Marine Environment & Ecology

Development of a qPCR assay for the Black-striped Mussel, *Mytilopsis sallei*

Nathan J. Bott[1], Danièle Giblot-Ducray[1] and Michael Sierp[2]

SARDI Publication No. F2012/000436-1
SARDI Research Report Series No. 676

SARDI Aquatics Sciences
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December 2012

Report prepared for Commonwealth Department of Agriculture, Fisheries and Forestry & Biosecurity SA

Government of South Australia

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This publication may be cited as:
Bott, N.J.[1], Giblot-Ducray, D.[1] and Sierp, M[2]. (2012). Development of a qPCR assay for the Black-striped Mussel, *Mytilopsis sallei*. Report prepared for Commonwealth Department of Agriculture, Fisheries and Forestry & Biosecurity SA. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2012/000436-1. SARDI Publication No. 676. 18pp.

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Printed in Adelaide: December 2012

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Date:	11 December 2012
Distribution:	Commonwealth Department of Agriculture, Fisheries and Forestry & Biosecurity South Australia , SAASC Library, University of Adelaide Library, Parliamentary Library, State Library and National Library
Circulation:	Public Domain

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Executive Summary

The *National System for the Prevention and Management of Marine Pests* requires tools for the detection and monitoring of marine pests. Specific, robust molecular assays for the identification and quantification of marine pests (including eggs and larvae) from environmental samples provide the rapid, low-cost surveillance required to support effective management and control of marine pests.

Marine pest surveillance is currently based on the system in the Australian Marine Pest monitoring Manual (the Monitoring Manual) (DAFF, 2010) which uses dive surveys, dredges, traps and plankton tows, with organisms being identified by traditional taxonomy. This process is slow and expensive. Quantitative polymerase chain reaction (qPCR) is suitable for marine pest surveillance because it allows testing very large numbers of samples and rapid identification of the genetic material of the target organism (referred to as high-throughput screening). PCR is an enzymatic technique used for the amplification of nucleic acids (e.g. DNA), and qPCR is a technique that monitors changes in fluorescence in the reaction in real time to quantify the amount of target DNA in a sample. The development of qPCR assays for marine pests will facilitate rapid testing of large numbers of plankton samples at relatively low cost, to assess if a pest species is present in a marine system.

In this study we developed a qPCR assay for the detection of the black-striped mussel, *Mytilopsis sallei* (Recluz). We have assessed the specificity of the assay with a range of other bivalve and marine species (in our collection) and showed that the assay is species-specific when tested against the DNA controls available to us. We tested DNA extracted from plankton samples from a range of different Australian localities for *M. sallei* using the qPCR assay developed in this study and found no positives. We conducted spiking experiments with *M. sallei* DNA added to a range of *M. sallei*-negative plankton DNA samples and determined the sensitivity of the qPCR assay in plankton samples, demonstrating our ability to successfully detect *M. sallei* DNA from a spiked plankton DNA sample using 454 pyrosequencing. This technique may prove useful in situations where the presence of a pest requires verification, and is carried out through matching the DNA sequences of a sample to known pest DNA sequences.

This assay, in conjunction with our other marine pest assays, quality control assays and plankton sampling method, provides a basis for surveillance of marine pests. There is still, however, a need to validate these methods in more localities and on different plankton assemblages to adequately troubleshoot issues with sampling procedures, calibrate the DNA extraction process on a wider range of samples and provide field validation of the complete sampling and analysis system.

Acknowledgements

Assistance from the following is gratefully acknowledged:

- Dr Herdina and Dr Alan McKay (SARDI Diagnostics) for assistance and advice in the laboratory
- Dr Marty Deveney (SARDI Aquatic Sciences)
- Dr Richard Willan (NT Museum), Prof. Brian Morton (Natural History Museum, London) and Prof. Carol Stepien (University of Toledo, Ohio) kindly provided specimens for analyses
- Karina Keast and Jenny Cupit (Department of Agriculture Fisheries and Forestry)
- Northern Territory Fisheries Reference collection for photos of Black-striped mussel, *Mytilopsis sallei* used in this report
- This study was funded by the Australian Government, Department of Agriculture, Fisheries and Forestry (DAFF).

