511F	LSU-rDNA	TTGTGGGGCTGCGTTGCTTCGTGT	PCR	This study
CGCS484F	LSU-rDNA	CGGGACCCACCAACAAACAGTTCAACC	PCR *G. catenatum*-specific	This study
CGCS721R	LSU-rDNA	ATTGGTCGGCCGCTGATGCTAAGG	PCR *G. catenatum*-specific	This study

The cysts were sorted under a compound microscope and washed twice with GSe medium (Loeblich 1975) to minimize inadvertent transfer of cells. Isolated cysts (1, 5, 8 or 13) transferred directly to PCR tubes with as little of ethanol carryover as possible. Residual ethanol was vacuum dried and rehydrated with 5µl of sterile Milli-Q water. The sample was then twice snap frozen at -80°C and thawed at 37°C to disrupt the cells. The above PCR cocktail was immediately added directly to the tubes and subjected to PCR amplification.

Environmental and ballast water samples were subjected to a two-step nested PCR to enhance sensitivity of the test. Primary enrichment PCR was conducted using the LSU-rDNA primer pair DinoLSU201F and DinoLSU836R (Table 3.2). PCR conditions were the same as the standard PCR described previously (in section DNA extraction and sequencing). The secondary *G. catenatum*-specific PCR was carried out using the primer pair CGCS484F and CGCS721R as described above with one twenty fifth the volume of the primary reaction as template.

A separate PCR reaction was carried out on all samples using universal small sub unit (SSU) ribosomal DNA primers (Table 3.2; NSF1179 and NSR 1642) to confirm suitability of each sample for PCR. PCR reactions were done in a 25µl volume containing 0.2 µM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions were: 94°C for 9 minutes then 35 cycles (94°C, 30s / 54°C, 30s / 72°C, 1 minute) followed by 72°C for 5 minutes.

Aerosol-resistant pipette tips were used with all PCR solutions and negative control reactions were performed with each PCR cocktail. The products of the *G. catenatum*-specific PCR and

the SSU positive control PCR corresponding to each of the samples were either mixed or run separately on a 1.8% agarose gel. All gels were stained with ethidium bromide and visualized under UV light and documented using a Nikon Coolpix digital camera.

Ballast water and environmental sampling

To validate the PCR technique and determine its minimum detection level in field conditions, *in situ* ballast water conditions were simulated. Ballast water samples that were PCR negative when tested with *G. catenatum* specific primers, were spiked with known numbers of *G. catenatum* cysts to demonstrate the feasibility of detection. The "blank" samples came from ballast tanks of Vessel #25 and Vessel #1 that had taken ballast water from Port of Devonport (Tasmania) and Port of Botany Bay (New South Wales) respectively. Sampling was undertaken three days post ballast uptake in October 2002. Although both ports of Devonport and Botany Bay have no known record of *G. catenatum* in plankton or benthic samples, carry over from previous loads of ballast water or their occurrence in these ports can not be ruled out.

A total of 8 ballast water samples were taken by filtering about 250 litres of ballast water through a 20 µm mesh phytoplankton net each time. Plankton samples were rinsed and stored in SET buffered (0.375M NaCl, 2.5mM EDTA, 40mM Tris HCl, pH 7.8) 80% ethanol fixative until required. A sub-sample (roughly about half) from each of the eight samples was pooled, in order to obtain uniform background composition. The pooled sample was then divided into 14 roughly equal parts. Each of these 14 simulated samples represents filtrate from ~72 litres of ballast water; the settled volume of plankton in each sample was approximately 1.2 millilitres. Twelve of the 14 samples were spiked with either 13 (n=1), 10 (n=2) 8 (n=2), 5 (n=2), or 1 (n=3) *G. catenatum* cysts, to simulate different cyst densities. Two samples were left unspiked to serve as negative controls. Filtration, DNA extraction and nested PCR for all ballast water samples were performed as described previously. The DNA was diluted to between 3 and 5 ng/µl for use in PCR.

Eight environmental samples were collected from the Derwent river estuary, southern Tasmania in September 2002, by filtering about 100 litres of water through 20 µm mesh plankton net. The filtrate was collected and fixed in SET buffered ethanol fixative. DNA extraction and nested PCR were carried out as described previously.

3.3 Results

Sequence analysis

Clustal alignment of the partial LSU rDNA sequence data (636 nucleotides) obtained from both in- house sequencing (four strains) and from the GenBank (six strains) of *G. catenatum* revealed that the strains had identical sequences in the region, with the exception of one strain (accession no. AF200672) that had four base mismatches. Sequences corresponding to this region were also obtained for 10 other species of *Gymnodinium* from the GenBank. The alignment of the primer binding region (50 nucleotides) of the species of *Gymnodinium,* showed a minimum of 6 nucleotide differences at the primer binding site between *G. catenatum* and the remaining species analysed, with *G. nolleri* exhibiting closest similarity (Table 3.3). The sequences from all the ten analysed strains of *G. catenatum* were identical at the primer binding site. The primer binding region not only exhibits sufficient variability between the different species of *Gymnodinium*, but also between other dinoflagellates suggesting a potential for its development as a species-specific PCR assays.

Specificity of the PCR assay

To test the specificity of the "*G. catenatum*-specific" PCR, amplifications were carried out on genomic DNA from 21 cultures representing 15 different species of dinoflagellates (Table 3.1). Based on the initial results (prior to testing *G. nolleri*), the primer pair CGCS511F and CGCS721R was identified as "*G. catenatum*-specific", however this pair was unable to discriminate between *G. catenatum* and *G. nolleri* even at an annealing temperature of 61°C (data not shown). As a consequence, a new forward primer (CGCS484F) that increased the mismatch at the primer binding site was designed and tested. PCR results following amplification of DNA from *G. catenatum*, *G. microreticulatum*, *G. nolleri* and two strains of *Gymnodinium sp*., using the new forward primer CGCS484F and the reverse primer CGCS721R are shown in Figure 3.1. As seen in Figure 3.1, the primer pair consistently generated the expected 253 bp amplicon only from the DNA of *G. catenatum* strains (Figure 3.1; lanes 7-10). Two undescribed strains of *Gymnodinium sp.* (Figure 3.1; lanes 2-3) held at the CSIRO Collection of Living Microalgae, two strains of *G. microreticulatum* (Figure 3.1; lanes 4-5) and a strain of *G. nolleri* (Figure 3.1; lane 6) tested in this study returned negative results. Additional PCR results carried out on several other species of dinoflagellates are summarised in Table 3.1. The PCR assay carried out using the universal SSU primer pair as internal control on all the 17 non-*G. catenatum* cultures returned positive (Table 3.1, column 4) implying that the template DNA was adequate for PCR reaction. None of these samples were PCR positive when assayed with "*G. catenatum*-specific" primers (Table 3.1, column 5). The results imply that the primer pair CGCS484F and CGCS721R is highly specific to *G. catenatum.* All further *G. catenatum* specific assays were done using the primer pair CGCS484F and CGCS721R at an annealing temperature of 61°C. The specificity of the primers in a two-step nested PCR approach was confirmed with each of the strains/species.

Table 3.3 *G. catenatum*-specific PCR primers aligned with corresponding sequence from 10 other species of *Gymnodinium* (a dot indicates the nucleotide is the same as in the top sequence). Numbers in parenthesis indicate number of clones from which the sequences were derived. The *G. catenatum* sequences include samples isolated from Australia, Japan, Spain and Portugal. Sequences from four cultures of *G. catenatum*, and one each of *G. nolleri*, *G. microreticulatum* and *G. galatheanum* were sequenced in house and the remaining derived from the GenBank.

Species	n	*G. catenatum* F →	← *G .catenatum* R
G. catenatum	10	**5'**GGGACCCACCAACAAACAGTTCAACC	ATTGGTCGGCCGCTGATGCTAAGG **3'**
G. nolleri	2	A................T.....T.A	......................TA
G. microreticulatum	2	A....T.G.TTGTGT..T.CATTCTT	..........T........A..TA
*G. mikimotoi**	4	T.A..T..TTTTTG...T.C..TCTG	.........TT....G...A..AA
*G. breve**	1	T.A..T..TTTTTG...T.C...CTG	.........TT....G...AG.TA
*G. galatheanum**	2	A.A..T..TGTCT....T.A.TTC.G	.........TT....G...A..TA
G. aureolum	2	A.A..T...TGT..T.TT.C..CT..	..C.......T..........GCA
G. palustre	1	A.T..T.G.TTG.GC.....G.CCT.	..C.......T........G..CA
G. chlorophorum	1	..A..T....TT.G...T.CAA.C.G	..C.......T........A...A
G. fuscum	1	..A.TTTG...C..TGTT.CGTCGA.	..C.......T........A.G.A
G. impudicum	1	A.A..GT...CC..G..GACC.CGGA	..C.......T........A.GCA

Recently reclassified: G. mikimotoi = Karenia mikimotoi; G. breve = Karenia breve; G. galatheanum = Karlodinium micrum

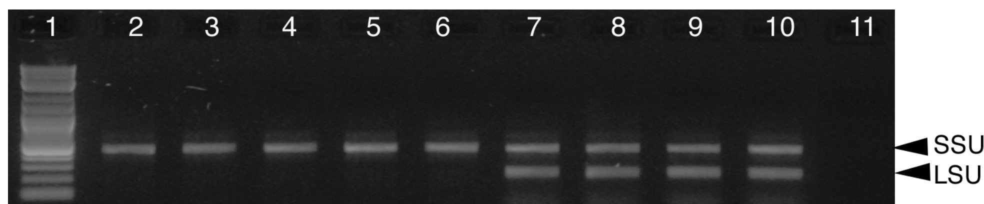


Fig. 3.1 A representative gel photograph showing "*G. catenatum*-specific" PCR products separated on a 1.8% agarose gel. The upper band (c468 bp) is the positive internal control reaction (SSU, arrowhead) and the lower band is the diagnostic *G. catenatum*-specific (LSU, arrowhead) PCR product (c253 bp). *G. catenatum*-specific PCR (primers CGCS484F and CGCS721R) and the internal control PCR (primers NSF 1179 and NSR 1642) were carried out separately and 5 µl (from 25 µl) of PCR products from each amplification corresponding to the target isolate were mixed and separated in the same lane. Lane 1, standard size markers (2-log DNA ladder, New England Biolabs); lane 2, *Gymnodinium* sp. (CS380-2); lane 3, *Gymnodinium* sp (CS381); lane 4-5, *G. microreticulatum* (isolates GMUR02 and NC01-2 respectively)); lanes 6, *G. nolleri* (GDKB03); lanes 7-10, *G. catenatum* (isolates GCDE08, GCHA01, GCJP01 and GCPT03 respectively); lane 11, negative control.

Sensitivity of the PCR assay

To test the sensitivity of the assay directly on isolated cysts, either 1(n=5), 5(n=2), 8(n=2) or 13(n=1) healthy looking cysts of *G. catenatum* were assayed. The *G. catenatum*-specific one-step PCR successfully amplified the expected 253 bp amplicon from all the samples (Figure 3.2; LSU, arrow head). More importantly, all the four replicates that had one cyst as target (Figure 3.2, labelled 1 above the lanes), generated a clear PCR product, suggesting that one can consistently amplify DNA directly from single cysts as templates.

Simulated ballast water testing

The average weight of the filtrate obtained from the 14 ballast water samples following vacuum filtration was 131 mg (SD ± 15 mg). The amount of DNA recovered from the samples ranged between 3.0 and 6.0 µg per sample. Amplification involving standard one-step PCR was unable to amplify the diagnostic PCR product from any of the samples that were spiked with 1, 5, 8, 10 or 13 cysts (data not shown). Therefore, a nested PCR approach as described in material and methods was adopted. As seen in Figure 3.3, both the unspiked control ballast water plankton samples were negative (Figure 3.3, lanes labelled 0).

The absence of *G. catenatum* specific PCR product in these cases was not due to poor quality of DNA or the presence of PCR inhibitors, as the same template DNA yielded abundant PCR product when universal SSU rDNA primer was used (Figure 3.3, all sample lanes; SSU, arrowhead). Among the spiked samples, all samples that had ≥5 cysts successfully amplified the expected PCR (c253 bp) product (Figure 3.3, lanes labelled ≥5). None of the samples spiked with one cyst were positive indicating a lower practical detection threshold of 5 cysts in the assay conditions. The lack of a PCR product in the unspiked control reactions indicates that non-specific amplification products may not be obtained from a group of estuarine planktonic organisms commonly found in the ballast water from these locations at this time of year.

Environmental samples testing

The ability of nested PCR to detect *G. catenatum* in background plankton biomass ranging from 25-150 mg was confirmed with environmental plankton samples collected from Derwent Estuary, Hobart, Tasmania. All the eight environmental samples tested were PCR positive for *G. catenatum* and DNA sequences of all eight PCR products were identical to the Derwent River isolate (GCDE08) held at CSIRO Collection of Living Microalgae as well as the corresponding sequences of the strain GC12V (Accession no. AF375855). These results further confirm the specificity of the probes.

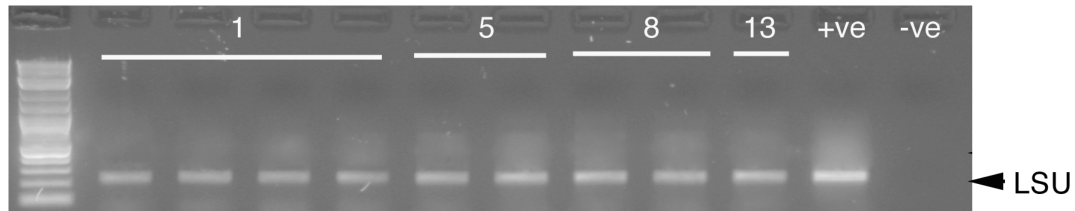


Fig. 3.2 **Results of the sensitivity trial carried out directly on the *G. catenatum* cysts involving single step "*G. catenatum*-specific" PCR. The left-hand most lanes contain standard size marker (2-log ladder, New England Biolabs). Numbers above the remaining lanes indicate PCR products from tubes that contained known numbers (1, 5, 8 or 13) of *G. catenatum* cysts (GCDE06 X GCHU11). LSU, (arrowhead) indicates the diagnostic (c253 bp) *G. catenatum*-specific amplicon; +ve, positive control; -ve, negative control.**

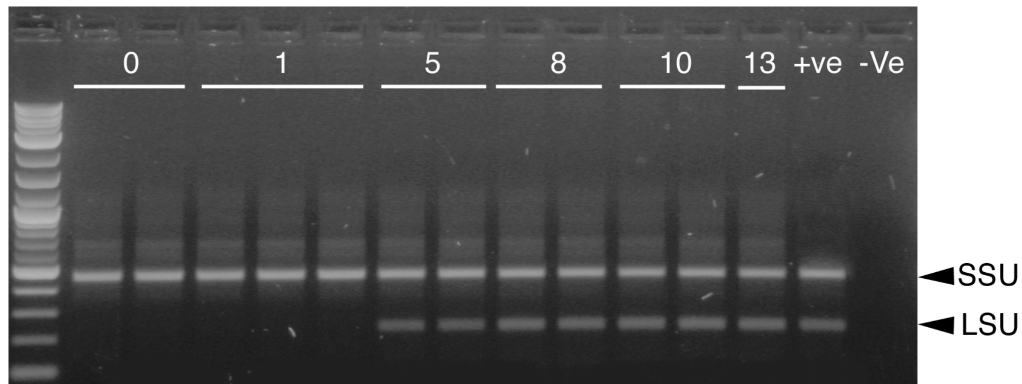


Figure 3.3 **Results of the nested "*G catenatum*-specific" PCR sensitivity trial carried out on spiked ballast water samples. The left-hand lane contains standard size marker (2-log ladder, New England Biolabs). Numbers above the remaining lanes are PCR products from mixed plankton samples spiked with known numbers (0, 1, 5, 8, 10 or 13) of *G. catenatum* cysts (GCDE06 X GCHU11). For each sample lane, the upper band corresponds to positive internal control (SSU, arrowhead) and the lower band corresponds to the diagnostic (c253 bp; LSU, arrowhead) *G. catenatum*-specific amplicon; +ve, positive control; -ve, negative control**

3.4 Discussion

An assay for detecting the toxic dinoflagellate *G. catenatum* in ballast water and environmental samples was developed to assess the risk posed by shipping and other vectors and to provide an early warning of an imminent bloom. This study took advantage of the availability of sequence data of nuclear rDNA loci in general, and that of LSU rDNA in particular, from relatively large number of dinoflagellates (species and strains) in the GenBank, in developing this "*G. catenatum*- specific" PCR assay. Several other studies have used specific PCR amplification as a rapid means for detection and sometimes quantification of morphologically cryptic dinoflagellate species based on SSU rDNA (e.g. Bowers *et al*. 2000, Saito *et al*. 2002) or LSU rDNA (e.g. Haley *et al*. 1999) loci. Our initial attempts to develop a "*G. catenatum* specific" PCR assay based on SSU rDNA locus had limited success. This may be explained by the fact that molecular evolution of the SSU rDNA gene is slower than the LSU rDNA (Daugjberg *et al.* 2000), providing less variability between species and hence a lack of, or limited, options for species-specific PCR probes. Moreover, the presence of highly variable regions intermixed with very conservative areas combined with almost twice the size of SSU rDNA, means that LSU rDNA provides greater flexibility in developing species-specific probes.

Taking advantage of the length and complexity of dinoflagellate LSU rDNA locus we designed PCR primers that appear to be specific for *G. catenatum.* The large sequence diversity at the primer binding site suggests that species- specific probes for other species of dinoflagellates in general and *Gymnodinium* in particular, may be made. Detection of a species by direct amplification of target DNA from a mixed pool of DNA, as achieved in this study eliminates the need for microscopic processing of samples. Most significantly, with suitable controls for specificity and reproducibility of the assay, additional characterization of the PCR products should be unnecessary. In contrast, assays that employ genus- or group-specific or universal primers require additional techniques such as restriction fragment length polymorphism (RFLP), sequencing, hetero duplex mobility (HMA) assay etc for species discrimination, which add to cost and labour. The simple "*G catenatum*-specific" PCR test described here overcomes these limitations, making it possible to identify not only isolated *G. catenatum* cysts and vegetative cells, but more importantly their detection in environmental and ballast water samples.