Introduction

Marine pests can cause substantial harm to endemic biodiversity and habitats (Galil, 2007; Wallentinus and Nyberg, 2007). Marine pests can be translocated and introduced by vectors including ship ballast, hull fouling, floating debris, transportable man-made structures and aquarium releases (Bax et al., 2003). Marine pest introductions continue to occur and threaten the marine environment, associated industries, communities and social amenity (Hayes and Sliwa, 2003). Increasing globalisation will intensify worldwide transport networks and air transport of live seafood and aquarium fish, shipping will become faster and trips between ports will become more frequent (Bax et al., 2003). The development of effective early detection strategies to monitor for increases in propagule pressure is an important step in prevention and control (Bott et al., 2010a; Darling and Mahon, 2011). Central to such strategies is the ability to rapidly detect and identify the presence of a pest species.

The early detection of newly arrived pests is essential to facilitate an effective response or containment. The Australian, State and Territory governments, along with marine industries and researchers, are collaborating to implement Australia's *National System for the Prevention and Management of Marine Pest Incursions* (the National System). The National System aims to prevent new marine pests arriving, respond when a new pest does arrive or a significant range extension occurs, and minimise the spread and impact of pests already established in Australia.

The National System requires tools for the detection and monitoring of marine pests. Specific, robust molecular assays for identification and quantification of marine pests (including eggs and larval stages) from environmental samples facilitate rapid, low-cost surveillance, and contribute to effective control strategies where marine pest incursions and range extensions are detected. The development and implementation of such diagnostic techniques for identification and surveillance of marine pests from environmental samples (e.g. sea water, sediments, and ship's ballast) is an essential step in early detection and control of marine pests, particularly in areas that are currently pest free.

Current marine pest diagnostics research at South Australian Research and Development Institute (SARDI) includes the development and refinement of quantitative polymerase chain reaction (qPCR) assays for the detection of a number of marine pest species (Bott et al; 2010b; Bott and Giblot-Ducray, 2011a; 2011b; 2012). PCR is an enzymatic technique used for the amplification of nucleic acids (e.g. DNA). qPCR is a PCR technique that enables

quantification of target DNA through real-time monitoring of changes in fluorescence in the reaction. Through consultation between SARDI Aquatic Sciences, Biosecurity SA and the Commonwealth Department of Agriculture, Fisheries and Forestry (DAFF), it was determined that a qPCR assay for the Black-striped mussel, *Mytilopsis sallei* should be developed. *Mytilopsis sallei* is listed on the now out of date Consultative Committee on Introduced Marine Pest Emergencies (CCIMPE) Trigger List, which was endorsed by the former National Introduced Marine Pest Coordination Group (NIMPCG), now the Marine Pest Sectoral Committee (MPSC) since 2011.

Black-striped mussel, *Mytilopsis sallei*

The Black-striped mussel, *Mytilopsis sallei* (Dreissenidae) (Figure 1) is native to tropical and sub-tropical waters of the eastern Pacific, the Gulf of Mexico and the northern parts of South America (Bax et al., 2002). *Mytilopsis sallei* has the potential to infest Australian marine waters from Fremantle, Western Australia across the north of the continent to Sydney, New South Wales. There is also potential for infestation of the warmer parts of Gulf St Vincent and Spencer Gulf in South Australia (DAFF, 2007). Black-striped mussels are sexually mature within one month of settlement and are highly fecund, but there is debate about their precise environmental spawning cues (Bax et al., 2002). Black-striped mussels are considered perhaps the most serious marine pest threat to tropical Australian waters (DAFF, 2007) and they can rapidly foul objects in the water, building a layer up to 15cm thick. It is believed that *M. sallei* is transported to non-endemic locations by vessel hull fouling and/or larvae carried in ballast water (DAFF, 2007).

There has been one documented incursion of *M. sallei* into Australian waters, at Cullen Bay Marina, Darwin Harbour, in 1998-99. The incursion was limited to the marina and eradication required pumping copper sulphate and chlorine into the site (Bax et al., 2002). The AUS$2.2 million cost of the emergency response (not including personnel) was considered minimal (Bax et al., 2002) in comparison to the potential cost to fishing and aquaculture industries in the region, and the unquantifiable costs to the surrounding ecosystem, if the incursion had not been detected early, had spread and was unable to be contained.

The Zebra mussel, *Dreissena polymorpha* and Quagga mussel, *Dreissena rostriformis bugensis,* both freshwater relatives of *M. sallei,* are invasive species in North America. It has been estimated that in the USA between 1993 and 1999 these two species cost

industries, businesses and communities in excess of US$5 billion in relation to cleaning and control exercises, with costs not abating in recent years (Western Regional Panel on Aquatic Nuisance Species, 2010).

Figure 1: Black-striped mussel, *Mytilopsis sallei*

Molecular testing methods for marine pests

Development of rapid testing methods has recently focussed on molecular techniques, and a broad range of these techniques have been assessed for marine pests (see Bott et al., 2010a and references therein). PCR has revolutionised many areas of biological research, including species and strain delineation. PCR can amplify minute amounts of template DNA, and its high specificity makes it effective for species and strain identification for a wide range of organisms. The relatively low cost of equipment and reagents makes PCR accessible to a wide range of laboratories. With a rapid analysis time (< 2 hours), qPCR offers high-throughput screening of samples and allows amplification of target DNA to be monitored in real-time. It allows linear quantification over a wide dynamic range (>6 orders of magnitude), and has the benefit of not requiring post-PCR handling ("closed-tube" format). It is now routinely used in numerous clinical applications for the detection of a wide range of bacterial, fungal, parasitic and viral diseases of humans (Espy et al., 2006). A number of studies have

successfully utilised qPCR-based techniques for the identification of marine pests (see; Galluzi et al., 2004; Pan et al., 2008 Bott et al., 2010a).

Development of PCR tests requires that the target organism is taxonomically unambiguous. Assessing assay specificity requires testing species closely related to the target organism, and environmental samples containing unknown taxa. Most test development achieves the first criterion, but it is also important to validate tests on samples exhibiting higher biological complexity, such as water and sediment, due to the high planktonic diversity inherent to these types of samples. These samples should be obtained from a variety of localities covering the biogeographic regions where the test will be used.

PCR-based tests are developed based on nuclear, ribosomal and mitochondrial gene sequences. A suitable DNA region should vary in sequence sufficiently to allow the identification of an individual organism to the taxonomic level required. For specific identification, the DNA marker should exhibit little or no genetic variation within a species but differ sufficiently between species so as to allow unequivocal delineation.

Genes evolve at different rates. In nuclear genes and spacers (ribosomal DNA (rDNA) genes, Internal Transcribed Spacers (ITS) and Intergenic Spacers (IGS)/ Non-transcribed Spacers (NTS)), there is typically little variation among individuals of a species within and between populations, making them particularly useful as species specific markers for marine pest assay development (Larsen et al., 2005; Livi et al., 2006). The mitochondrial genome is also utilised for diagnostic purposes; mitochondria are generally inherited maternally, making them particularly useful as a species-specific marker for the delineation of closely related species (Blair et al., 2006, Kamikawa et al., 2008).

454 Pyrosequencing Ecogenomics

Ecogenomics is broadly defined as the examination of genetic materials from the environment. Ecogenomic techniques typically target genes of interest which are present in all organisms. Once the sequence of a given gene is known, we can match it against extensive online databases to determine species. In cases where the gene cannot be matched precisely, some level of taxonomic information can be obtained through comparison to similar genes in the databases (e.g. identify to genus or family level). Until relatively recently, ecogenomic methods were costly and extremely time consuming, but the advent of pyrosequencing technology (www.454.com) enables targeted genes within a complex mixture (i.e. plankton or sediments) to be sequenced simultaneously, producing up

to 100,000 sequences per run. Previous studies (e.g. Charition et al., 2010; Stoeck et al., 2010) have utilised these methods to conduct biodiversity assessments. We anticipate that pyrosequencing will allow us to gain a better understanding of floral and faunal assemblages in ports, and allow the verification of positive qPCR results through obtaining matching sequences to known pest species.

Methods

Assay design

All assays at SARDI Diagnostics (see Figure 2) are developed as qPCR, using TaqMan® minor groove binder (TaqMan MGB) chemistry. DNA sequences of the desired genetic marker of target and related organisms were imported into the sequence manipulation software Bioedit© (available from http://www.mbio.ncsu.edu/bioedit/bioedit.html), and aligned using Clustal W (Larkin et al., 2007). The genetic marker of choice is defined by the ability for that marker to delineate the target from heterologous species, as well as the availability of sequences of the chosen genetic marker for related species from publicly available databases. The National Centre for Biotechnology Information (NCBI), as a division of the National Library of Medicine (NLM) at the National Institutes of Health (NIH), has developed databases to deal with molecular data, and facilitates the use of molecular databases by the research and medical community. Genbank® (http://www.ncbi.nlm.nih.gov/genbank/) is one of these databases and is an annotated collection of all publicly available nucleotide and amino acid sequences. A range of DNA sequences of *Mytilopsis sallei* and related taxa were obtained from GenBank, and were aligned to infer sequence regions that appeared to be useful diagnostically. DNA sequences common to the target taxa but distinguishable from related taxa were identified. Specific PCR primers and TaqMan MGB probes were identified by eye, and checked to ensure that they displayed suitable thermodynamic properties and nucleotide content for efficient amplification, using OligoCalc (Kibbe, 2007).