The specificity of the assay was empirically tested against several species of dinoflagellates notably against the taxonomically most closely related species *G. nolleri* (Daugbjerg *et al.* 2000) to confirm the species-specificity implied by the sequence divergence at the primer binding site. Ideally, specimens of all members of the genera *Gymnodinium* and other dinoflagellates would have been tested to validate the assay. It is, however, not feasible to obtain DNA sequence information on, or test, all related taxa, particularly as some may be closely related and as yet undescribed. It is more likely that as yet undescribed members of cyst (hypnozygote) producing *Gymnodinium* will be genetically similar to *G. catenatum* and hence produce a false positive result. Nonetheless, possibility of a false positive arising from other species appears unlikely as the primer binding site between the dinoflagellate is significantly variable and that of the *G. catenatum* is distinct from the very closely related *G. nolleri* (Table 3.3). Further, even when we lowered the stringency of the test (by lowering the PCR annealing temperature by 4ºC), negative results were obtained in the tested dinoflagellate cultures. It would, however, be prudent to confirm the identity of a subset of positive results by sequencing or other techniques such as RFLP, HMA, Denaturing gradient gel electrophoresis (DGGE) or

southern hybridisation. We have previously demonstrated the utility of DGGE and HMA to discriminate between three different species of *Asterias* (Deagle *et al.* 2003). Such secondary confirmation may assist in discovery of novel or cryptic strains/sub-species of *G. catenatum*. Three undesignated (to species level) strains of *Gymnodinium* spp. (CS380-2, CS381 and CS409) tested in this study did not yield the diagnostic PCR band implying that they are unlikely to be *G. catenatum.*

Although, PCR Detection of microscopic organisms in environmental samples has previously been achieved (Morgan and Rogers 2001, Rublee *et al.* 2001), it is considerably more difficult than genetic identification of pure samples (Godhe *et al.* 2001). One of the problems with environmental samples can be low concentration of target DNA. A common approach to enhance sensitivity while dealing with a low concentration of target DNA is the use of nested PCR, especially in environmental microbial studies (e.g. Miserez *et al.* 1997). However, the nested-PCR requires samples to be transferred to a new tube part way through the amplification increasing chance of cross contamination. The risk of contamination may be minimized by using single-tube nested PCR that use immobilized internal primer pairs (Abath *et al.* 2002).

It has previously been shown that cysts can be used directly as templates for PCR amplification without purification of DNA (Bolch 2001). Similarly, PCR amplification of target DNA using single cysts as templates was observed in this study. Our approach to determine the lower level of detection measured the number of *G. catenatum* cysts that produced a positive PCR result when mixed with approximately 130 mg of plankton. Cysts were preferred over vegetative cells as they were easier to handle and are more likely to survive the rigours of a ballast water journey, and thereby pose a greater invasion risk. With a nested PCR approach, our results suggest that five or more cysts can be detected. This sensitivity is expected to be lower for vegetative cells as they are haploids in relation to the cysts that are formed as a result of fusion of two cells. The above sensitivity estimates are slightly lower than those that were obtained with *Asterias amurensis* larvae (Deagle *et al.* 2003) wherein single bipinnaria larvae could be detected at times. It must, however, be noted that being multicellular, *A. amurensis* larvae are likely to present higher template DNA, compared to single celled *G. catenatum*. Sensitivities lower than those achieved in this study could be found in environmental samples where chemicals and detritus in the water may affect extraction efficiency and or the performance of the PCR test. As the composition and quality of ballast water samples are expected to vary, appropriate use of positive control reactions (i.e. spiking and testing of sub-samples) will be critical for accurate detection of *G. catenatum* in environmental and ballast water samples.

It is known that the *G. catenatum* generally blooms between December-January (early summer) until June-July (autumn-early winter) in southern Tasmanian waters and when in full bloom cell densities range between 10^4 to 10^6 cells l^{-1} (Hallegraeff *et al.* 1989). It is believed that the cells do persist in the water column through out the year, albeit in low numbers during colder months. In this study, *G. catenatum* were successfully detected in environmental planktonic samples collected in September (early spring) 2002 from the Derwent Estuary further supporting that they indeed persist in the water column even when water temperatures are suboptimal for their growth. Although detection of a planktonic species would be sufficient for some applications, it should be possible to extend this assay to quantify the abundance or biomass of the target species using real time PCR (Bowers *et a* . 2000). Quantification would be particularly useful in an environmental monitoring system for predicting bloom formation.

In summary, we have developed a sensitive and specific assay for detection of the toxic dinoflagellate *G. catenatum* that can be used to explore the biology of the organism and its

bloom dynamics. *In situ,* rapid and unequivocal detection of alien species in ballast water provides an early warning signal of their potential translocation, and can be used to establish their transportation routes, frequency and where management intervention is most needed. These genetic probes were developed to detect *G. catenatum* in ballast water samples collected as part of an Australian ballast water demonstration project at the Port of Hastings, where *G. catenatum* has yet to arrive. The aim of the larger study is to determine the accuracy of predictions of the biological risk assessment (Hayes and Hewitt 2000), used by the Australian Quarantine Inspection Service (AQIS) to manage ballast water. Of particular interest, are cases where the target pest species is found in ballast tanks predicted to be free of this species. The assay developed here has been deployed to measuring the reliability of Australia's compulsory ballast water management program and the level of compliance. This is the second of the high profile alien marine species in Australia for which we have developed PCR detection assay. Our long-term aim is to develop an automated, high throughput and cost-effective method for routinely screening domestic and foreign ballast water for the identified alien species that have the potential to cause environmental and economic damage to Australia's marine environment.

4. CRASSOSTREA GIGAS

4.1 Introduction

Among oysters, the Pacific oyster *Crassostrea gigas*, is not only the most farmed species in the world, but also enjoys vast geographical distribution owing largely to deliberate introduction for either establishing new fisheries or to augment collapsed native oyster fisheries (Shatkin *et al.* 1997). From a commercial point of view, *C. gigas* has a formidable track record of successful introductions from Japan to the west coast of North America, Europe, Australia and New Zealand (Korringa 1976; Chew 1990; Menzel 1991). The Pacific oyster was deliberately introduced in Australia at Pittwater, southern Tasmania (Figure 4.1) to establish a sustainable fishery, as well as oyster harbour, Albany, Western Australia but this introduction apparently failed (Thompson 1952). Although the Pittwater introductions performed poorly, subsequent introduction at Port Sorell, northern Tasmania resulted in established populations from Tamar River in the east to the Mersey Estuary of Port Sorell in the west (Thompson 1959). A thriving Pacific oyster industry worth millions to the economy of Tasmania has developed from this initial introduction.

An illegal introduction of Pacific oyster to Port Stevens, New South Wales (NSW), in the early 1980s led to the rapid establishment of a large breeding population that quickly spread to other NSW estuaries. The Pacific oyster was declared a noxious fish by the NSW government in 1985, but after several years of attempted eradication it was clear that the Pacific oyster was there to stay (Holliday and Nell 1987). Pacific oysters have had a devastating effect on the farming and wild fishery of the native Sydney rock oyster (*Saccostrea glomerata*) considered superior in flavour to *C. gigas* (Holliday and Nell 1985). Some Sydney rock oyster farmers in the region have been forced to exit the industry, abandoning their leases in the process. Ecological impacts include partial displacement of the native *Saccostrea* species and the occupation of much of the available habitat by the exotic oyster (Holliday and Nel 1985; Chew 1990).

Concern has also been increasing over the impact that feral Pacific oyster populations are having in Tasmania, including the loss of native oyster beds (*Ostrea angasi* and *Saccostrea glomerata*) and coastal amenity values. A recent survey found feral populations widespread along the north and east coasts of Tasmania, with some individuals exceeding 250 mm in length (Mitchell *et al.* 2000).

Cognisant of the potential negative economic and ecological impacts, the State of Victoria currently bans the farming of Pacific oyster, despite the State being the largest market for the Tasmanian grown Pacific oysters (DNRE 1996). While Victoria has banned farming, thus reducing the risk of intentional introduction, the threat of natural advection from *Crassostrea* populations in its three neighbouring States (Tasmania, New South Wales and South Australia) is ever present.

There is a large shipping traffic between ports in the four States, often involving journeys of less than a day. Ballast water discharge from these vessels is seen as a major threat by Victoria. Australia introduced compulsory ballast water management for ships arriving in Australia from July 2001 and compulsory ballast water management for domestic shipping is currently being

put in place. Ballast water that is considered at high risk of containing listed pest species will be required to be exchanged at sea; ballast water that is considered to have a low risk of containing any of the listed pest species may be discharged in port (Hayes and Hewitt 2000). If ballast water management is to adequately protect Victorian ports from unwanted pests from this source, it must be demonstrated that the ecological risk assessment underpinning the management decisions is correct. This requires the reliable detection and identification of species of concern in ballast water. Unfortunately, and similarly to larvae of most marine organisms, the Pacific oyster larvae cannot be reliably identified using conventional morphological observations, especially when mixed with equally cryptic larvae of other species. Even if it were possible, routine microscopic screening based on morphology is laborious, time-consuming and requires highly skilled expertise. Even the morphology of adult oysters is often of limited value for unambiguous identification due to the large level of phenotypic plasticity as a whole (Boudry *et al.* 2003). The detection, species discrimination, systematics and knowledge of biogeography of molluscs in general, and oysters in particular, have greatly benefited from the development of molecular probes and markers in recent years.

For example, molecular DNA studies have helped to group oyster taxa initially described in separate geographical areas into one single species (Anderson and Allard 1994; Kenchington *et al.* 2002). Molecular markers have also been used to correct misidentification of oyster species and to confirm their geographic range (Ó Foighil *et al.* 1999; Lapègue *et al.* 2002). In another instance it has been suggested, based on the analysis of both mitochondrial and nuclear DNA loci that a new species of *Crassostrea* may exist in Hong Kong (Boudry *et al.* 2003). Molecular tools have helped to differentiate the three species of Asian cupped oysters, *C. gigas*, *C. ariakensis* and *C. sikamea,* (Banks *et al.* 1993; Ó Foighil *et al.* 1995), been used to identify genetically pure stocks of *C. sikamea* introduced into USA (Gaffney *et al.* 1998) and to confirm their presence in Ariake sea (Hedgecock *et al.* 1999). More recently, COI and 16S mitochondrial DNA markers were used to confirm the presence of the European flat oyster *Ostrea edulis* in Oyster harbour, Western Australia (Morton *et al.* 2003).

Despite this widespread development of genetic tools for oysters, there are no genetic tools for the detection and identification of oyster larvae in unsorted plankton samples, such as would be collected in environmental studies including ballast water monitoring. Geller *et al.* (1994) used a polymerase chain reaction (PCR)-based detection method to identify mussel larvae in ballast water but this necessitated the difficult process of isolating and culturing bivalve larvae, prior to PCR analysis. Other studies have demonstrated the feasibility of developing family- (Bell and Grassle 1998), genus- (Frischer *et al.* 2000), and species-specific (Hare *et al.* 2000; Frischer *et al.* 2002) oligonucleotide probes and/or PCR primers for detection of bivalve larvae in plankton samples. Recently we have shown that a two-step, nested PCR approach targeted at the mitochondrial (mt) COI locus of the sea star *Asterias* (Deagle *et al* 2003) and nuclear large sub unit (LSU) locus of the dinoflagellate *Gymnodinium catenatum* (Patil *et al.* in review) can specifically and reliably detect larvae of *Asterias* and cysts and cells of *G. catenatum*, respectively from ballast water samples.

In this study, we have developed PCR-based probes to specifically amplify DNA of *C. gigas* in mixed plankton samples. Results show that *C. gigas* DNA can be specifically amplified and that by using two-step nested PCR it is possible to boost the detection level in complex environmental/ballast water samples.

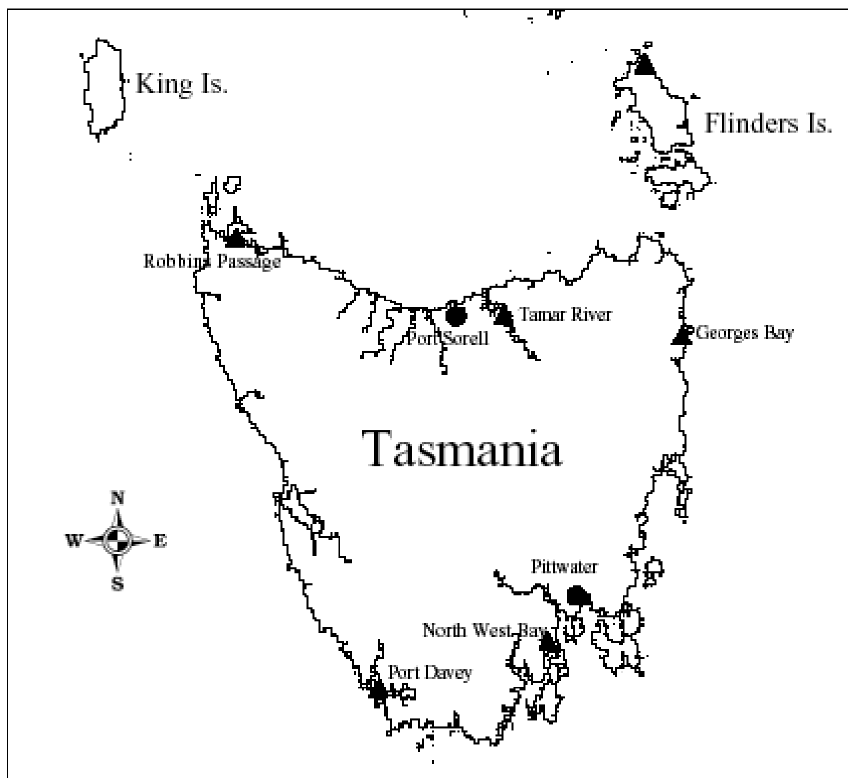


Figure 4.1 Map of Tasmania showing locations of Pacific oyster introductions (Adopted from Thompson 1952 and Mitchell *et al.* 2000)

4.2 Materials and Methods

Sample collection

Adult specimens of the Pacific oyster, *C. gigas* were collected from three different commercial oyster farms along the east coast of Tasmania (n=14; Table 4.1). Additional frozen tissue samples of *C. gigas* (n=12*), Saccostrea glomerata* (n=5) and *Pinctada maxima* (n=1*)* were obtained from Drs S. Appleyard and B.Ward (CSIRO, Australia). Native mud oysters *Ostrea angasi* (n=5) were obtained from a commercial oyster farm in southern Tasmania (Shell Fish Culture Ltd.). Adults of the mussel *Mytilus galloprovincialis* (n=5) were harvested from Derwent estuary, Hobart, Tasmania. The larvae of *C. gigas* were collected 6 (ciliated blastula) and 20 (D-hinge) hours post fertilization (HPF), from a commercial hatchery located in St Helens, Tasmania (Geordy River Aquaculture) and fixed in SET buffered (0.375 M NaCl, 2.5mM EDTA, 40mM Tris HCl, pH 7.8) 80 % ethanol fixative, until required. DNA samples of *C. ariakensis* (n=8) from two populations in China, *C. belcheri* (n=2) from Thailand, and *C. iredalei* (n=2) from China were kindly provided by Dr K Reece (VIMS, USA), and those of *C. virginica* (n=4) by Dr X . Guo (Rutgers University, USA).

DNA extraction and sequencing

Genomic DNA was extracted directly form the adult mantle tissue (30-60 mg) using the DNeasy tissue kit (QIAGEN). Planktonic samples were concentrated by vacuum filtration through a 5µm pore-sized hydrophilic Durapore Filter (Millipore). The filtrate was allowed to air dry briefly, transferred to a 1.5ml tube and DNA was extracted using the DNeasy Plant Kit (QIAGEN) following supplier's instructions. DNA was retrieved in 200µl elution buffer and stored at 4°C.

Amplification and sequencing of the mitochondrial cytochrome oxidase c subunit I (COI) was carried out using the universal primers LCO1490 and HCO2198 (see Table 4.2 for sequence and references). The ~700 bp amplicon corresponds to sequence numbers 15599-16298 of the published *Crassostrea gigas* mitochondrial genome (Accession No. AF177226*)*.

Standard PCR reactions were done in a 25µl volume containing 0.2 µM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer, and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions were: 94°C for 9 minutes then 35 cycles (94°C, 30s / 54°C, 30s / 72°C, 1 minute) followed by 72°C for 5 minutes.

PCR products were purified using the QIAquick PCR purification kit (QIAGEN). Sequencing reactions were carried out on both strands, using the original amplification primers, with the ABI Big Dye dideoxy terminator cycle sequencing kit (Applied Biosystems). Electrophoresis was carried out on an ABI-377 automated DNA sequencer and sequence data were edited with Sequence Navigator software (Applied Biosystems). Sequence data were aligned using CLUSTAL_X (Thompson *et al.* 1997). These sequences along with additional sequences from GenBank were used to assess the level of COI variation within *C. gigas* and between this species and other species of oysters, including the native oysters *O. angasi* and *S. glomerata*.

"C. gigas-specific" primer design

Regions of 20-25 bp that varied between species of oysters, but were conserved and unique to *C. gigas* were identified in the ~700 bp region of the mt COI locus. Following initial identification, the primer sites were analysed for secondary structures and self-complimentarity using OLIGO (Rychlik 1996). Each of the identified oligonucleotide was checked for uniqueness against the COI sequences in the GenBank. Four primer pairs considered to be "*C. gigas*-specific" were synthesised for empirical validation. In preliminary trials, two pairs (CCGS3F; CCGS3R and CCGS4F; CCGS4R) seemed promising. However, the latter pair of CCGS4F and CCGS4R (Table 4.2) turned out to be the more specific of the two pairs and was used in all subsequent work.

C. gigas-specific PCR reactions were done in a 25µl volume containing 0.4 µM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions for the *C. gigas*-specific primers (CCGS4F; CCGS4R) were as follows: 94°C for 9 minutes then 35 cycles (94°C, 30s / 64°C, 30s / 72°C, 15s) followed by 72°C for 5 minutes.

Larvae isolated under a compound microscope were transferred directly to PCR tubes with a minimum of ethanol carryover. Residual ethanol was vacuum dried, then rehydrated with 5µl of sterile Milli-Q water. The sample was then twice snap frozen at -80°C and thawed at 37°C to disrupt the cells. Immediately the PCR cocktail (as above) was added directly to the tubes and subjected to PCR amplification.

A two-step nested PCR was used for environmental samples to enhance the sensitivity of the test. Primary enrichment PCR was conducted using the universal primer pair LCO1490 and HCO2198 (Table 4.2). PCR conditions were same as the standard PCR described previously (in section DNA Extraction and Sequencing). The secondary *C. gigas*-specific PCR was carried out using the primer pair CCGS4F; CCGS4R as described above with one twenty fifth the volume of the primary reaction as template.

A separate PCR reaction was carried out on all samples using universal 18S ribosomal DNA primers (Table 4.2; NSF1179 and NSR 1642) to confirm suitability of each sample for PCR. PCR reactions were done in a 25µl volume containing 0.2 µM of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1X AmpliTaq Gold® buffer, and 0.625 units AmpliTaq Gold® (Applied Biosystems). Thermal cycling conditions were: 94°C for 9 minutes then 35 cycles (94°C, 30s / 54°C, 30s / 72°C, 1 minute) followed by 72°C for 5 minutes.

Aerosol-resistant pipette tips were used with all PCR solutions and negative control reactions were performed with each PCR cocktail. The products of the *C. gigas*-specific PCR and the 18S positive control PCR corresponding to each of the samples were either mixed or run separately on a 1.8% agarose gel. All gels were stained with ethidium bromide, exposed under UV light and documented with a Nikon Coolpix digital camera.

Simulation of environmental samples

To validate the PCR technique and determine its minimum detection level in field conditions, *in situ* controlled environmental conditions were simulated. Plankton samples were collected from the Derwent river estuary, in early September before the recorded time for *C. gigas* spawning in Southern Australia. In all 20 plankton hauls were made with a plankton net, filtering a total water volume of approximately 30,800 l. Plankton samples were rinsed and

stored in SET buffered 80% ethanol fixative. All samples were pooled and then divided into 21 equal parts. Each of these 21 reconstituted samples represents filtrate from about 1460 litres of water sample; the settled volume of plankton in each sample was approximately 1.7 millilitres. Nine samples each were spiked with known numbers (5, 10 or 50) of either the 6HPF (ciliated blastula) or 20HPF (D-hinge) larvae, to simulate different cell densities. Three samples were left un-spiked to serve as negative controls. Filtration, DNA extraction and nested PCR for all spiked samples were performed as described above. The DNA was diluted to between 3 and 5 ng/µl for use in PCR.

4.3 Results

Sequence analysis

Clustal alignment of the partial mt-COI sequence data (nucleotides) of *C. gigas* obtained from both in house sequencing (n=5) and from GenBank (n=7) revealed that the region is highly conserved within the species. All the five individuals we sequenced, as well as four other previously published sequences, were identical to the corresponding region of the published *C. gigas* complete mitochondrial genome sequence (Accession no. AF177226). However two previously published sequences (Accession no. AF280608 and AJ553910) exhibited a single base pair mismatch, while a third sequence (Accession no. AJ553911) exhibited two.

When sequences corresponding to the mt-COI region of *C .gigas* and 14 other species of oysters were aligned, it was possible to identify short sequences that were unique for *C. gigas* to serve as target specific PCR primers, despite the overall high similarity of the mt-COI gene sequence between the oyster species. Alignment of the primer binding region from different species of oysters*,* including the native (to Australia) rock oyster, *S. glomerata* and the mud oyster, *O. angasi* is given in Table 4.3. Over this 50 bp region, the target sequence shows a minimum 4 nucleotide difference between *C. gigas* and the remaining species analysed, with *C. angulata* being most similar. One (Accession no. AJ553910) of the 12 sequences analysed within *C. gigas* exhibited a single nucleotide mismatch at the primer binding site. The native oyster *S. glomerata* and *O. angasi* sequences were distinctly different (14 and 15 bp mismatches respectively) from *C. gigas*. The relatively high sequence difference (8-30%) at the primer binding site between *C. gigas* and the other species of *Crassostrea* may permit development of species specific PCR assay for other members of the genera.

Specificity of the PCR assay

The specificity of the predicted "*C. gigas*-specific" primers was empirically tested by PCR amplification of genomic DNA of several bivalve species. Amplifications were carried out on 33 samples representing 8 species of oysters and a single mussel species (Table 4.1). Although four pairs of primers were designed and tested, three of them were considered less suitable as they had very little difference (1 or 2 bp mismatch) when compared with *C. angulata* sequences, at the primer binding site.

Representative PCR results following amplification of DNA from five different species of *Crassostrea*, two species of native oysters and a species of bivalve*,* using the fourth primer pair (CCGS4F and CCGS4R) are shown in Figure 4.2. The primer pair amplified the expected 339 bp amplicon only from the DNA of *C. gigas* (Figure 4.2; lane 1, bottom band). Samples from five other species of *Crassostrea* (Figure 4.2; lanes 2-6) and a sample each of *O. angasi* (Figure

4.2; lane 7), *S. glomerata* (Figure 4.2; lane 8), and the mussel *Mytilus galloprovincialis* (Figure 4.2; lane 9) returned negative results. In concurrent PCR amplification studies, a universal primer pair targeted at the 18S rDNA generated an expected fragment size of about 460 bp (Figure 4.2; lanes 1-9, top band) from all the samples, indicating that adequate quantity and quality of template DNA was supplied in each PCR reaction.

Additional PCR results giving the number of individuals of each of the species shown in Figure 4.2 and a species of pearl oyster, *Pinctada maxima* tested are presented in Table 4.1. All the 26 samples of *C. gigas* were PCR positive when amplified with the primer pair CCGS4F and CCGS4R (Table 4.1, column 5). In contrast all the remaining 33 samples representing five other species of *Crassostrea, O. angasi, S. glomerata*, *M. galloprovincialis, P. maxima* were PCR negative (Table 4.1, Column 5) when tested with the primers CCGS4F and CCGS4R. Again all the samples successfully amplified the 18s rDNA internal control fragment (Table 4.1, Column 4), further corroborating the specificity of the primer pair CCGS4F and CCGS4R to selectively amplify target DNA derived only from *C. gigas.* This specificity was further tested and confirmed in a two-step nested PCR approach on all the species listed in Table 4.1

Simulated plankton sample testing

It was possible to amplify the *C. gigas* mt COI amplicon using isolated larvae (20 HPF) as templates. Detection accuracy was 100% and 90% when ≥2 and 1 larva were used as templates, respectively (Table 4.4). However, the detection level in the presence of the background sample is of more concern when dealing with unsorted environmental samples. Therefore the ability to detect the *C. gigas* specific mt-COI DNA from plankton samples spiked with known numbers of *C. gigas* larvae was determined*.* The average weight of filtrate from 21 reconstituted environmental water samples used in spiked experiment was 146 mg (SD ±15 mg). The amount of genomic DNA extracted from these samples ranged between 32 and 57 ng/µl and samples were diluted to a concentration of 3-5 ng/µl for PCR reaction. Amplification involving standard PCR was unable to amplify the diagnostic PCR product from any of the samples that were spiked with 5, 10 or 50 of both the 6 and 20 HPF stage larvae (data not shown). Therefore a nested PCR approach as described in material and methods was adopted. Results of the *C. gigas*-specific nested PCR carried out on these samples are summarized in Figure 4.3. The unspiked control ballast water plankton samples were consistently negative (Figure 4.3, lanes labelled 0). The absence of *C. gigas*-specific PCR product in these cases was not due to poor quality of DNA or the presence of PCR inhibitors, as the same template DNA yielded abundant PCR product when universal 18S rDNA primers were used (Figure 4.3, all sample lanes). In samples spiked with 6 HPF larvae, only samples with 50 larvae successfully amplified the expected PCR (339 bp) product (Fig 3, lanes labelled 6HPF; 50). None of the samples spiked with <50 larvae were positive. In contrast, all the samples spiked with 5, 10 or 50 of the 20 HPF larvae were PCR positive when amplified with the *C. gigas* specific primer set (Figure 4.3; lanes labelled 20 HPF; ≥5). The results imply that the detection level of the PCR test varies depending on the developmental stage of the larvae. This was expected as the number of cells per larvae of any multicellular organism increase as larval development progresses. Consequently, the advanced larvae (20HPF in this case) would have yielded more total genomic DNA and hence increased the sensitivity of the assay from 50 down to 5 larvae per sample.

Table 4.1 Species of bivalves that were tested with "*C. gigas* specific" COI primers and universal 18S-rDNA internal control primers.

Species	Sample Location	Sample size	18S-rDNA internal control PCR	"*C.gigas* specific" PCR results
Crassostrea gigas	Various locations, Tasmania	26	+	+ (64°C)
Crassostrea ariakensis	Dafen River, Beihai, Guangxi Province, China	4	+	- (64°C)
Crassostrea ariakensis	Yamen River, Nanshui Town, Guangdong Province, China	4	+	- (64°C)
Crassostrea belcheri	Suratthani, Thailand	2	+	- (64°C)
Crassostrea virginica	USA	4	+	- (64°C)
Crassostrea iredalei	Fujian Province, China	3	+	- (64°C)
Ostrea angasi	Oyster Hatchery, Tasmania	5	+	- (64°C)
Saccostrea glomerata	Port Stevens, NSW	5	+	- (64°C)
Mytilus galloprovincialis	Derwent estuary, Tasmania	5	+	- (64°C)
Pinctada maxima	Western Australia	1	+	- (64°C)

Table 4.2 Sequences of primers used in this study

Name	Gene	Sequence (5'-3')	Application	Reference
LCO 1490 (F)	COI	GGTCAACAAATCATAAAGATATTGG	PCR -Universal	Folmer *et al.* 1994
HCO 2198(R)	COI	TAAACTTCAGGGTGACCAAAAAATCA	PCR -Universal	Folmer *et al.* 1994
NSF 1179	18S-rDNA	AATTTGACTCAACACGGG	PCR –Universal positive control	Wuyts *et al.* 2001
NSR 1642	18S-rDNA	GCGACGGGCGGTGTGTAC	PCR –Universal positive control	Wuyts *et al.* 2001
CCGS4F	COI	TATTCGTTGGAGACTTTATAACCCT	PCR and sequencing *C. gigas* specific	This study
CCGS4R	COI	AAGGCTTAGAATTGCAAGGTCTATA	PCR and sequencing *C. gigas* specific	This study

Table 4.3 *Crassostrea gigas* specific PCR primers aligned with corresponding sequences from 15 other species of Ostreidae (a dot indicates the nucleotide is the same as in the top sequence). Numbers in parenthesis indicate number of individuals from which the sequences were derived.

Species	n	*C.gigas* F →	← *C.gigas* R
C.gigas	11	**5'**TATTCGTTGGAGACTTTATAACCCT	AAGGCTTAGAATTGCAAGGTCTATA **3'**
AJ553910	1	....	........T................
C.angulata	3	............G...........C	........A...............G
C.ariakensis	4	.........A..TT.G....C...A	........................G
	1	.........A..TT.G....C...A	....................T...G
C.nippona	1	.........A...T.G.....T..A	T.......A.........A......
C.sikamea	1	............G..A..C......	T.AA....A............C..G
C.iredalei	1	......C..A..G..G........G	C..C....A...A.....A......
C.virginica	2	......C..A..T.......CT...	T.AC....A...G....AA..C..G
C.belcheri	3	.........A..GT.G........A	T..T..C.A.........A..C..G
O.edulis	***2***	***A........A..GT.A.T...T...***	T.AT....A......C.AA..C..G
O.chilensis	2	A........A...T.G.T.......	C.AT....A......C..A..C..G
	1	A..C.....A..GT.G.T...T...	C.AT....A......C..A..C..G
O.angasi	2	A........A..GT.A.T...T...	T.AT....A......C..A..C..G
	1	A........A..GT.A.T...T...	T.AT....A......C..A..C...
O.aupouria	1	G.....G..A..G..G.T......G	...C...........C.AA.....G
S.glomerata	1	A.....A..A..GT.A.T...T..G	...C..C.....A..T.A.......
S.cucullata	1	A.....A..A..T....T...T..A	C..A....A...A..C.A...C...
*S.kegaki**	1		...C..C.A...A..T..A......

* COI sequence corresponding to the forward primer was unavailable

Table 4.4. Accuracy of the "*C. gigas* specific" PCR assay when 1-5, 20 HPF (D-hinge) larvae were used directly as template in PCR reaction.

No. of 20 HPF larvae	No. tested for PCR	No. of positives
1	20	18 (90%)
2	20	20 (100%)
3	20	20 (100%)
4	20	20 (100%)
5	20	20 (100%)

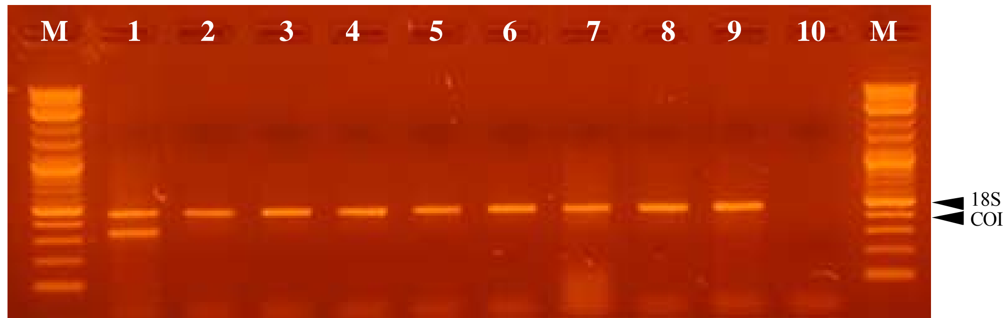


Figure 4.2. A representative gel photograph showing "*C. gigas*-specific" PCR products separated on a 1.8% agarose gel. The upper band (~460 bp) is the positive internal control reaction (18S, arrowhead) and the lower band (~339 bp) is the diagnostic *C. gigas*-specific (COI, arrowhead). *C. gigas*-specific PCR (primers CCGS4F and CCGS4R) and the internal control PCR (primers NSF 1179 and NSR 1642) were carried out separately and 5 µl (from 25 µl) of PCR products from each amplification corresponding to the target isolate were mixed and separated in the same lane. Lane M, standard size markers (2-log DNA ladder, New England Biolabs); lane 1, *C. gigas*; lane 2, *C. virginica*; lanes 3-4, *C. ariakensis* (Dafen and Yamen rivers, China respectively); lanes 5, *C. belcheri;* lane 6, *C. iredalei*; lane 7, *O. angasi*; lane 8, *S. glomerata*; lane 9, *Mytilus galloprovincialis*; lane 10, negative control.

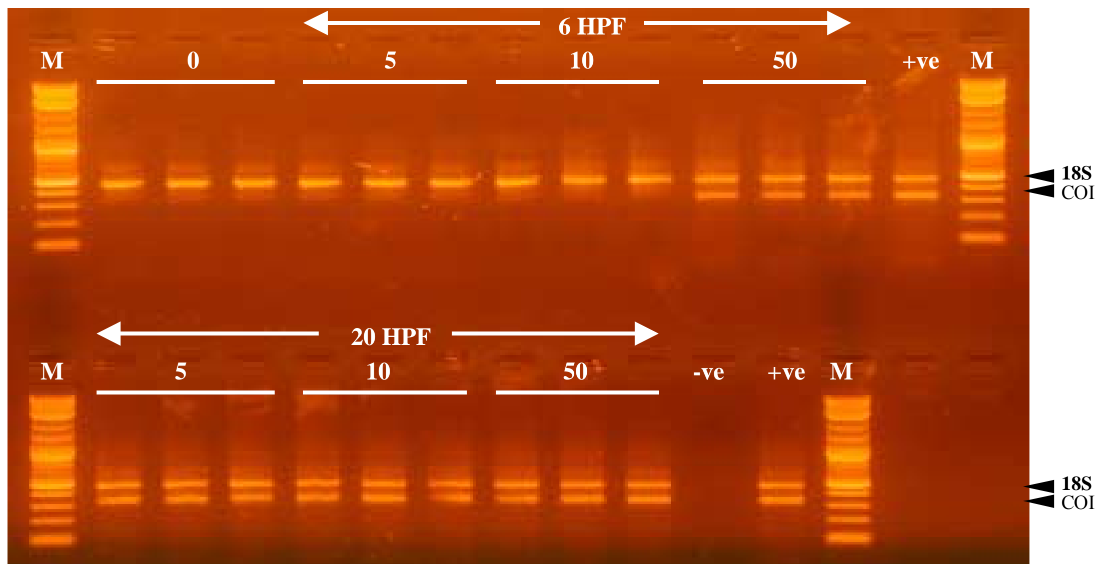


Figure 4.3. Results of the nested "*C. gigas*-specific" PCR sensitivity trial carried out on spiked plankton samples. Lane marked M contains standard size marker (2-log ladder, New England Biolabs). Numbers above the remaining lanes are PCR products from mixed plankton samples spiked with known numbers (0, 5, 10, 50) of *C. gigas* 6HPF (ciliated blastula; top panel) or 20 HPF (D-hinge; bottom panel) stage larvae. For each sample lane, the upper band corresponds to positive internal control (~ 460 bp; 18S, arrowhead) and the lower band corresponds to the diagnostic (~339 bp; COI, arrowhead) *C. gigas*-specific amplicon; +ve, positive control; -ve, negative control.

4.4 Discussion

Rapid and accurate identification of Pacific oyster larvae increases the options available to scientists and managers concerned with the Pacific oyster, either for commercial fisheries and aquaculture, or because of its success in establishing feral populations that are detrimental to native fauna and flora. In this study, we used a combination of genetic sequence comparison and empirical testing to develop *C. gigas* specific PCR primers targeted at the mt-COI locus. These primers can be used to identify *C. gigas* larvae individually or in unsorted plankton samples. The feasibility of specific PCR amplification as a rapid means for detection and or quantification of larvae has been previously demonstrated for the bay scallop (Frischer *et al.* 2000) and the zebra mussel *Dreissena polymorpha* (Frischer *et al*. 2002), based on 18S rDNA loci, and for the sea star *Asterias amurensis* (Deagle *et al.* 2003), based on the mt-COI locus.

High sequence variation at the mt-COI locus between bivalve species in general (Hare *et al.* 2000 and references there in) and Ostreidae in particular (this study) allowed us to design PCR primers that appear to be specific for *C. gigas.* Sequence variation was particularly evident at both primer binding sites (Table 4.3), making it possible to design both primers to be specific for *C. gigas*. Two species specific primers provide improved discrimination than one conserved primer with an opposing species-specific primer (Rocha-Olivares 1998). In an approach similar to ours, the use of two opposing species-specific primers has been shown to provide higher specificity of detecting bivalve larvae, although sorting or isolation of larvae was required prior to PCR amplification (Hare *et al*. 2000). The large interspecific sequence diversity at the primer binding site (Table 4.3) suggests that it might be possible to custom make species-specific probes for other species of oysters. However, including other bivalves than used in this study, especially those from the geographical region where it is intended to use the PCR assay, would be advised to ensure species specificity in a new area.

The PCR assay developed in this study, successfully amplified the target DNA in all the 26 samples of *C. gigas* tested. However, sequence alignment suggested that one (Accession No. AJ553910) of the 12 sequences had a single base pair mismatch at the primer binding site (Table 4.3). It is possible that such intraspecific polymorphism at the primer site might preclude amplification in some samples, producing a false negative result. Genetic characterization of the mt COI locus of *C. gigas* throughout its natural range in Asia as well as its *de novo* range, where it has been introduced, would be required to test the possibility of false negative results. However, the number of false negatives is expected to be small, or zero, as COI seems to have low levels of intraspecific polymorphism in marine bivalves (Hare *et al.*2000 and references there in).