Twelve qPCR assays were designed; 4 targeting 28S rDNA and 8 targeting cox1 mitochondrial DNA. The sensitivity of all putative qPCR assays was tested by determining the cycle threshold at which each could detect known *M. sallei* genomic DNA (gDNA) concentrations. Cycle threshold, (Ct) represents the PCR cycle number at which the fluorescence signal passes a fixed threshold; the lower the Ct value the earlier and more sensitive the qPCR assay. Specificity testing was conducted on the *M. sallei* qPCR assay/s that exhibited the best sensitivity using a set of heterologous gDNA controls including related and unrelated species. All DNA mass per volume amounts in this report are expressed as

femtograms per microlitre (fg/µl); femtograms are 10^{-15} g (further details in glossary). The limits of detection of the *M. sallei* assays were determined using dilutions of target DNA ranging from 200,000 to 2 fg/µl. For each DNA concentration, two replicate qPCRs were conducted.

Samples

Preserved specimens of *Mytilopsis sallei* from the Cullen Bay Marina outbreak were obtained from the Museum of the Northern Territory. Fresh specimens were collected in Hong Kong by Prof. Brian Morton. Specimens of zebra mussel, *Dreissena polymorpha* and quagga mussel, *Dreissena rostriformis bugensis* were obtained from the University of Toledo, Ohio USA. Non-dreissenid samples are from the SARDI marine invertebrate DNA collection and have been collected for numerous past marine pest research projects.

DNA extractions

Genomic DNA was extracted from target and non-target tissues using either of two methods. The first method was the Root Disease Testing Service (RDTS) commercial DNA extraction method, a service provided by SARDI Diagnostics, while the second method used was the QIAGEN DNeasy Blood and Tissue kit, following the manufacturer's instructions. DNA concentration was estimated by fluorometry (Wallac® 1420 multilabel counter) using Quant-iT™ PicoGreen® (Invitrogen). For assessment of the sensitivity of the assay, *M. sallei* gDNA dilutions ranging from 200,000 fg/µl to 2 fg/µl were used. For specificity experiments, gDNA from *M. sallei* and other marine species was tested at 200,000 fg/µl.

Figure 2: SARDI Diagnostics Laboratory

Plankton samples

Plankton samples collected from Port Hedland (WA) (n=16) and Christmas Island (n=8) using a sampling technique described in Giblot-Ducray and Bott (2012), were used for testing the *M. sallei* qPCR assay. These samples are from localities that are within the expected invasive range of *M. sallei* in Australia. DNA was extracted using the RDTS DNA extraction methodology, and all samples were analysed with the SARDI PCR inhibition and brine shrimp qPCR sample quality controls (see Giblot-Ducray and Bott, 2012).

Spiking experiments

Plankton DNA samples from a range of different localities that did not exhibit any evidence of PCR inhibition based on the SARDI PCR inhibition control were first analysed with the *M. sallei* qPCR assay to determine if any of the samples were positive for *M. sallei* (n=3 from each of Port Adelaide, Kangaroo Island, Western Port (Victoria), Dampier (WA), Fremantle (WA), Swan River (WA) and Christmas Island). When they were found to be negative for *M. sallei,* individual samples were spiked with a serial dilution of *M. sallei* DNA (2000 fg/µl to 0.2 fg/µl) and tested to determine the detection limit of the *M. sallei* qPCR assay in an otherwise natural plankton DNA sample.

454 pyrosequencing ecogenomics

Mytilopsis sallei-spiked plankton samples previously determined to be negative for *M. sallei* and subsequently spiked with a serial dilution of *M. sallei* DNA (2000 fg/µl to 0.2 fg/µl) were subjected to 454 pyrosequencing on a GS Junior sequencer (Roche) using the methodology described by Chariton et al. (2010). This experiment was designed to examine if 454 pyrosequencing could be utilised to confirm (through sequence matching) that a plankton sample that is qPCR positive for a pest species actually contains the pest species DNA sequence and is not a false positive. Briefly, the spiked plankton samples prepared previously were PCR amplified using universal fusion primers from Chariton et al. (2010) to amplify the DNA of a wide range of organisms. The PCR products were sequenced using 454 GS Junior Lib-A chemistry (Roche) and a Roche 454 GS Junior pyrosequencer. Sequence data were analysed using Geneious 5.3.6 software (Biomatters, NZ).

Results

Development of *Mytilopsis sallei* qPCR assay

Of the 12 assays designed, two of the 28S rDNA and one of the cox1 mtDNA assays showed the best *M. sallei* detection sensitivity. These assays were further tested and their specificity assessed against a wide range of related and unrelated species (see Table 1 for species list). The cox1 assay, using the primers and probe listed in Table 2, was selected because the 28S rDNA assay cross-reacted with more species.

Specificity of the *Mytilopsis sallei* cox 1 qPCR assay

The result of a qPCR analysis is given as a Ct value (Table 2), which represents the PCR cycle number at which the fluorescence signal passes a fixed threshold. This threshold, displayed as a horizontal green line in plots, is manually set to be the point where DNA amplification rises above the baseline (Figure 3). The lower the Ct value, the more target DNA has been detected.

The cox1 qPCR assay with best sensitivity was tested on a range of related and unrelated non-target taxa to assess if it was specific and did not detect non-target species. This experiment included other dreissenid species (zebra mussel, *Dreissena polymorpha,* and quagga mussel, *Dreissena rostriformis bugensis*), a range of other bivalve species, as well as more distantly related taxa. All four extracts of *M. sallei* were detected as shown by the four amplification lines to the left of Figure 3, with Ct values between 20.1 and 22.4 (Table

1). However, one out of four DNA samples (only one shown in Table 1) extracted from *Dreissenia rostriformis bugensis,* a freshwater species also exotic to Australia, exhibited late amplification (amplification line to the extreme right of Figure 3) with a Ct of 35.7 (Table 1).

Table 1: Results of specificity testing for *Mytilopsis sallei* qPCR assay

Phylum	Class	Genus	Species	Conc. (fg/µl)	RT-PCR Ct thold 0.2
Mollusca	Bivalvia	*Mytilopsis*	*sallei (Hong Kong)*	200,000	20.1
		Mytilopsis	*sallei (Hong Kong)*	200,000	20.9
		Mytilopsis	*sallei (Hong Kong)*	200,000	21.4
		Mytilopsis	*sallei (NT)*	200,000	22.4
		Dreissenia	*polymorpha*	200,000	UD
		Dreissenia	*rostriformis bugensis*	200,000	35.7
		Saccostrea	*glomerata*	200,000	UD
		Ostrea	*angasi*	200,000	UD
		Crassostrea	*gigas*	200,000	UD
		Limnoperna	*securis*	200,000	UD
		Musculus	*miranda*	200,000	UD
		Musculus	*cummingianus*	200,000	UD
		Perna	*viridis*	200,000	UD
		Modiolus	*micropterus*	200,000	UD
		Trichomya	*hirsutus*	200,000	UD
		Musculista	*senhousia*	200,000	UD
		Perna	*canaliculus*	200,000	UD
		Corbula	*gibba*	200,000	UD
Echinodermata		*Asterias*	*amurensis*	200,000	UD
		Asterias	*amurensis*	200,000	UD
		Asterias	*amurensis*	200,000	UD
Chordata		*Ciona*	*intestinalis*	200,000	UD
		Ascidiella	*aspersa*	200,000	UD
Heterokontophyta	Phaeophyceae	*Undaria*	*pinnatifida*	200,000	UD
Arthropoda		*Carcinus*	*maenas*	200,000	UD
Annelida	Polychaeta	*Sabella*	*spallanzanii*	200,000	UD
		NTC			UD

Key: NTC-No Template Control, UD-undetected, NT- DNA from preserved specimen from the Cullen Bay Marina outbreak, Northern Territory

Table 2: Primers and probe of the *Mytilopsis sallei* assay

	Name	Sequence
Forward primer	MytF	GYTAGTTCCRATGATGTTAGCTG
Reverse primer	MytR	ACCTATTGAAACAGGCAACACTC
Probe	Mytilopsis	CCTCGGCTTAATAATGTTAGT