When tested against several species of oysters, notably several species of *Crassostrea,* no false positives were obtained (Figure 4.2 and Table 4.1), implying species specificity of the probes. However, closely related species not initially available during primer design and empirical validation may have enough sequence similarity at the PCR primer site, which would cause a false positive result. Of the described species of oysters, *C. angulata* seems taxonomically most closely related to *C. gigas* (e.g. Ó Foighil *et al.*1998) and also it had the least DNA sequence difference at the primer binding site. It would have been ideal to test the probes on DNA of *C. angulata*, but unfortunately we were unable to acquire the DNA during the course of this study. Despite the absence of empirical data on *C. angulata*, we expect that the probes will not cross react for the following reason. Primer specificity in PCR is mostly conferred by the last few nucleotides at the 3' end of oligonucleotide, so even a single unique nucleotide can be used to direct species-specific PCR (Newton *et al.* 1989; Bottema *et al.*1993). The *C. gigas* specific

primers used here, not only had a two base pair mismatch with that of the corresponding *C. angulata* sequence, but the 3' end nucleotide of both the primers were mismatched.

We consider the primers to be species-specific, not because they have been tested on all potential congeners, but because congeners of the target species have not been recorded from Australian waters. The primers may be species specific outside of this geographic region, but further study would be needed to verify this possibility. In any case, if the primers were used for screening environmental samples, it would be prudent to confirm the identity of a subset of positive results by sequencing or other techniques such as RFLP, HMA, Denaturing gradient gel electrophoresis (DGGE) or southern hybridisation.

Genetic identification of microscopic organisms in environmental samples is more difficult than identifying pure or isolated samples (see Godhe *et al.* 2001). At least two reasons have been proposed to explain this difference. First, low concentration or dilution of target DNA in the background of environmental DNA samples might reduce the success of amplification. Second, PCR inhibitors such as humic material in the environmental samples could compromise the efficiency of PCR reactions. A common approach to enhance sensitivity of gene probes in analysing environmental samples is the use of nested PCR. Our studies indicate that nested PCR increases sensitivity by at least 100 times over standard PCR when dealing with environmental samples; others have demonstrated 10 000 times higher sensitivity when compared with standard PCR (Miserez *et al.* 1997). However the nested-PCR requires samples to be transferred to a new tube part way through the amplification increasing chance of cross contamination. The risk of contamination may however be minimized by adopting stringent laboratory practices and by use of single-tube nested PCR that uses immobilized internal primer pairs (Abath *et al.* 2002).

Detection levels achieved using purified target DNA are of little significance when dealing with mixed environmental samples, and therefore we determined the number of larvae that can be consistently detected when mixed with approximately 150 mg of plankton. It was possible to detect 50 or more of 6HPF and 5 or more of 20HPF larvae. The discrepancy in detection levels for different larval stages can be attributed either to the varying amounts of total DNA associated with each developmental stage or susceptibility of the early stage larvae to loss during pre sample processing (i.e. filtration). The former appears most likely as all post spiked samples were filtered through 5µm pore-sized hydrophilic Durapore Filter, making loss of larvae (40-50 µm) very unlikely.

Relative to several previously described molecular methods, the nested PCR approach described here reduces processing time as it does not involve manual pre sorting or isolation of larvae. Most significantly with suitable controls for specificity and reproducibility of the assay, additional characterization of the PCR products should be unnecessary. In contrast, assays that employ genus or group specific or universal primers require additional technique such as restriction fragment length polymorphism (RFLP; e.g. Banks *et al.*1993; Ó Foighil *et al.*1995), for species discrimination. By using fluorescently labelled primers, the assay developed here can be easily automated from standard PCR to visualization of PCR products using appropriate robotic facilities. Automation would reduce the cost of labour and consumables by minimizing reaction volumes and hence the reagent costs. In the future this assay may be used to quantify larval densities using real time PCR.

4.5 Conclusion

Genetic probes were primarily developed to detect *C. gigas* in ballast water samples collected as part of an Australian ballast water demonstration project at the Port of Hastings. The aim of the larger study is to determine the accuracy of predictions of the biological risk assessment (Hayes and Hewitt 2000), used by the Australian Quarantine Inspection Service (AQIS) to manage ballast water. This is third in a list of high profile alien marine species in Australia for which we have developed PCR detection assay. Our long-term aim is to provide a rapid, high throughput and cost-effective method for routine simultaneous monitoring of marine pest larvae to establish their potential translocation vectors and routes and to put in place management practices that will mitigate their spread to pristine environment. Additionally we believe this assay will contribute towards progress in larval ecology, sustained fishery and aquaculture of this commercially important bivalve. Regions of relatively high sequence diversity flanked by conserved regions at the COI locus enabled the development of this nested PCR assay for detection of *C. gigas* larvae in plankton samples. The relatively high efficiency of this assay stems from its ability to circumvent the need for sorting or isolating larvae prior to PCR analysis or post characterisation of PCR products.

5 EVALUATION OF TYPE II ERROR

5.1 Sample Population

Sampling design

This study was designed to assess the probability of Type II errors arising from three sources:

- the donor port was infected, but listed as uninfected;
- the vessel picked up exotic species, despite local data indicating that the species should have been seasonally absent; or
- the species survived the journey although journey duration is considered long enough for it to have died.

These 3 binary variables lead to 2^3 or 8 combinations that need to be sampled (Figure 5.1). Given an available sampling intensity of ~70 ballast tanks, it was determined that 9 vessels should be identified that satisfy each of the eight combinations. Consultants (Marine and Freshwater Resources Institute) were contracted by the EPA Victoria to sample vessels meeting the criteria using methods provided by CSIRO (Appendix B).

Samples taken

The Hastings project sampled 63 different vessels. Eight vessels were sampled twice, three vessels were sampled on three occasions and one vessel was sampled four times, giving a total of 80 vessel-tank or -sample date combinations (Appendix A). No ancillary data, however, were recorded for one vessel (Emerald Bunker) so the sample results for that vessels cannot be included in this analysis.

Table 5.1 summarises the number of vessels sampled under each of the tank selection rules for the three target species. Tank selection rules 2, 3, and 7 are well represented by more than thirty samples each. Tank selection rules 1 and 8 are only marginally represented by twenty and fourteen samples respectively. The analysis is unable to comment on tank selection rules 4, 5 and 6 because these are not adequately represented within the sample population.

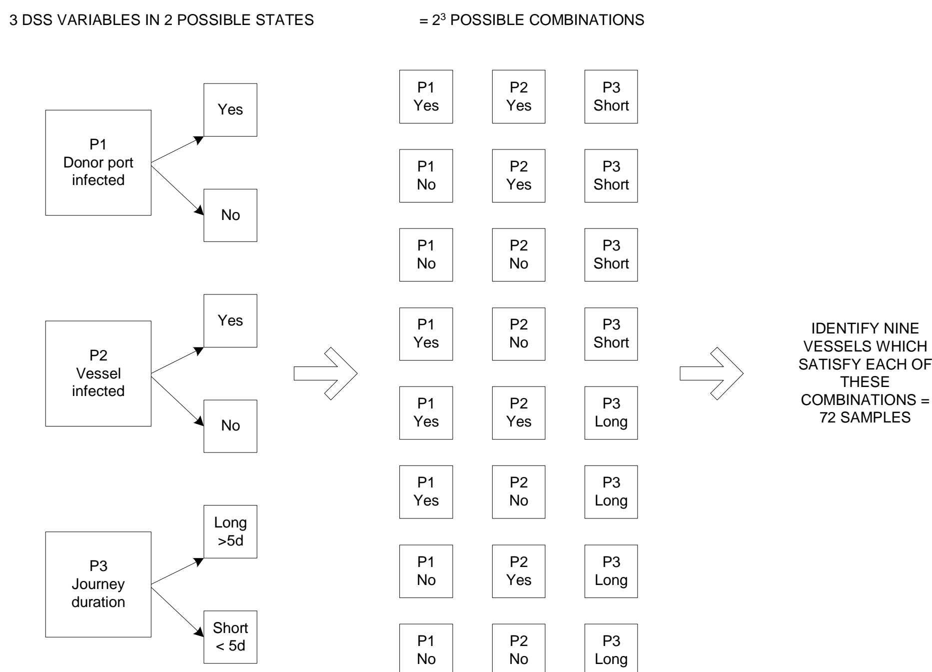


Figure 5.1 Sampling strategies for the Port of Hastings ballast water evaluation project

Table 5.1 Sample population and tank selection rules

Donor port infected	Vessel infected	Journey duration	TSR	Aa samples	Gc samples	Cg samples	Total
1	1	Short	1	2	0	18	20
0	1	Short	2	35	35	43	113
0	0	Short	3	23	25	0	48
1	0	Short	4	0	1	0	1
1	1	Long	5	0	0	1	1
1	0	Long	6	0	0	0	0
0	1	Long	7	16	4	15	35
0	0	Long	8	2	12	0	14
No data				2	3	3	8
Total				80	80	80	240

TSR = Tank selection rule
Aa = *Asterias amurensis*
Gc = *Gymnodinium catenatum*
Cg = *Crassostrea gigas*

For the purposes of this analysis Type II error is defined as the situation where a target species is detected in any one of the samples taken from a vessel whereas the ballast water risk assessment predicted the target species to be absent at the end of the vessel's journey. The presence of the species at the end of the journey does not necessarily represent a high risk. Under the current DSS a high risk occurs only if the species can also survive in the recipient port. This part of the DSS calculations was not tested in this project. In this analysis Type II errors can occur through incorrect information in the DSS:

- scenario 1 - the target species is recorded as absent from the donor port when in reality it is present;
- scenario 2 - the target species is recorded to be absent from the water column (and therefore unavailable to the vessel) when in reality it is present;
- scenario 3 - the target species is predicted to die prior to arrival in the recipient port when in reality it survives.

Type II errors can also occur through errors in the ballast water testing, background error and incorrect data reporting, leading to seven scenarios in all:

- scenario 4 - the sampling equipment is not thoroughly washed and dried between samples and is contaminated with water or sediment residues from a previous vessel;
- scenario 5 - cross-contamination occurs in the laboratory during DNA extraction and analysis;
- scenario 6 – the target species is present in the ballast water because it is completing (or attempting to complete) its life-cycle in the vessel's ballast tank; or,
- scenario 7 – information about the source of the ballast used in the risk assessment is incorrect.

It is possible that the DNA of recently dead cells could be amplified resulting in another Type II error scenario – the detection of non-viable organisms. The likelihood of this type of error, however, is very remote because the DNA of dead cells is subject to autolysis immediately after the death of the cell, unless preserved (mummified) under exceptional environmental conditions.

The information used in this analysis was collected by the sample teams with the co-operation of the ship's master and engineers. In this context it is important to note that ship's masters are not currently required to keep a log of domestic ballast water uptake and discharge. Most vessels do, however, maintain accurate logs of both international and domestic ballast water uptake and discharge.

The remaining sections of this chapter assess the incidence of Type II errors against each of these scenarios. It is important to note that this assessment represents our best-guess as to the most likely explanation for the error. In the many cases the assessment can only be confirmed by collecting additional information and/or a deeper analysis of existing data (e.g. sequencing of archived samples). We also assume throughout the assessment that each of the probes only tests positive for the target species – i.e. we assume no false positives (See chapters 2, 3 and 4 for a discussion of the validity of this assumption).

5.2 *Asterias amurensis*

Table 5.2 summarises the gene probe results for the presence/absence of *Asterias amurensis* relative to the predictions of the first three modules of the ballast water risk assessment. The ballast water risk assessment predicted that *A. amurensis* would not be present in 97% (76/78) of vessels at the end of their journey. The probe confirmed that in 84% (67/78) of cases *A. amurensis* was not detected in the ballast water samples taken at the end of the vessel's journey.

Table 5.2 Risk assessment predictions and gene probe results – *Asterias amurensis*

	Prediction	
Reality	Present	Absent
Present	1	10
Absent	1	66

Thirteen percent (10) of samples predicted to be absent tested positive for *Asterias amurensis*. These samples represent (apparent) Type II errors in the risk assessment. Table 5.3 summarises the ballast characteristics and sample results associated with these apparent errors. The discussion that follows describes the most likely scenario associated with each of the apparent Type II errors recorded here.

Table 5.3 Apparent Type II errors in the risk assessment – *Asterias amurensis*

Vessel name	Sampled	Donor port	Uptake	JD	BE	n	n_+
Vessel #25	01.10.02	Devonport	September	3	No	10	3
Vessel #1	03.10.02	Port Botany	September	4	nd	7	1
Vessel #53	08.10.02	Townsville	September	9	nd	9	6
Vessel #53	08.10.02	MacKay	October	6	nd	9	4
Vessel #61	14.10.02	Burnie	October	1	nd	9	1
Vessel #62	17.10.02	Bluff, New Zealand	October	8	No	9	2
Vessel #58	12.12.02	Bell Bay	December	2	No	9	2
Vessel #50	17.12.02	Portland	December	1	nd	9	1
Vessel #34	21.01.03	Sydney	January	2	nd	6	1
Vessel #57	15.05.03	Burnie	May	2	nd	9	3

JD = Journey duration in days
BE = Ballast exchanged prior to arrival in Melbourne or Geelong
n = number of samples taken from the vessel
n_+ = number of sample that tested positive for *Asterias amurensis*
nd = data was not collected from the vessel by survey personnel

Vessel #25

Vessel #25 was sampled on three occasions and tested positive on the second occasion after being sampled in Geelong on the 1st October, 2002. Positive replicates were recorded on three out of ten replicate samples (90 µm and 20 µm plankton samples, ballast tank #3 port side). The vessel did not exchange the ballast that was sampled prior to arrival in Geelong. The ballast log shows the donor port as Devonport, but also records Esperance as a previous source of ballast water in the sample tank. Lloyds Maritime Information Unit, however, records the vessel's previous ports of call as Hobart, Geelong and Esperance, departing Hobart on the 26th September, 2002.

In this instance ambiguous source information (scenario 7) seems the most likely explanation for the Type II error – i.e. the water sampled was in fact sourced from Hobart five or six days earlier. Another possible explanation is that the vessel did in fact source ballast from Devonport and that *A. amurensis* is present in this port (scenario 1). All other Type II error scenarios are unlikely or not applicable. Scenario 4 seems particularly unlikely due to the six month delay between the sampling of Vessel #25 and the last positive vessel, Vessel #61 (Appendix A).

Vessel #1

Vessel #1 was sampled in Melbourne on the 3rd October, 2002, and tested positive for one sample out of seven (20 µm plankton sample, ballast tank 6WS). According to the vessel log, the ballast water that was sampled was sourced from Port Botany on the 29th September, 2002. It is not clear whether or not the vessel exchanged this ballast prior to arrival in Melbourne. The vessel's previous ports of call were Brisbane, Port Botany and Melbourne, departing the latter on the 20th September, 2002 (LMIU data).

The most likely explanation for the Type II error here is that the sampled ballast water was carried-over from the vessel's previous visit to Melbourne two weeks earlier (scenario 7). For the vessel to be positive, however, would require *Asterias amurensis* larvae to survive for at least 13 days and remain in the ballast tank despite all previous (de)ballasting events in Brisbane and Port Botany – i.e. scenario 3 must also be true. Another possible explanation is that *A. amurensis* is present in Botany Bay (scenario 1). Scenario 4 is possible because Vessel #25 was sampled only two days earlier. Other unlikely explanations are that cross-contamination occurred laboratory (scenarios 5), or *A. amurensis* is present in the ballast tank (scenario 6).

Vessel #53

Two separate tanks (ballast tanks 2 and 5 on the starboard side) of Vessel #53 were sampled in Geelong on the 8th October, 2002. All of the plankton samples from tank 2S were positive; whilst four of the six plankton samples (90 µm and 20 µm) taken from tank 5S were positive. The vessel records the source of the ballast water in these tanks as Townsville and Mackay respectively. The vessels previous ports of call and ballast exchange history for these tanks were not recorded in this instance. LMIU records the vessel's previous ports of call as Incheon (Korean Republic) and Mackay, departing Incheon on the 30th of August, passing Thursday Island on the 25th September and arriving at Mackay on the 1st October, 2002.

In this instance there a number of possible reasons for the Type II error. To start with, scenario 7 seems likely: LMIU records the vessel's previous ports of call as Incheon and Mackay, not

Townsville and Mackay. Incheon is within the native range of *Asterias amurensis* (IUCN bioregion NWP – 4a) and it is recorded as present in the port in the DSS (hence scenario 1 is not applicable). Interestingly, however, this information would not have changed the DSS low risk assignation for this vessel because *A. amurensis* is not thought to spawn in the (Northern hemisphere) summer months of July, August and September, and the journey duration is very long (38 days). Hence scenario's 2 and 3 must also have been true for this to be the case. Scenario 6 is also possible, however, which would explain these apparent anomalies if true. Scenarios 4 seems unlikely – the vessel sampled and analysed before Vessel #53 (Vessel #1) was positive for *A. amurensis* but the five day delay between the sampling of two vessels mitigates against cross contamination of field equipment. Scenario 5 is also unlikely.

Vessel #61

The Vessel #61 trades exclusively across the Bass Strait between Burnie, Tasmania and Melbourne, Victoria. It was sampled in Melbourne on the 14th October, 2002. Only one of the nine samples taken from the vessel (20 µm plankton sample) was found to be positive. The most likely explanation for the Type II error here is the carry-over of ballast water from the vessel's previous visit to Melbourne two days earlier (scenario 7). Scenarios 1 and 6 are also possible reasons for the Type II error in this case. Scenario 6 is particularly worth investigating further because of the nature of this vessel's activities. Scenario 7 is not applicable in this case due to the vessel's trading patterns. Scenario 2 is not applicable because the sample date is within the *Asterias amurensis* larval season - assumed to be June to January in the DSS. Scenario 3 is not applicable because of the short journey duration. Scenario's 4 and 5 are also unlikely in this instance.

Vessel #62

A wing tank and double bottom tank (# 4 port side) of Vessel #62 were sampled in Westernport on the 17th October, 2002. The vessel log indicated that the ballast water in these tanks was sourced from Bluff in New Zealand 8 days earlier and had not be exchanged enroute to Westernport. Two of the nine samples taken from the vessel tested positive for *Asterias*, one planktonic sample and one benthic sample sourced from the sounding pipe. LMIU records the vessel's previous ports of call (and departure dates) as Taichung, Taiwan (15th September), Gove, Australia (26th September) and Bluff, New Zealand (12th October). Taichung lies within the native range of *Asterias amurensis* (IUCN Bioregion NWP-3a) and is recorded as present in the port in the DSS.

The circumstances surrounding the Type II error associated with Vessel #62 are almost identical to those associated with Vessel #53. In both cases the vessel previously visited a port within the native range of *Asterias amurensis*. In both cases the journey durations are very long (30 days for Vessel #62) and ballasting occurred outside of the presumed Northern winter spawning season. In the case of the Vessel #62, however, it is also possible that *A. amurensis* is present in the temperate port of Bluff, New Zealand (scenario 1).

Vessel #58

Vessel #58 was sampled in Melbourne on the 12th December, 2002. Two 20 µm benthic samples taken from the forepeak tank tested positive for *Asterias amurensis*. The vessel indicated that this water was drawn from Bell Bay (Launceston) 2 days earlier and that prior to this, water was sourced from Melbourne and Port Kembla (no dates given). LMIU records the

vessel's last ports of call as Launceston, Australia (departed 10th December), Tauranga (departed 4th December) and Lyttleton, New Zealand (departure date unknown), Sydney (departed 20th November), Port Kembla and Melbourne (departed 17th November).

It is possible that the Type II error in the case of Vessel #58 is the same as that of Vessel #61 and Vessel #1 (scenario 7). In all cases the vessels had drawn ballast water from a port known to be infected with *Asterias amurensis*. This case is less clear cut than that of Vessel #61, however, because the asteroid larvae would have to of survived 25 days in the ballast tank and (presumably) numerous ballasting/de-ballasting events for the water to test positive for *A. amurensis* on the 12th December. The fact that none of the planktonic samples tested positive in this vessel suggests that the larvae, if indeed they were present, were distributed in the bottom of the tank – suggesting that they may have settled in or on the tank. Scenario 6 is therefore possible. It is possible that *Asterias* is present in Lyttelton or Launceston (scenario 1) - both are temperate water ports but the ballast water was sourced very late in the southern hemisphere larval season of *A. amurensis*. Scenarios 4 and 5 are unlikely.

Vessel #50

Vessel #50 was sampled in Melbourne on the 17th December 2002. One plankton sample from the starboard wing tank (#1) was found to be positive. The vessel indicated its last three ports of call as Portland, Brisbane and Mackay, taking on ballast in Portland on the 16th December. No information regarding ballast water exchange is provided.

LMIU records Vessel #50's previous ports of call as Portland, Newcastle, Port Kembla and Mackay – arriving in Mackay on the 28th November. According to LMIU the last time the vessel was in Brisbane was on the 30th May, 2002. It is possible, however, that the LMIU data is in error in this instance – for the week of the 4th to the 11th December, LMIU records the vessel as traveling between Newcastle and Newcastle, whereas the vessel reported that it took ballast on in Brisbane on the 7th December.

The reason for the Type II error in this instance is unclear. It is possible that *Asterias amurensis* is present in Portland (scenario 1) but again the ballast was sourced late in the southern hemisphere larval season. Scenario 3 is not applicable in this context because the ballast water is only a day old. Scenario 4 is unlikely because the last positive vessel to be sampled before Vessel #50 was Vessel #58 five days earlier (Appendix A). Scenario 7 is possible and in the absence of a likely explanation, scenario 5 is also possible. Scenario 6 is considered to be unlikely. It is also possible that the vessel exchanged ballast in the vicinity of an infected port (e.g. Port Phillip Bay) but we are unable to confirm this.

Vessel #34

Vessel #34 was sampled in Melbourne on the 21st January, 2003. One plankton sample from the port topside ballast tank #4 was found to be positive for *Asterias amurensis*. The vessel indicated that this ballast water was sourced from Sydney on the 19th January but that prior to this ballast was sourced from Pusan, Republic of Korea and Osaka, Japan, 15 to 17 days earlier. Pusan and Osaka lie within the native range of *Asterias amurensis* (IUCN bioregion NWP-3b), the ballast was sourced during the northern hemisphere spawning season of *A. amurensis*, and would have been 15-17 days old when it was sampled in Melbourne. Hence the most likely reason for the Type II error here is the carry over of ballast water from either Pusan or Osaka (Scenario 7) and survival of the larvae over a relatively long journey duration (scenario 3).

Vessel #57

The Vessel #57 operates in an identical fashion to the Vessel #61 – it trades exclusively between Burnie and Melbourne. Three of the nine samples taken from sample tank – two 20 µm planktonic samples and one 20 µm benthic sample – tested positive for *Asterias amurensis*. In this case the most likely reason for the Type II error is similar to that of Vessel #61 – carry-over of ballast from the vessel's previous visit to Melbourne. In this instance, however, the ballast was sourced from Melbourne in May – a month before the presumed larval season of *A. amurensis* in Port Phillip Bay[3]. Hence the most likely reasons for the Type II error in this context are scenarios 2 and 7.

It is interesting to note that Vessel #61 and Vessel #57 were sampled on three other occasions, during the assumed larval season of *Asterias amurensis*, and tested negative. In each case the source of the ballast was Burnie, hence these results did not raise concerns. If, however, ballast water carry-over is common on these vessels, as these Type II error results suggest, then we would expect to see more positive results on these vessels. These results highlight the difficulties associated with ballast water sampling and underline the fact that ballast water samples may not be representative of the ballast tank community (Gollasch *et al*. 2003).

5.3 *Crassostrea gigas*

Table 5.4 summarises the gene probe results for the presence/absence of *Crassostrea gigas* relative to the predictions of the first three modules of the ballast water risk assessment. The ballast water risk assessment predicted that *C. gigas* would be not be present in 75% (58/77) of vessels at the end of their journey. The probe confirmed that *C. gigas* was indeed absent in the ballast water of 32% (31/77) of vessels.

Table 5.4 Risk assessment predictions and gene probe results – *Crassostrea gigas*

	Prediction	
Reality	Present	Absent
Present	13	33
Absent	6	25

Forty three percent (25/58) of samples predicted to be negative tested positive for *Crassostrea gigas*. These samples represent (apparent) Type II errors in the risk assessment. Table 5.5 summarises the ballast characteristics and sample results associated with these apparent errors.

[3] The larval season of *Asterias amurensis* in Port Phillip Bay has since been confirmed to be May to October - see Chapter 2.

Table 5.5 Apparent Type II errors in the risk assessment – *Crassostrea gigas*

Vessel name	Sampled	Donor port	Uptake	JD	BE	n	n+
Vessel #1	03.10.02	Port Botany	September	4	nd	7	2
Vessel #53	08.10.02	Townsville	October	9	nd	9	2
Vessel #53	08.10.02	Mackay	October	6	nd	9	2
Vessel #41	22.10.02	Sydney	October	3	No	6	1
Vessel #10	08.11.02	Port Botany	November	4	No	8	2
Vessel #21	09.11.02	Sydney	November	2	No	9	1
Vessel #13	12.11.02	Newcastle	November	3	No	9	3
Vessel #5	15.11.02	Sydney	November	3	nd	9	5
Vessel #51	24.11.02	Melbourne	November	2	nd	9	5
Vessel #48	28.11.02	Port Kembla	November	7	No	9	7
Vessel #12	29.11.02	Brisbane	November	5	No	6	1
Vessel #14	04.12.02	Fremantle	November	4	nd	9	1
Vessel #38	04.12.02	Port Kembla	November	4	nd	9	1
Vessel #52	05.12.02	Kwinana	November	7	nd	6	1
Vessel #16	11.12.02	Sydney	December	2	No	9	6
Vessel #33	13.12.02	Portland	December	2	No	8	3
Vessel #50	17.12.02	Portland	December	1	nd	9	2
Vessel #15	18.12.02	Brisbane	December	3	nd	7	4
Vessel #11	19.12.02	Newcastle	December	?	nd	9	7
Vessel #3	07.01.03	Newcastle	January	4	nd	6	6
Vessel #25	11.01.03	Townsville	January	6	nd	9	8
Vessel #60	14.01.03	Newcastle	January	2	nd	6	4
Vessel #34	21.01.03	Sydney	January	2	nd	6	3
Vessel #12	04.02.03	Newcastle	January	4	nd	8	6
Vessel #44	05.02.03	Port Kembla	February	2	nd	8	3
Vessel #55	10.02.03	Brisbane	January	15	nd	9	7
Vessel #55	10.02.03	Newcastle	February	10	nd	9	7
Vessel #24	14.02.03	Newcastle	February	4	nd	9	7
Vessel #45	18.02.03	Sydney	February	4	nd	9	9
Vessel #11	28.02.03	Newcastle	February	3	nd	9	7
Vessel #9	22.03.03	Newcastle	March	3	nd	9	9
Vessel #17	01.05.03	Sydney	April	3	nd	9	7
Vessel #45	08.05.03	Port Stanvac	May	2	nd	6	4

Of the 33 vessels predicted to be negative but shown to be positive for the presence of *Crassostrea gigas*, 22 (67%) sourced their ballast in the New South Wales ports of Sydney, Port Botany, Newcastle and Port Kembla (Table 5.5). Recent surveys of these ports - Newcastle: 1997, Botany Bay: 1998, Port Kembla: 2000, Sydney 2001 - did not detect the presence of *C. gigas* (Hewitt *et al*. 1998a; Pollard and Pethebridge 2002a; b; Australian Museum Business Services 2002). Anecdotal evidence gathered during this analysis, however, suggests that *C. gigas* is in fact present in these ports. Furthermore, the majority of these positive results (91%) occur in ballast water sourced from these ports between mid-October and late April, coinciding with raised water temperatures and phytoplankton blooms that trigger the summer-autumn spawnings of the oyster and hence larvae would be expected to be in the water column (Gouletquer *et al* 1997; Shatkin *et al.* 1997).

In the early 1980s there was an illegal introduction of *Crassostrea gigas* from Tasmania into Port Stephens (just north of Newcastle) in New South Wales. This resulted in the establishment of a large breeding population that quickly spread to other estuaries. In 1985 the New South Wales Agriculture and Fisheries Department declared the Pacific oyster a noxious fish, making culture and presence of *C. gigas* on a shellfish lease illegal. After several years of trying to eradicate the Pacific oyster, because it rapidly outgrows the native rock oyster *Saccostrea glomerata*, the government ended eradication attempts and allowed the cultivation of *C. gigas* (Holliday and Nell 1987). Established populations of *C. gigas* are now thought to exist in all oyster farming estuaries from Wallis Lake (just north of Port Stephens) south. Recent observations in the Botany Bay/Georges River area by NSW Fisheries found that *C. gigas* accounted for up to 90% of all oysters present and this has been the situation for many years. *Crassostrea gigas* has also been observed in the Hunter River (Newcastle Harbour) and large numbers have established themselves on the foreshore of Sydney Harbour (*pers. comm.*, J. Nell, NSW Fisheries). This analysis has not unearthed similar information for Port Kembla but it appears highly likely that *C. gigas* is also present in this port.

In summary these results suggest that at least three accredited port surveys failed to detect the presence of *Crassostrea gigas*. Thus the most likely explanation for the Type II errors associated with these 22 vessels is scenario 1 - the target species is recorded as absent from the donor port when in reality it is present. Other possible, but less likely, explanations are that *C. gigas* is present in the ballast tanks, piping or sea chests of a large number of vessels (scenario 6), or that *C. gigas* is present in the water column as larvae advected from adjacent coastal waters, but is not established in any of the ports (scenario 1).

For all of these vessels scenario 2 (the target species is recorded to be absent from the water column when in reality it is present) is not applicable because the DSS does not currently make any predictions in this regard, rather it simply records *Crassostrea gigas* as present in the water column all year round due to uncertainties surrounding its reproductive cycle. Information gathered during this analysis suggests a larval season in the southern hemisphere of October to April. However, the positive record from Vessel #1, that sourced water from Port Botany in September, suggests that the larval season may start before October in some areas.

Scenario 3 (the target species is predicted to die prior to arrival in the recipient port when in reality it survives) is also inapplicable because the DSS currently only holds species-specific journey survival data for *Asterias amurensis*. Information collected during this study indicates that larvae are present in the water column for at least 4 weeks.

In light of the discussion above it seems unnecessary to assume that scenario 6 – the target species is present and completing (or attempting to complete) its life-cycle in the vessel's ballast tank - applies to any of these vessels. This is impossible to confirm, however, without additional field effort. Scenario 4 is unlikely in all but four of these 22 vessels because the delay between sampling consecutively (positive) vessels is 3 days or more. Scenario 5 is also considered to be unlikely because of the careful laboratory procedures adhered to through-out this project. The reasons for the Type II error associated with remaining 11 vessels are less obvious – each vessel is discussed individually below.

Vessel #53

Two separate tanks (ballast tanks 2 and 5 on the starboard side) of Vessel #53 were sampled in Geelong on the 8th October, 2002. Two of the nine (20 µm plankton samples) from tank 2S were positive two of the six samples (20 µm benthic samples) taken from tank 5S were also positive. As previously discussed for *Asterias amurensis*, the vessel records the source of the ballast water in these tanks as Townsville and Mackay respectively. The vessel's previous ports of call and ballast exchange history for these tanks were not recorded in this instance. LMIU records the vessel's previous ports of call as Incheon (Korean Republic) and Mackay, departing Incheon on the 30th of August, passing Thursday Island on the 25th September and arriving at Mackay on the 1st October, 2002.

It is unclear from this voyage history how *Crassostrea gigas* came to be on board this vessel. Scenario 7 (ambiguous ballast source) is possible but this does not explain the error well in this case. *Crassostrea gigas* is not recorded from Mackay and is unlikely to survive there (scenario 1). Incheon, however, is within the native range of *C. gigas* (IUCN bioregion NWP – 4a) and the oysters are thought to spawn in the (northern hemisphere) summer months of July, August and September. It seems unlikely, however, that larvae could survive in the ballast tank for six weeks unless they had settled in the tank, or were are able to delay settlement during unfavorable conditions. Scenario 4 seems likely – the vessel sampled and analysed before Vessel #53 (Vessel #1) was positive but the five day delay between the sampling of the two vessels mitigates against cross contamination of field equipment. Cross contamination may have occurred on board the vessel, between the two tanks, if the equipment was not thoroughly washed between samples. Scenario 5 is considered unlikely. In the absence of a likely cause, scenario 6 is considered possible.

Vessel #51

Vessel #51 was sampled in Geelong on the 24th November, 2002, and tested positive for five samples out of nine (20 µm and 90 µm plankton, and 20 µm benthic, samples). LMIU data records that this vessel entered Australian waters via Indonesia from Japan, arriving in Brisbane on the 13th of November, after which it visited Botany Bay (20th November), Melbourne (22nd November) and finally Geelong where it was sampled. The vessel log indicates that the ballast water that was sampled was taken up in Melbourne on the 22nd of November and prior to that from the ocean near Java.

The most likely explanation for the Type II error in this case is that the sampled ballast water was in fact sourced from the vessel's stop over in Botany Bay prior to its arrival in Melbourne (scenarios 1 and 7), as there is no record of *Crassostrea gigas* in the port of Melbourne. Scenario 4 is unlikely because the last positive vessel to be sampled before Vessel #51 was

Vessel #5 nine days earlier (Appendix A). Scenarios 2 and 3 are not applicable, and 4, 5 and 6 are considered unlikely.

Vessel #12

Vessel #12 was sampled in Melbourne on the 29th November, 2002 and tested positive for one sample out of six (90 µm plankton sample, ballast tank #2 port side). The most likely explanation for this positive result is scenario 7 and scenario 1. In this instance the data collected by the sample team states that the vessel's ballast water was sourced from Brisbane on the 23rd and 26th of November. Lloyds Maritime Information Unit, however, records the vessel's previous ports of call as Newcastle (departing 27th November) then Brisbane (departing 23rd November). Hence, it appears that this vessel picked up ballast water in Newcastle after leaving Brisbane, 3 days before being sampled in Melbourne.

Vessel #12 has a regular schedule and every month visits the ports of Brisbane, Newcastle, Melbourne, Adelaide, Hobart, Port Kembla, Newcastle, then leaves for Asia, re-entering Australian waters via Brisbane. It is possible that this result could be caused by *Crassostrea gigas* larvae being picked up in either the NSW or Tasmanian ports during this schedule, if the ballast water is not completely exchanged. Scenario 4 is also possible because the last positive vessel to be sampled before Vessel #12 was Vessel #48 the previous day (Appendix A).

Vessel #14

Vessel #14 was sampled in Melbourne and tested positive in one (20 µm benthic sample) out of nine samples. The LMIU voyage history for this vessel agrees with the recorded log. This vessel left Australia from Fremantle on the 13th of October, 2002, bound for Singapore. It passed through the Suez Canal on the 11th of November, re-entered Australian waters at Fremantle on the 29th before arriving in Melbourne on the 4th of December where it was sampled that day.

The cause of this Type II error in this instance is unclear because we have no record of the vessel's voyage history prior to its passage through the Suez Canal. *Crassostrea gigas* is not recorded from Fremantle but there are two vessels in this study that have shown positive results for *C. gigas* from ballast sourced in Fremantle/Kwinana in November. It is possible, therefore, that *C. gigas* is present in Fremantle (scenario 1). It seems unlikely that the ballast water sourced in Asia in the six weeks before the vessel re-entered Australia would contain *C. gigas* larvae because October is outside the larval season in the northern hemisphere. Scenario 4 is unlikely because the last positive vessel to be sampled before Vessel #14 was Vessel #12 six days earlier (Appendix A). Scenarios 5 and 6 are also considered to be unlikely.

Vessel #52

Vessel #52 was sampled in Melbourne on the 5th of December, 2002, and tested positive for one sample out of six (90 µm plankton sample). The vessel log indicates that the sampled ballast water was sourced from Kwinana on the 28th of November, and before this from Merak, Indonesia (22nd November) and Singapore (16th November). This information agrees with LMIU records of the vessel's movements during this period. This is the second vessel that has tested positive for *Crassostrea gigas* with ballast water sourced from Fremantle/Kwinana suggesting that *C. gigas* may be present in the port (scenario 1). Scenario 4 is also possible, however, because the last positive vessel to be sampled before Vessel #52 was Vessel #38 the previous day (Appendix A).

Vessel #33

Vessel #33 was sampled in Melbourne on the 13th December, 2002, and recorded positive results for three out of eight samples including 20 µm and 90 µm plankton, and 20 µm benthic, samples. The vessel indicated that this water was sourced from Portland two days earlier. The vessel's previous ports of call in Australia were Portland (departed 7th December) and Geelong (departed 4th December). Prior to this its last port of call was Longview, USA from which it departed on the 16th of November.

The cause of the Type II error in this instance is unclear. *Crassostrea gigas* is not recorded from Portland or Geelong. It may be possible that the vessel took up oyster larvae during its ballast operations overseas but we lack information on its voyage history prior to its arrival in Longview. Scenario 4 is possible because the last positive vessel to be sampled before Vessel #33 was Vessel #16 two days earlier (Appendix A). Scenarios 5 and 6 are considered unlikely.

Vessel #50

Vessel #50 was sampled in Melbourne on the 17th December, 2002. Two 20 µm plankton samples from the starboard wing tank (#1) were found to be positive. The vessel indicated its last three ports of call as Portland, Brisbane and Mackay, taking on ballast in Portland on the 16th December. No information regarding ballast water exchange was collected by the sample team. LMIU records Vessel #50's previous ports of call as Portland, Newcastle, Port Kembla and Mackay – departing Mackay on the 29th of November. The most likely explanation for this Type II error is scenario 1 and 7 – i.e. the vessel picked up ballast water in either Newcastle or Port Kembla before arriving in Melbourne. Scenario 4 is unlikely because the last positive vessel to be sampled before Vessel #50 was Vessel #33 four days earlier (Appendix A).

Vessel #15

Vessel #15 was sampled in Melbourne on the 18th December, 2002, and tested positive in four out of seven samples (20 µm and 90 µm plankton samples and 20 µm benthic samples). The vessel log records Port Kembla as the last port of call, but notes Brisbane (ballasted 15th December) as the last source of the ballast water that was sampled, and Shanghai, China (ballasted 2nd December) before that. The most likely explanation for the Type II error here is that the ballast water that was sampled was actually sourced from Port Kembla a day or two before the vessel arrived in Melbourne (scenario 7). As noted above there is a very high likelihood that *Crassostrea gigas* is present in Port Kembla (scenario 1), and oyster larvae are expected to be in the water column in December. It is unlikely that the positive ballast water was sourced whilst the vessel was in Asia because November and December lie outside *C. gigas*' spawning season in the northern hemisphere. Scenario 4 is possible because the last positive vessel to be sampled before Vessel #15 was Vessel #50 the previous day (Appendix A).

Vessel #25

Vessel #25 was sampled in Geelong and recorded eight out of nine positive results from 20 µm and 90 µm plankton samples and 20 µm benthic samples. The vessel records its ballast water sources as Townsville (ballasted 6th January 2003), Devonport (ballasted 21st December) and Brisbane (ballasted 12th December). LMIU records for 2002 show that this vessel travels regularly between the ports of Brisbane, Sydney/Botany, Geelong, Devonport, Hobart, Townsville and Esperance. During the last month of 2002 it visited Devonport (departing 21st

December), Geelong (departed 25th December), Sydney (departed 29th December) and Townsville prior to its arrival in Geelong on the 11th of January, 2003.

The most likely explanation for the Type II error here is that the sampled ballast water was sourced and/or carried-over from the vessel's previous visit to Devonport three weeks earlier (scenario 7)[4] or Sydney two weeks earlier (scenarios 1 and 7). For this to be true, however, *Crassostrea gigas* larvae would have to survive the long journey duration and the various ballasting/de-ballasting events that took place between Devonport and Melbourne. Scenario 4 is unlikely because the last positive vessel to be sampled before Vessel #25 was Vessel #3 four days earlier (Appendix A). Scenario 2 is not applicable. The DSS currently does not hold any information on journey survival of *C. gigas* larvae in ballast water; hence scenario 3 is also not applicable.

Vessel #55

Vessel #55 was sampled in Melbourne on the 10th February, 2003, and tested positive for seven samples out of nine. The vessel log records the ballast sources as Brisbane (ballasted 26th January), Rotterdam, Holland (ballasted 1st December) and Gulfport, USA (ballasted 28th October, 2002). The vessel, however, also took ballast on in Newcastle on the 1st of February although this was not purportedly the source of the ballast water in this instance. In the absence of the LMIU data for 2003, the most likely cause of the Type II error in this case is that the ballast water that was sampled here was mixed with ballast sourced from Newcastle (scenarios 1 and 7).

Vessel #45

Vessel #45 was sampled in Melbourne on the 8th May, 2003, and tested positive for four samples out of six (20 and 90 µm plankton samples). The vessel log states that the ballast water was sourced from Port Stanvac (6th May), Melbourne (1st May) and Lyttelton New Zealand (26th April). We lack the LMIU shipping data for 2003 but records for 2002 show that this vessel travels regularly around southern Australia between the ports of Port Stanvac, Port Lincoln, Adelaide, Melbourne, Geelong, Sydney and Botany Bay with occasional visits to Launceston and Burnie.

It is possible that the vessel actually sourced ballast from one of the ports in NSW where *Crassostrea gigas* is present (scenario 1). Indeed the same vessel was sampled during this project in February carrying ballast water sourced from Sydney. We cannot, however, confirm this without LMIU data for 2003. It is also interesting to note that May is outside *C. gigas'* larval season in the southern hemisphere, which may merit further analysis. Scenario 4 is unlikely because the last positive vessel to be sampled before Vessel #45 was Vessel #17 seven days earlier (Appendix A). Scenario 6 is possible given this vessel's trade routes.

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[4] *Crassostrea gigas* occurs throughout the Port of Devonport where it is a common on the wharf piles and river bank (Martin *et al.* 1996).

5.4 *Gymnodinium catenatum*

Table 5.6 summarises the gene probe results for the presence/absence of *Gymnodinium catenatum* relative to the predictions of the first three modules of the ballast water risk assessment. The risk assessment predicted that *G. catenatum* would not be present in any of the vessels at the end of their journey (because of the very restricted geographical distribution of *G. catenatum* in Australia). The probe confirmed that *G. catenatum* was not detected in 60% (46/77) of samples taken from these vessels. Forty percent (31/77) of samples predicted to be negative, however, tested positive for *G. catenatum*. These samples represent (apparent) Type II errors in the risk assessment. Table 5.7 summarises the ballast characteristics and sample results associated with these apparent errors.

Table 5.6 Risk assessment predictions and gene probe results – *G. catenatum*

	Prediction	
Reality	Present	Absent
Present	0	31
Absent	0	46

All of the ballast water samples in this study were taken between September 2002 and May 2003 but the majority (62%) of the samples that tested positive for *Gymnodinium catenatum* were taken in November 2002. All *G. catenatum* samples were collected from ballast tank water and sediments using 20um plankton nets. Positive results occurred with both the planktonic and benthic samples with no obvious pattern.

Gymnodinium catenatum can infect vessels in one of two ways: as cysts recently re-suspended from harbour sediment or as cysts and vegetative cells taken from the water column during a bloom. (Hayes and Hewitt 2000; Hayes 2002). Scenario 2 is therefore not applicable to this part of the analysis because the DSS is currently unable to distinguish between cysts derived from the sediments and vegetative cells (or cysts) derived from the water column. Furthermore, while high cell densities occur during a bloom, low levels of vegetative cells are thought to persist in the water column throughout the year (Chapter 3).

The vegetative cells of *Gymnodinium catenatum* are very sensitive and are unlikely to survive more than a few days in a ballast tank. By contrast the cysts are very resistant and capable of surviving in sediments (including ballast sediments) for many years so long as they do not germinate (Hallegraeff 1998). This ability to survive for prolonged periods of time produces a high-likelihood of "carry-over" between ports, confounding the predictions of risk assessment made on the basis of the last few ports of call, and makes scenario 3 highly applicable to this part of the analysis: the DSS currently holds no journey survival information for *G. catenatum*. The resistant nature of the cysts also increases the likelihood of scenario 4 – cross contamination of the sampling equipment in the field - if equipment is not thoroughly washed between vessels (or tanks).

Table 5.7 Apparent Type II errors in the risk assessment – *Gymnodinium catenatum*

Vessel name	Sample	Donor port	Uptake	JD	BE	n	n_+
Vessel #53	08.10.2002	Mackay	October	6	nd	6	1
Vessel #53	08.10.2002	Townsville	September	9	nd	6	1
Vessel #61	14.10.2002	Burnie	October	1	nd	6	2
Vessel #43	19.10.2002	Port Kembla	October	3	No	6	1
Vessel #37	06.11.2002	Hobart	November	3	No	6	6
Vessel #10	08.11.2002	Port Botany	November	7	No	6	2
Vessel #21	09.11.2002	Sydney	November	2	nd	6	1
Vessel #42	11.11.2002	Brisbane	November	10	No	6	2
Vessel #6	12.11.2002	Port Botany	November	3	nd	6	4
Vessel #13	12.11.2002	Newcastle	November	3	No	6	4
Vessel #7	14.11.2002	Port Botany	November	3	nd	6	2
Vessel #5	15.11.2002	Brisbane	November	6	nd	6	2
Vessel #5	15.11.2002	Sydney	November	3	nd	6	2
Vessel #39	15.11.2002	Adelaide	November	2	nd	6	4
Vessel #61	19.11.2002	Burnie	November	1	nd	6	2
Vessel #51	24.11.2002	Melbourne	November	2	nd	6	2
Vessel #28	28.11.2002	Sydney	November	6	nd	6	2
Vessel #48	28.11.2002	Port Kembla	November	7	nd	6	2
Vessel #14	04.12.2002	Fremantle	November	4	nd	6	2
Vessel #38	04.12.2002	Port Kembla	November	4	nd	6	1
Vessel #52	05.12.2002	Kwinana	November	7	nd	6	1
Vessel #45	10.12.2002	Bell Bay	November	17	nd	6	1
Vessel #16	11.12.2002	Sydney	December	2	nd	6	2
Vessel #50	17.12.2002	Portland	December	1	nd	6	1
Vessel #15	18.12.2002	Brisbane	December	3	nd	5	2
Vessel #11	19.12.2002	Newcastle	December	?	nd	6	2
Vessel #46	05.01.2003	Bell Bay	January	2	nd	6	2
Vessel #25	11.01.2003	Townsville	January	6	nd	6	2
Vessel #57	29.04.2003	Burnie	April	1	nd	6	2
Vessel #47	06.05.2003	Newcastle	May	3	nd	6	1
Vessel #45	08.05.2003	Port Stanvac	May	2	nd	3	1

Unless inhibited by anoxic conditions in sediments, *Gymnodinium catenatum* cysts will usually mature and germinate within 2 weeks of formation (Blackburn *et al.* 1989). Water in ballast tanks is often agitated due to bad weather or mixing during ballasting and de-ballasting procedures. This could encourage cysts that are buried in (possibly anoxic) ballast tank sediments to become entrained or, if mature, germinate into the overlying water column from which they are subsequently sampled. Germinated cysts would help to explain the positive plankton samples recorded here from vessels whose ballast water is older than 3 days.

The only locations in Australia where *Gymnodinium catenatum* has been reported are the ports of Hobart and Triabunna, through-out southeastern Tasmania, Lorne in Victoria, and the Hawkesbury River in New South Wales (Aquenal Pty Ltd 2002; Bolch and Reynolds 2002; *pers comm.* G. Hallegraeff, University of Tasmania; *pers comm.* A. Turnbull, Tasmanian Quality Assurance Program). The dinoflagellate has also been recorded around the Port Phillip Bay heads and along the open coast but has not been recorded during the surveys of the ports of Geelong or Melbourne (Sonneman and Hill 1997; Cohen *et al.* 2001a). Based on this information and the information provided by the vessel, only one of the Type II errors recorded here (Vessel #37) can be attributed to the (currently) known distribution of *G. catenatum* in Australia.

Vessel #37 was sampled on the 6th November 2002 and six of the nine replicate samples (planktonic and benthic) recorded positive results. This vessel had sourced its ballast water in Hobart three days earlier; hence the Type II error is likely to be due to scenario 1[5]. Hobart is a relatively deep port but simple propeller wash calculations suggest that it is possible for cysts to be resuspended from the commercial berths (Hayes and Hewitt 2000). Alternatively cysts or vegetative cells may have been ballasted during a bloom but we are unaware of a bloom event in Hobart during November 2002.

Almost half (13) of the vessels that tested positive for *Gymnodinium catenatum* sourced ballast water from ports where *Gymnodinium* species are known to be present, namely: Botany Bay (3), Sydney (4), Port Kembla (3), Adelaide (1), Melbourne (1) and Portland (1) (Table 5.7). Unidentified Gymnodinoid spp. cysts and motile cells, thought to be native non-toxic *Gymnodinium spp*., have been collected from Geelong, Hastings, Melbourne, Portland, Adelaide, Botany Bay and Port Kembla (Currie *et al.* 1998; Currie and Crookes 1997; Cohen *et al.* 2001a; Parry *et al.* 1997; Cohen *et al.* 2001b; Pollard and Pethebridge 2002a; b). Cysts of *Gymnodinium spp*. were also found during the Sydney port survey but they could not be germinated to determine if they were *Gymnodinium catenatum* (Australian Museum Business Services 2002)[6]. Bolch and Reynolds (2002), however, recorded cysts of *G. catenatum* in the nearby Hawkesbury River. If unidentified Gymnodinoid samples were actually *G. catenatum* this would explain 42% of the Type II errors recorded here (scenario 1).

Extensive surveys throughout NSW, however, have failed to detect to *Gymnodinium catenatum* (*pers comm.*, G. Hallegraeff, University of Tasmania). Thus, if scenario 1 is true, these results would represent a significant change in the accepted biogeography of *G. catenatum* in Australia. It is important to note, however, all of these positive results could also be attributed to the "carry-over" of cysts in ballast water sediments (scenario 6).

[5] Hobart was surveyed, and found to be infected with *Gymnodinium catenatum*, in April 2002 (Aquenal Pty Ltd 2002), well after the Hastings project was initiated. The survey results were only recently entered in the DSS and were not available when the Hastings analysis started.
[6] *Gymnodinium catenatum* has very distinctive microreticulate cysts that (usually) do not have to be germinated to be identified (*pers comm.* G. Hallegraeff, University of Tasmania).

A small form of cyst with identical reticulate patterns to those of the *Gymnodinium catenatum* (referred to as *Gymnodinium* sp. A) were encountered at the port of Newcastle and MacKay (Hewitt *et al.* 1998a; b). Similar cysts have also been reported from south-east Tasmania, present in low concentrations in areas where larger *G. catenatum* cysts are found (Bolch and Hallegraeff 1990), and are also known from Bunbury, Albany and Port Lincoln (Hewitt *et al.* 1997a; b; c).

Gymnodinium sp. A has recently been confirmed to be a new species, *Gymnodinium microreticulatum*, genetically and morphologically distinct from the larger *G. catenatum* (Bolch *et al.* 1999b). The toxicity of *G. microreticulatum* is currently unknown, there is no reason to suspect that this species produces PSP toxins, and is not therefore considered to be of concern with the possible exception of its frequent co-occurrence with toxic "true" *G. catenatum* (Bolch 1997). The genetic probe developed for this project has been tested against *G. microreticulatum* and found to be negative (Chapter 3).

Of the 31 vessel/tank combinations that tested positive for *Gymnodinium catenatum*, 16 had sourced ballast water from Australian ports surveyed to accredited standards and either: a) found to be free of any *Gymnodinium* species; or, b) contained *Gymnodinium* species which have since been identified as *Gymnodinium microreticulatum*. These ports are: Newcastle (3); Brisbane (3), Townsville (2), Mackay (1); Fremantle/Kwinana (2); Burnie (3) and Bell Bay (2) (Hewitt *et al.* 1998a; Fearon and O'Brien 2001; Neil *et al.* 2001; Hewitt *et al.* 1998b; Hewitt *et al.* 2000; Aquenal Pty Ltd 2001; *pers comm.* K. Parsons, Aquenal). Nine of these vessels, however, had recently sourced ballast water in Asia (Japan, Korea, China and Vietnam) or Europe (Spain) where *G. catenatum* is known to be present (Chapter 3). The voyage histories of all these 16 vessel/tank combinations are discussed below. One other positive vessel (Vessel #45) sourced ballast water from Port Stanvac, which has yet to be surveyed.

Vessel #53

Two separate tanks (ballast tanks 2 and 5 on the starboard side) of Vessel #53 were sampled in Geelong on the 8th October, 2002. Only one benthic 20µm sample out of the six taken from each tank was positive. The vessel records the source of the ballast water in these tanks as Townsville and Mackay respectively. The vessels previous ports of call and ballast exchange history for these tanks were not recorded in this instance. LMIU records the vessel's previous ports of call as Incheon (Korean Republic) and Mackay (see section 5.3).

Scenarios 6 and 7 seem the most likely explanation for the Type II error in this instance. LMIU records the vessel's previous ports of call as Incheon and Mackay, not Townsville and Mackay, and Incheon is within the native range of *Gymnodinium catenatum* (IUCN bioregion NWP – 4a). The fact that only a small proportion of the samples taken from each tank were positive, and these the fact that these were benthic samples, suggests that a low level prevalence within the ballast sediments of this vessel. This explanation is consistent with the "carry-over" hypothesis – i.e. the survival and carry-over of cysts from Incheon.

Vessel #61 & Vessel #57

Vessel #61 and Vessel #57 are vessels that trade exclusively between Burnie and Melbourne. Vessel #61 was sampled on the 14th October and 19th November, 2002 and positive results were found for two benthic 20 µm samples out of six, and two planktonic 20 µm samples out of six respectively. Vessel #57 was sampled on the 29th April, 2003 and two planktonic 20 µm

samples out of six were found to be positive for *Gymnodinium catenatum*. The ballast water log for both vessels notes Burnie as the last ballast water source. *G. catenatum* was not found in a recent survey of Burnie (Aquenal Pty Ltd 2003), hence the most likely reason for the Type II error is the carry-over of ballast from the vessels previous visits to Melbourne (scenarios 1, 6 and 7). The restricted trading pattern of these vessels strongly suggests that the *Gymnodinium* spp recorded in Port Phillip Bay include *G. catenatum*.

Vessel #42

Vessel #42 was sampled in Melbourne on the 11th of November, 2002, and tested positive for two out of six samples (one benthic and one plankton). The vessel log indicates that the sampled ballast water was taken up in Brisbane on the 1st of November and before that at Yokohama, Japan on the 20th of August. LMIU data shows that the vessel visited Sydney (departed 7th November), Newcastle (departed 3rd November) and Brisbane (arrived 1st November) before its arrival in Melbourne, and that it's first port of call after leaving Australia was Yokohama.

Gymnodinium catenatum is present in the bioregion that Yokohama is situated (noted as cryptogenic, IUCN bioregion NWP-3b). It is possible therefore that the Type II error in this instance is due to "carry-over" from Yokohama (scenarios 6 and 7) or mixing of ballast water sourced from Sydney (scenarios 1 and 7). If scenario 1 is correct, however, then scenario 2 may also be correct as the DSS notes that *G. catenatum* does not bloom in November, although it is possible that cysts were sourced from the sediment not the water column and low numbers of vegetative cells may persist in the water column all year round (see above). Scenario 3 is not applicable because the DSS does not currently hold data on *G. catenatum* survival in ballast tanks. Scenario 4 is possible given the resistant nature of the cysts but scenario 5 is considered to be unlikely.

Vessel #13

Vessel #13 was sampled in Melbourne on the 12th of November, 2002, testing positive for one 20 µm plankton sample (out of three). The vessel recorded the source of sampled ballast as Newcastle (ballasted 9th November), Port Kembla (ballasted 11th September) and Melbourne (ballasted 30th August). LMIU data confirms these dates and locations, but also notes that the vessel visited Hobart on the 8th of September. Hobart is known to be infected with *Gymnodinium catenatum* and there is a strong likelihood that Melbourne is also infected with *G. catenatum* in light of the positive results from Vessel #61 and Vessel #57. Scenarios 1, 6 and 7 therefore seem likely.

Vessel #5

On the 15th November, 2002, two separate tanks of Vessel #5 were sampled in Melbourne. The vessel records the source of the ballast water in the starboard and port number four ballast tanks as Brisbane (9th November) and Sydney (12th November) respectively. For each tank, one 20 µm plankton and one benthic 20µm sample out of the six taken were positive. The vessel log indicates that prior to this ballast water was taken up in mid-ocean on the 4th of November and before that at Melbourne on the 19th of October. LMIU data shows that the vessel departed Adelaide on the 20th October for Singapore and returned to Australia via Port Kelang in Malaysia and reached Brisbane on the 6th of November.

Gymnodinium catenatum is currently recorded in the DSS as unknown from Malaysia and Singapore - the DSS records both bioregions in which these ports are situated in (EAS-VI) as uninfected. The global distribution of *G. catenatum*, however, has recently been redefined to include Malaysia and Singapore (Bolch and Reynolds 2002). Hence the Type II error in this case is most likely due to the carry-over of cysts from either Malaysia or Singapore (or other intervening infected ports in Asia - scenarios 1, 6 and 7). It is also possible that *G. catenatum* cysts were carried over from the vessel's previous visit to Melbourne or Sydney (if indeed these ports are infected).

Vessel #14

Vessel #14 was sampled in Melbourne and tested positive in two 20 µm benthic samples out of six. The LMIU voyage history for this vessel agrees with the recorded log (see section 5.3). During the Fremantle/Kwinana port survey in 1999 over 700 cysts were examined from a wide range of locations in Fremantle and Cockburn Sound and no *Gymnodinium catenatum* cysts were recorded (Hewitt *et al.* 2000). It is possible that the vessel sourced ballast water containing *G. catenatum* in Asia in the two months between it last departure and last arrival in Australia. We do not, however, hold LMIU data for this period and cannot therefore confirm the vessel's movements. The reason for the Type II error is therefore unclear in this instance.

Vessel #52

Vessel #52 was sampled in Melbourne on the 5th of December, 2002, and tested positive for one sample out of six (20 µm plankton sample). The vessel log indicates that the sampled ballast water was sourced from Kwinana on the 28th of November, and before this from Merak, Indonesia (22nd November) and Singapore (16th November). This information agrees with LMIU records of the vessel's movements during this period. *Gymnodinium catenatum* has not been recorded from Fremantle (Hewitt *et al.* 2000) and is recorded in the DSS as unknown from Merak and Singapore - the DSS records both bioregions in which these ports are situated (EAS-VII and EAS-VI respectively) as uninfected. The global distribution of *G. catenatum*, however, has recently been redefined to include Singapore (Bolch and Reynolds 2002). Hence, the Type II error in this case is most likely due to the Asian source of the ballast water (scenarios 1 and 6).

Vessel #45

Vessel #45 was sampled in Geelong on the 10th December, 2002, and in Melbourne on the 8th May, 2003. It tested positive for one 20 µm plankton sample out of six on both occasions. On the first occasion the vessel log indicated the ballast water was sourced in Bell Bay (23rd November) and no data was provided prior to this. LMUI records show that in November this vessel visited Melbourne three times, Port Stanvac twice and Sydney, Botany Bay and Bell Bay once each prior to reaching Geelong. On the second occasion the vessel log states that the ballast water was sourced from Port Stanvac (6th May), Melbourne (1st May) and Lyttelton New Zealand (26th April). We lack the LMIU shipping data for 2003 but records for 2002 show that this vessel travels regularly around southern Australia between the ports of Port Stanvac, Adelaide, Melbourne, Geelong, Sydney, and Botany Bay with occasional visits to Port Lincoln, Launceston and Burnie.

Scenarios 6 and 7 seem likely if the vessel traded its usual routes in 2003 as *Gymnodinium catenatum* is recorded from Port Lincoln (Hewitt *et al*. 1997c) or if indeed *G. catenatum* is

present in Melbourne (scenario 1) as previously discussed. *G. catenatum* is also known to be widespread around the North Island of New Zealand. A large extensive bloom was recorded in May 2000, after which a few cells were detected in the Marlborough sounds at the north end of South Island (Irwin *et al.* 2003; *pers comm.* A. Turnbull, Tasmanian Quality Assurance Program). *Gymnodinium catenatum* is recorded in the DSS as cryptogenic in the waters around Lyttelton (IUCN bioregion NZ-IV). Weekly phytoplankton samples from Lyttelton have not detected *G. catenatum* cells but harbour sediments have not been tested for cysts (*pers comm.* J. Sim, New Zealand Food Safety Authority).

Vessel #15

Vessel #15 was sampled in Melbourne on the 18th of December, 2002, and tested positive in two 20 µm benthic samples (out of six). There is a discrepancy between the data recorded by the sample team and LMIU data for this vessel. The sample team notes Port Kembla as the last port of call, but recorded Brisbane (ballasted 15th December) as the last source of the ballast water that was sampled, and Shanghai, China (ballasted 2nd December) before that. According LMIU, however, the vessel left Pohang, Republic of Korea on the 27th of November bound for Brisbane (arriving on the 10th December). From here is visited Port Kembla (arriving 12th December) before finally arriving in Melbourne.

It is possible that the Type II error here is due to ballast water being sourced from Port Kembla (scenarios 1 and 7), however, both Shanghai and Pohang are recorded in the DSS as infected with *Gymnodinium catenatum* (cryptogenic in bioregions NWP-3a and NWP-4a respectively. Hence scenarios 6 and 7 seem most likely.

Vessel #11

The forepeak tank of Vessel #11 was sampled on the 19th December, 2002, in Melbourne. One 20 µm plankton and one benthic 20 µm sample out of the six taken were positive. The sample team indicates that this ballast water was sourced in Newcastle in December and prior to that a 10% ballast water exchange occurred in the Pacific Ocean on the 2nd December. LMIU data, however, records this vessel in Newcastle from the 3rd to the 19th of November after which it sailed to Singapore and returned to Australian waters via Jakarta, Indonesia arriving in Brisbane on the 3rd December. It then traveled to Melbourne on the 14th December. This vessel also visited Hobart on the 28th October, 2002. *G. catenatum* is not known from Indonesia but is now known to be present in the nearby regions of Singapore, Malaysia and the Philippines (Bolch and Reynolds, 2002). Scenarios 1, 6 and 7 are therefore all likely.

Vessel #46

One 20 µm plankton and one benthic 20 µm sample out of the six samples taken from Vessel #46 in Melbourne on the 5th January, 2003, recorded positive results. The vessel history indicated that the water was sourced from Bell Bay (3rd January), Melbourne (1st January) and Port Stanvac (29th December, 2002). Again there is a discrepancy between the data recorded by the sample team and LMIU data for the dates of the Port Stanvac visit as the LMUI records this vessel present in Port Stanvac (12-14th December) then Botany Bay (14-21st December), prior to sailing to Melbourne and Bell Bay.

It is likely that the Type II error here is due to ballast water being sourced from Melbourne or from the yet to be surveyed Port Stanvac (scenario 1), as one other vessel that sourced ballast water from that port also gave a positive result (Vessel #45, May 2003).

Vessel #25

Vessel #25 was sampled in Geelong and recorded two out of six positive results from 20 µm benthic samples. The vessel records its ballast water sources as Townsville (ballasted 6th January, 2003), Devonport (ballasted 21st December) and Brisbane (ballasted 12th December). LMIU records for 2002 show that this vessel travels regularly between the ports of Brisbane, Sydney/Botany Bay, Geelong, Devonport, Hobart, Townsville and Esperance. During the last month of 2002 it visited Devonport (departing 21st December), Geelong (departed 25th December), Sydney (departed 29th December) and Townsville prior to its arrival in Geelong on the 11th of January, 2003. It last visited Hobart on the 11th of November. Likely Type II error scenarios are therefore 6 and 7 if the vessel sourced ballast water from Hobart.

In this context it is interesting to note that ballast water was taken from this vessel in October 2002 (sourced from Devonport) and used as a negative control during the development of the *Gymnodinium catenatum* probe (Chapter 3). Clearly in this instance the ballast water did not contain *G. catenatum* cells or cysts, which suggests that a) ballast water sampling does not always detect species when they are present in the tank; or, b) carry-over of cysts between ports (or ballast tanks) does not always occur.

Vessel #47

Vessel #47 was sampled in Melbourne on 6th May 2003 and tested positive for one 20 µm plankton sample out of six. The vessel history states that the ballast water was sourced in Newcastle on 3rd May and prior to this in the Netherlands in March and Korea in January, 2003.

The species of *Gymnodinium* in northern Europe is now believed to be the non toxic species, *G. nolleri* (Ellegaard and Moestrup 1999) so it is unlikely that the positive result is due to ballast water retained from the Netherlands. The genetic probe has been tested against this species and gives a negative result (Chapter 3). It is possible that the vessel sourced ballast water containing *G. catenatum* in Asia more than the two months before its arrival in Australia - the “carry-over” hypothesis. Since we do not hold LMIU data for this period we cannot confirm the vessel’s movements and therefore the reason for the Type II error is unclear in this instance.

6 SUMMARY & RECOMMENDATIONS

6.1 *Asterias amurensis*

A high proportion of vessels (84%) sampled in the Hastings project were correctly identified to be free of *Asterias amurensis* at the end of the vessel's journey. These results coupled with the final model of the ballast water risk assessment (survival in the recipient port) suggest that the DSS will provide effective risk mitigation for *A. amurensis* on most vessels. Nine vessels (13%) predicted to be negative at the end of journey, however, tested positive for *A. amurensis* indicating that there is still considerable room for improvement in the DSS. Table 6.1 summarises our best estimate of the likelihood of the Type II error scenarios associated with these vessels.

Asterias amurensis is a conspicuous sub-tidal species whose Australian and world-wide distributions are relatively well defined. It is perhaps unsurprising that none of the nine instances of Type II error discovered during this project are likely to be due to its unknown presence in a port (Scenario 1), although they cannot be unequivocally ruled out.

What is surprising, however, is that on three occasions vessels which had visited infected ports more than three weeks earlier tested positive for *A. amurensis* (Scenario 3). Data collected by CSIRO personnel on board the *MV Iron Sturt* suggests that zooplankton densities in ballast tanks decline exponentially to zero within twenty days (Figure 6.1). Similarly, simple models of *A. amurensis* life-expectancy in ballast tanks suggest that there is a less than a one in five chance that *A. amurensis* larvae could survive for more than 30 days in a ballast tank (Hayes 2003). These results suggest that *A. amurensis* may be more resilient to ballast water transport than previously thought. Additionally, reballasting of infected tanks might prolong tank survival by providing a fresh plankton supply.

It is also possible that these apparently anomalous results are in fact caused by the presence of adult *Asterias amurensis* in the ballast tanks of the vessels sampled (Scenario 6). It is important to note, however, that whilst adult *A. amurensis* have been detected in sea chests (Coutts *et al.* 2003) they have never been reported in ballast tanks and on the whole this is considered to be an unlikely scenario.

Another important lesson provided by these results is the importance of accurate ballast water source reporting and the potential carry-over of ballast water from infected sources (Scenario 7). In all but two of the nine Type II errors reported here, it appears likely that the incorrect information was entered into the DSS. Clearly it is imperative that all sources of ballast water, particularly in tanks that are mixed, are accurately recorded in the vessel's ballast log and that this information is entered into the DSS.

Table 6.1 Summary of the likelihood of Type II error scenarios – *Asterias amurensis*

Vessel	Type II error scenarios						
	1: Present in port	2: Present in vessel	3: Survives voyage	4: Sample contaminated	5: Laboratory contamination	6: Present in tank	7: Incorrect data
Vessel #25	Possible	NA	NA	Possible	Unlikely	Unlikely	Likely
Vessel #1	Possible	NA	Likely†	Unlikely	Unlikely	Unlikely	Likely†
Vessel #53	Unlikely	Possible*	Possible*	Unlikely	Unlikely	Possible	Likely
Vessel #61	Possible	NA	NA	Unlikely	Unlikely	Possible	Likely
Vessel #62	Possible	Possible*	Possible*	Unlikely	Unlikely	Possible	Likely
Vessel #58	Possible	NA	Possible‡	Unlikely	Unlikely	Possible	Possible
Vessel #50	Possible	NA	NA	Unlikely	Possible	Unlikely	Possible
Vessel #34	Unlikely	NA	Likely†	Unlikely	Unlikely	Unlikely	Likely†
Vessel #57	Possible	Likely	NA	Unlikely	Unlikely	Possible	Likely

Likely† = seems to be the most likely scenario(s) but would require larvae to survive in the ballast tank for more than 10 days.

Possible‡ = is possible but would require larvae to survive in the ballast tank for more than 20 days

Possible* = is possible but would require larvae to survive in the ballast tank for more than 30 days and an amended larval season.

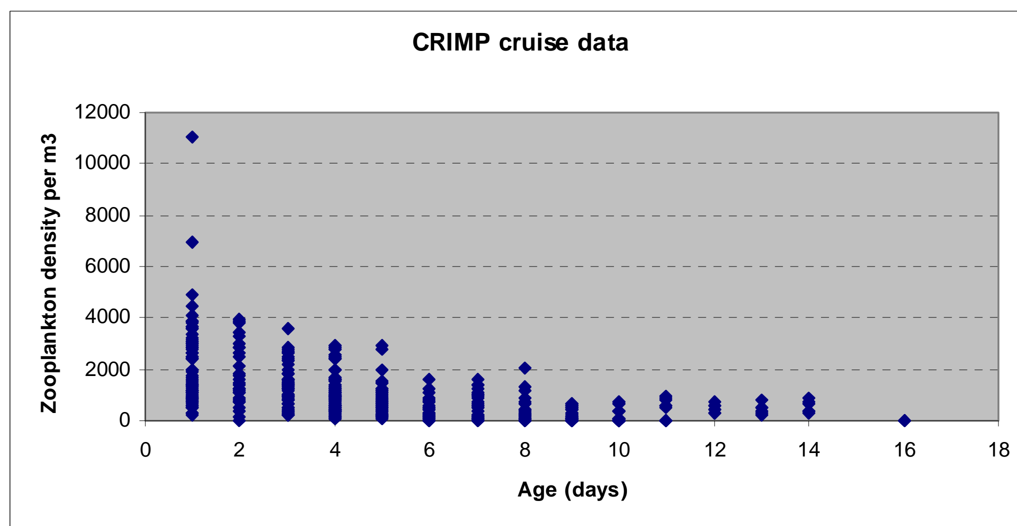

Figure 6.1 Data collected on board the *MV Iron Sturt* showing an exponential decline in the density of zooplankton in voyages around south eastern Australia.

These results also point to potential errors in the timing and/or duration of the larval season of *Asterias amurensis* in the Northern Hemisphere (Scenario 2). On two occasions the positive results recorded here suggest that the spawning period of *A. amurensis* in Korea and Taiwan may not be accurately reflected in the DSS.

Based on this analysis, we make the following recommendations to improve the accuracy of the risk assessments for *Asterias amurensis* provided by the DSS:

- routinely sample and test a proportion of low risk vessels for the presence/absence of *A. amurensis* to continually increase the sample size of Type II error results and assist in the on-going development of the DSS;
- collect and analyse additional field samples (using the gene probe developed during this project) to provide a more accurate determination of the life-expectancy of larval *A. amurensis* in the ballast tanks of infected vessels, both with and without ballast exchange;
- periodically inspect low risk vessels that routinely trade between infected ports, or consistently test positive in routine sampling regimes, for the presence of adult *A. amurensis* in the ballast tanks; and
- collect additional literature and field samples (if necessary) to verify the spawning and larval season of *A. amurensis* in Korea and Taiwan.

6.2 *Crassostrea gigas*

An unacceptably high proportion (43%) of vessels predicted to be free of *Crassostrea gigas* tested positive at the end of the vessel journey. Table 6.2 summarises our best estimate of the likelihood of the Type II error scenarios associated with these vessels.

Crassostrea gigas is a prominent inter-tidal fouling organism. It is, therefore, very surprising that the vast majority of the Type II errors recorded here (91%) are most likely attributable (directly or indirectly) to Scenario 1 – the species is recorded as absent in the donor port when in fact it is present. In 63% of these cases, the port in question was correctly entered into the DSS. In the remaining cases ballast water appears to have been carried over from a port not entered into the DSS, but confirmed or suggested by LMIU data. More importantly, however, all of the ports in question – Sydney, Port Botany, Newcastle and Port Kembla - were surveyed to accredited standards using CRIMP survey protocols.

Table 6.2 Presumed likelihood of Type II error scenarios – *Crassostrea gigas*

Vessel	1: Present in port	2: Present in vessel	3: Survives voyage	4: Sample contaminated	5: Laboratory contamination	6: Present in tank	7: Incorrect data
Vessel #1	Likely	NA	NA	Possible	Unlikely	Unlikely	Unlikely
Vessel #53	Unlikely	NA	NA	Likely	Unlikely	Likely	Likely
Vessel #53	Unlikely	NA	NA	Likely	Unlikely	Likely	Likely
Vessel #41	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #10	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #21	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #13	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #5	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #51	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Likely
Vessel #48	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #12	Likely	NA	NA	Possible	Unlikely	Unlikely	Likely
Vessel #14	Possible	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #38	Likely	NA	NA	Possible	Unlikely	Unlikely	Unlikely
Vessel #52	Possible	NA	NA	Possible	Unlikely	Unlikely	Unlikely
Vessel #16	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #33	Possible	NA	NA	Possible	Unlikely	Unlikely	Possible
Vessel #50	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Likely
Vessel #15	Likely	NA	NA	Possible	Unlikely	Unlikely	Likely
Vessel #11	Likely	NA	NA	Possible	Unlikely	Unlikely	Unlikely
Vessel #3	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #25	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Likely
Vessel #60	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #34	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #12	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #44	Likely	NA	NA	Possible	Unlikely	Unlikely	Unlikely
Vessel #55	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Likely
Vessel #55	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Likely
Vessel #24	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #45	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #11	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #9	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #17	Likely	NA	NA	Unlikely	Unlikely	Unlikely	Unlikely
Vessel #45	Possible	NA	NA	Unlikely	Unlikely	Possible	Possible

Anecdotal evidence gathered during this analysis suggests that *Crassostrea gigas* is prevalent in most estuaries of New South Wales, including the ports of Sydney, Port Botany, Newcastle and Port Kembla. The fact that 91% of these Type II errors also occurred in ballast water sourced between mid-October and late April, when oyster larvae are expected to be present in the water column, add additional strength to this argument. If this argument is true, these results indicate repeated failure of the port surveys to detect a prominent fouling organism – indicative of low statistical power in the sample design or inattention to Quality Assurance issues. In this context it is interesting to note that two Type II errors recorded here were from vessels that had sourced their ballast water from Fremantle – which has also been surveyed and declared free of *C. gigas*.

On three occasions (9%) the source of the Type II error associated with *Crassostrea gigas* is unclear. On two of these occasions ballast was sourced from an infected port but the age of the ballast water (38 days) mitigates against a positive result. These positive results came from separate tanks of the same vessel – benthic samples from one tank, plankton samples from the other. On this occasion it seems likely that the oysters had settled in the tank (hence the positive benthic samples) and the sampling equipment was contaminated (hence the positive plankton samples).

Based on this analysis we make the following recommendations to improve the accuracy of the risk assessments for *Crassostrea gigas* provided by the DSS:

- confirm the presence or absence of *C. gigas* in Sydney, Port Botany, Newcastle, Port Kembla and Fremantle as soon as possible, and amend the DSS database accordingly;
- review the port survey protocols, placing particular emphasis on the power of the resultant survey and the use of ancillary data and associated Quality Assurance issues, and where necessary re-design survey and/or monitoring methods, together with arrangements for their accreditation;
- routinely sample and test a proportion of low risk vessels for the presence/absence of *C. gigas* to continually increase the sample size of Type II error results and assist in the on-going development of the DSS;
- sample ballast tanks for vessels ballasting in areas of known *C. gigas* abundance, to determine whether it can complete its life cycle in ballast tanks;
- gather all available information on the life-cycle of *C. gigas* and amend the DSS database accordingly; and
- collect and analyse additional field samples (using the gene probe developed during this project) to provide a more accurate determination of the life-cycle and life-expectancy of larval *C. gigas* in the ballast tanks of infected vessels.

6.3 *Gymnodinium catenatum*

Gymnodinium catenatum is believed to have a very limited distribution in Australia – restricted to only two locations outside of southeastern Tasmania: Lorne, Victoria and the Hawkesbury River in New South Wales. Neither of these locations, or indeed the infected Tasmanian locations, are major ports. As a result relatively few domestic vessels are predicted by the DSS to become infected with *G. catenatum*. In this project, for example, the DSS predicted all of the vessels that were sampled would test negative. In reality, however, almost half of the vessels (40%) tested positive. Table 6.3 summarises our best estimate of the likelihood of the Type II error associated with these vessels.

Our interpretation of the *Gymnodinium catenatum* results is clouded by the possibility of "carry-over" of cysts in ballast sediments. For example, it is virtually impossible to distinguish between Scenario 1 (unexpected presence in port) and Scenario 6 (carry-over of cysts) if, as previous researchers suggest, *G. catenatum* cysts are capable of surviving for several years in ballast tank sediments.

Thirteen (42%) of the vessels listed in Table 6.3 sourced ballast water from ports where *Gymnodinium* species are known to be present but thought to be native non-toxic species. These ports are Botany Bay, Sydney, Port Kembla, Adelaide, Melbourne and Portland. Extensive surveys of these locations have repeatedly failed to detect *G. catenatum*. If these positive samples were in fact sourced from these ports, rather than carried over from other ports, then these results they would point to a significant change in the accepted biogeography of *G. catenatum* in Australia.

Whilst it is difficult to distinguish between cyst carry-over and Scenario 1, the positive results associated with two vessels – Vessel #61 and Vessel #57 – provide strong evidence that the *Gymnodinium* spp. discovered in Melbourne may in fact be *Gymnodinium catenatum*. Vessel #61 and Vessel #57 trade exclusively between Burnie and Melbourne. It is difficult to see how these vessels became infected with *G. catenatum* unless Burnie or Melbourne were also infected, and Burnie has recently been surveyed and found to be free of any *Gymnodinium* spp.

Sixteen (52%) of the vessels that tested positive for *Gymnodinium catenatum* recorded their ballast water sources as ports that are thought to be free of any *Gymnodinium* spp or contain species which have since been identified as *Gymnodinium microreticulatum*. Eight of these vessels, however, were known to have sourced ballast water from overseas locations where *G. catenatum* is known to exist. The remaining vessels had all visited ports in Australia known (e.g. Hobart) or suspected (e.g. Melbourne) to be infected with *G. catenatum*. Hence, again carry-over of cysts (Scenario 6) is considered to be likely or at least possible for all of these vessels. Two positive vessels had recently sourced ballast water from Port Stanvac (yet to be surveyed), which may, therefore, be infected with *G. catenatum* as well.

Table 6.3 Presumed likelihood of Type II error scenarios – *Gymnodinium catenatum*

Vessel	1: Present in port	2: Present in vessel	3: Survives voyage	4: Sample contaminated	5: Laboratory contamination	6: Present in tank	7: Incorrect data
Vessel #53	Unlikely	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #61	Possible	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #43	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #37	Likely	NA	NA	Possible	Unlikely	NA	Unlikely
Vessel #10	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #21	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #42	Possible	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #6	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #13	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #7	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #5	Likely	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #39	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #61	Likely	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #51	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #28	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #48	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #14	Unlikely	NA	NA	Possible	Unlikely	Possible	Possible
Vessel #38	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #52	Likely	NA	NA	Possible	Unlikely	Likely	NA
Vessel #45	Possible	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #16	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #50	Possible	NA	NA	Possible	Unlikely	Possible	Unlikely
Vessel #15	Possible	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #11	Likely	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #46	Likely	NA	NA	Possible	Unlikely	Likely	Unlikely
Vessel #25	Possible	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #57	Likely	NA	NA	Possible	Unlikely	Likely	Likely
Vessel #47	Unlikely	NA	NA	Possible	Unlikely	Possible	Possible
Vessel #45	Possible	NA	NA	Possible	Unlikely	Possible	NA

Based on this analysis we make the following recommendations to improve the accuracy of the risk assessments for *Gymnodinium catenatum* provided by the DSS:

- confirm the presence or absence of *G. catenatum* in Botany Bay, Sydney, Port Kembla, Adelaide, Melbourne, Port Stanvac and Portland as soon as possible, either by applying the probes to the port survey material collected from these ports, or by collecting additional samples (and amend the DSS database accordingly);

- review the efficacy of ballast water exchange as a risk management strategy for dinoflagellate, and other cyst producing, species, and collect additional ballast sediment samples to quantify the incidence of the "carry-over" of cysts between ports; and,

- routinely sample and test a proportion of low risk vessels for the presence/absence of *G. catenatum* to continually increase the sample size of Type II error results and assist in the on-going development of the DSS.

ACKNOWLEDGEMENTS

This study was completed as part of the Port of Hastings National Demonstration project, jointly funded by Victoria and the Commonwealth Natural Heritage Trust. We would like to thank Teresa Hatch and Vicki Barmby who have managed this project on behalf of the Victorian Government consortium and its partners and Tony Sheehan who arranged for collection of the ballast water samples.

We would especially like to thank ship' masters, agents and companies for their willingness and co-operation in supporting this project. We would also like to thank Gustaaf Hallegraeff and Chris Bolch (University of Tasmania), Alison Turnbull (Tasmanian Department of Health and Human Services), Jim Sim (New Zealand Food Safety Authority) and John Nell (New South Wales Fisheries) for their assistance during the completion of this project, and Leanne Armand for helping to produce the report.

The project's steering committee members were: John Hirst (AAPMA), Michael Drynan (AFFA), Tim O'Hara (AMCS), Jennifer Taylor (ASA), Andy Carroll (AQIS), Nic Bax (CSIRO), Mark Curry (DOI), Warren Geeves (DEH), Chris Bell (EPA Victoria), Rod Gowans (DSE Victoria)

The project's technical committee members were: Jennifer Taylor (ASA), Helen Gannon (AQIS), Peter Cassells (DAFF), Chad Hewitt (CSIRO), John Barker (DOI), Kelly O'Shanassy (EPA Victoria), Sarah Walters (EPA Victoria), Don Hough (DSE Victoria), Capt. Dick Cox (Toll Western Port), Siri Gunawardina (Toll Geelong Port).

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APPENDIX A OVERVIEW OF RESULTS

Vessel ref.	Sample date	Aa TSR	Gc TSR	Cg TSR	Aa +	Gc +	Cg +
Vessel #25	1/04/2002	3	2	2	N	N	N
Vessel #61	26/04/2002	3	2	1	N	N	N
Vessel #25	1/10/2002	2	3	1	Y	N	Y
Vessel #1	3/10/2002	2	3	2	Y	N	Y
Vessel #53	8/10/2002	7	8	7	Y	Y	Y
Vessel #53	8/10/2002	7	8	7	Y	Y	Y
Vessel #61	14/10/2002	2	3	1	Y	Y	N
Vessel #57	17/10/2002	2	3	1	N	N	N
Vessel #62	17/10/2002	7	no data	no data	Y	N	N
Vessel #43	19/10/2002	2	3	2	N	Y	N
Vessel #43	19/10/2002	7	8	7	N	N	N
Vessel #41	22/10/2002	2	3	2	N	N	Y
Vessel #54	23/10/2002	2	3	2	N	N	N
Vessel #31	24/10/2002	2	3	2	N	N	N
Vessel #20	25/10/2002	no data	no data	no data	N	N	N
Vessel #35	27/10/2002	2	3	2	N	N	N
Vessel #22	31/10/2002	2	3	1	N	N	N
Vessel #59	1/11/2002	2	3	1	N	N	N
Vessel #27	5/11/2002	7	8	7	N	N	N
Vessel #37	6/11/2002	1	4	1	Y	Y	N
Vessel #10	8/11/2002	2	3	2	N	N	Y
Vessel #10	8/11/2002	7	8	7	N	Y	N
Vessel #21	9/11/2002	2	3	2	N	Y	Y
Vessel #42	11/11/2002	7	8	7	N	Y	N
Vessel #6	12/11/2002	2	3	2	N	Y	N
Vessel #13	12/11/2002	2	3	2	N	Y	Y
Vessel #7	14/11/2002	2	3	2	N	Y	N
Vessel #5	15/11/2002	2	3	2	N	Y	N
Vessel #5	15/11/2002	7	8	7	N	Y	Y
Vessel #39	15/11/2002	2	3	2	N	Y	N
Vessel #61	19/11/2002	2	3	1	N	Y	N
Vessel #4	22/11/2002	2	3	1	N	N	N
Vessel #51	24/11/2002	1	3	2	N	Y	Y
Vessel #18	27/11/2002	7	no data	no data	N	N	N
Vessel #28	28/11/2002	7	8	7	N	Y	N
Vessel #48	28/11/2002	7	8	7	N	Y	Y
Vessel #12	29/11/2002	2	3	2	N	N	Y
Vessel #26	29/11/2002	7	8	7	N	N	N
Vessel #57	2/12/2002	2	3	1	N	N	N
Vessel #14	4/12/2002	2	3	2	N	Y	Y
Vessel #38	4/12/2002	2	3	2	N	Y	Y
Vessel #52	5/12/2002	7	8	7	N	Y	Y
Vessel #45	10/12/2002	7	8	5	N	Y	N
Vessel #16	11/12/2002	2	2	2	N	Y	Y
Vessel #58	12/12/2002	2	2	1	Y	N	N
Vessel #33	13/12/2002	2	2	2	N	N	Y

Vessel ref.	Sample date	Aa TSR	Gc TSR	Cg TSR	Aa +	Gc +	Cg +
Vessel #50	17/12/2002	2	2	2	Y	Y	Y
Vessel #15	18/12/2002	2	2	2	N	Y	Y
Vessel #11	19/12/2002	no data	2	2	N	Y	Y
Vessel #46	5/01/2003	2	2	1	N	Y	N
Vessel #3	7/01/2003	2	2	2	N	N	Y
Vessel #25	11/01/2003	7	7	7	N	Y	Y
Vessel #60	14/01/2003	2	2	2	N	N	Y
Vessel #49	16/01/2003	2	2	2	N	N	N
Vessel #34	21/01/2003	2	2	2	Y	N	Y
Vessel #8	3/02/2003	3	2	1	N	N	N
Vessel #12	4/02/2003	2	2	2	N	N	Y
Vessel #44	5/02/2003	3	2	2	N	N	Y
Vessel #55	10/02/2003	7	7	7	N	N	Y
Vessel #55	10/02/2003	8	7	7	N	N	Y
Vessel #24	14/02/2003	3	2	2	N	N	Y
Vessel #30	17/02/2003	3	2	2	N	N	N
Vessel #45	18/02/2003	3	2	2	N	N	Y
Vessel #11	28/02/2003	3	2	2	N	N	Y
Vessel #19	5/03/2003	3	2	2	N	N	N
Vessel #36	12/03/2003	8	7	7	N	N	N
Vessel #8	14/03/2003	3	2	1	N	N	N
Vessel #29	15/03/2003	3	2	1	N	N	N
Vessel #9	22/03/2003	3	2	2	N	N	Y
George T	1/04/2003	3	2	2	N	N	N
Vessel #2	8/04/2003	3	2	2	N	N	N
Vessel #56	17/04/2003	3	2	1	N	N	N
Vessel #40	24/04/2003	3	2	2	N	N	N
Vessel #57	29/04/2003	3	2	1	N	Y	N
Vessel #17	1/05/2003	3	2	2	N	N	Y
Vessel #47	6/05/2003	3	2	2	N	Y	N
Vessel #45	8/05/2003	3	2	2	N	Y	Y
Vessel #32	11/05/2003	3	2	2	N	N	N
Vessel #57	15/05/2003	3	2	1	Y	N	N
Vessel #63	17/05/2003	3	2	2	N	N	N

APPENDIX B SAMPLING PROTOCOLS

Ballast water sampling methods

In order to efficiently and consistently collect samples for the Hastings Demonstration Project, a standardised set of sampling methods was deemed necessary. While these are not the definitive suite of sampling methods useful for identifying the flora and fauna in ballast tanks, the methods outlined below are designed explicitly to *increase the likelihood* of detecting the target species. Compromises due to time constraints are noted.

A general field data sheet should be developed that includes:

1. Vessel Name and Risk Assessment Number
2. Location (port, berth)
3. Date and Time (in 24hr format)
4. Ballast Tank information:
    - locations (sketch of ship)
    - access (manholes, sounding pipes, etc...)
    - type of tank (wing tanks, bottom tanks, etc..)
    - Samples collected (methods, volumes, etc)
    - Temperature and Salinity measurements
    - Miscellaneous comments

Method 1 (plankton)

Many planktonic organisms, including the meroplanktonic larvae of *Asterias amurensis* (northern Pacific oyster) and *Crassostrea gigas* (Pacific oyster) and vegetative planktonic cells of the toxic dinoflagellate *Gymnodinium catenatum*, positively respond to the presence of light. In most ballast water sampling efforts these biases are avoided, however our intent is to detect (even at low densities) those target species. Consequently, the following criteria should be incorporated into the sampling activity

1. Sampling should occur on high risk vessels (with a risk of the target species being present);
2. Sampling should proceed through manhole or hatch covers to ballast tanks;
3. Exposure of light for at least 15 minutes prior to sampling (daylight or artificial light);
4. The use of a plankton pump (ASA diaphragm pump either petrol or electric) with flow rates of 100 - 130 l min^{-1}

5. or alternately a pneumatic pump capable of flow rates of 40 l min^{-1}

6. (**NOTE: the flow rates will need to be calibrated in order for accurate calculations of volume);

7. The inflow hose should be placed at less than 0.5 m from the water surface in order to gain further advantage from the behaviour of the plankton;

8. Outflow of the pumps through a plankton net (100µm mesh);

9. A total volume of water to be sampled should be at least 1000 l, providing a detection threshold of at least 1 individual in this volume. While larger volumes are preferable, the trade-offs in time and number of tanks per vessel to be sampled preclude the option of larger samples;

10. At least three replicates per ballast tank,

11. Remove excess water from the filtered material and preserve in SET buffered fixative (See below for recipe)

12. Labels should be made of waterproof paper and be printed with indelible ink. Information should include:

    - Vessel identifier (name or risk assessment number)
    - Date and time in 24hr format
    - Ballast Tank
    - Replicate Number (numbered in the order of sampling)
    - Sampled volume (time pumped and flow rate)
    - Sample equipment
    - Sample Type (planktonic/benthic)

Method 2 (benthic):

While the vegetative planktonic cells of the toxic dinoflagellate *Gymnodinium catenatum* are planktonic in nature, this organism will produce resting cysts that fall to the sediment. The most direct method of sampling has been to collect sediment from the tanks after discharge, however this is neither practical nor safe. Cysts of toxic dinoflagellates have been collected from the bottom of tanks using sounding pipes. These pipes are generally of small diameter (30 - 50 mm) and require slightly different equipment due to the increased head:

1. Sampling should occur on high risk vessels (with a risk of the target species being present);

2. For heads of less than 6m - a Mono pump (flow rate ~ 20 l min^{-1}) attached to a flexible 12 to 20mm internal diameter hose,

3. For heads greater than 6m - a Waterra pump attached to a flexible 12 to 20mm inner diameter hose with a Waterra foot valve (**NOTE: the flow rates will need to be calibrated in order for accurate calculations of volume);

4. Outflow of the pumps through a plankton net (53µm mesh) sitting in a 20 l bucket that is allowed to overflow;

5. A total volume of water to be sampled should be at least 250 l providing a detection threshold of at least 1 cyst in this volume;

6. At least three replicates per ballast tank,

7. Remove excess water from the filtered material and preserve in SET buffered fixative (See below for recipe)

8. Labels should be made of waterproof paper and be printed with indelible ink. Information should include:

   - Vessel identifier (name or risk assessment number)
   - Date and time in 24hr format
   - Ballast Tank
   - Replicate Number (numbered in the order of sampling)
   - Sampled volume (time pumped and flow rate)
   - Sample equipment
   - Sample Type (planktonic/benthic)

Buffer preparation

Preparation of 25X SET buffer

Reagent	Stock in 400ml	To make 400ml of 25X SET	Final Molarity
3.75 M NaCl		87.66g	3.75 M
0.5M Na_2 EDTA (pH8)	74.4g	20 ml	25mM
0.8 M Tris HCl (pH 7.8)	50.42g	200ml	0.4 M

Adjust pH of stock EDTA (pH8) and Tris HCl (pH7.8). Note EDTA does not go into solution unless close to a pH of 8.

Preparation of SET buffered, EtOH fixative (Made fresh from stock)

95% EtOH (25ml), use absolute EtOH directly from the bottle, no need do make up 95%, Milli-Q-water (2ml), 25XSET(3ml). Add reagents in the given order.