Note: Y=C or T nucleotide, R=A or G nucleotide

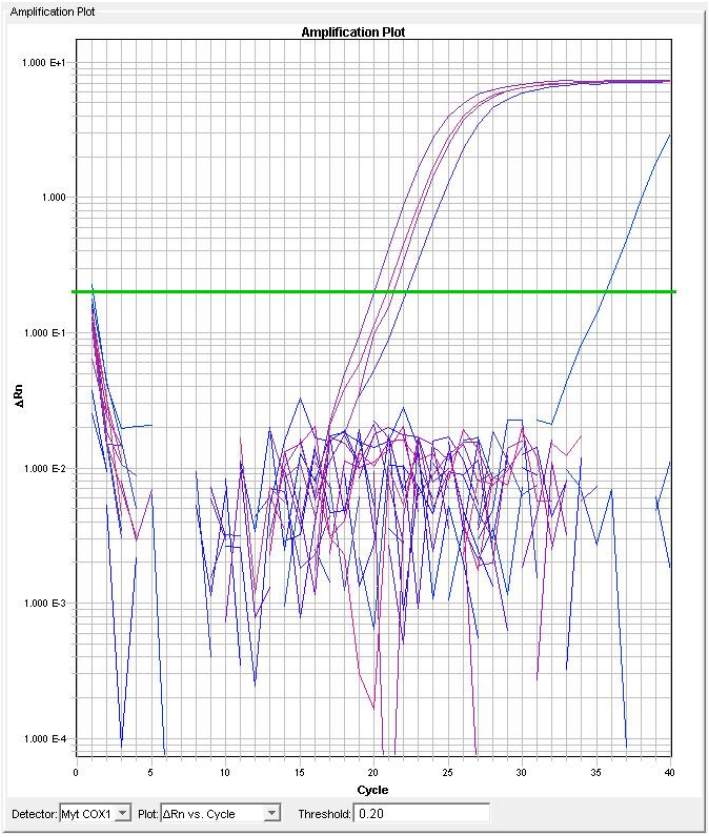

Figure 3: Amplification plot for specificity testing of *Mytilopsis sallei* qPCR assay

Sensitivity of the *Mytilopsis sallei* qPCR assay

The assay can reliably detect 20 fg/µl of *M. sallei* DNA and, less reliably, as little as 2 fg/ul (Figure 4). The assay proved to be linear over at least 6 orders of magnitude (R^2=0.999; slope=-3.3973) (Figure 5) and the efficiency (E) of the assay calculated based on these calibration data is 96.94% (E= ($10^{(-1/slope)}$ -1) x 100). qPCR assays should have an E between 90-100% (Espy et al., 2006), which indicates that amplification is occurring close to exponentially.

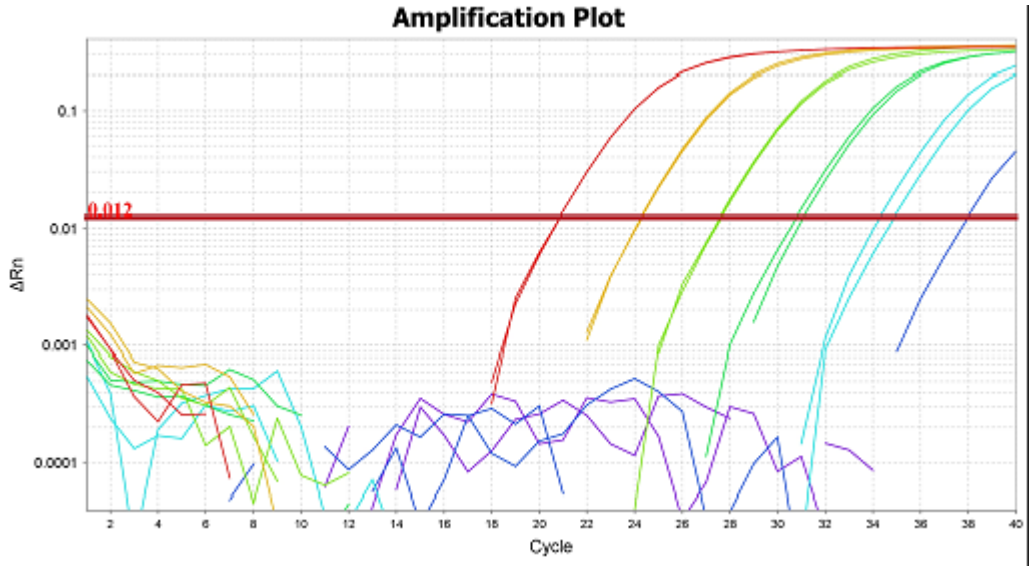

Figure 4: Sensitivity testing for *Mytilopsis sallei* qPCR assay Note: The dark blue line on the far right indicates the 2 fg/µl replicates

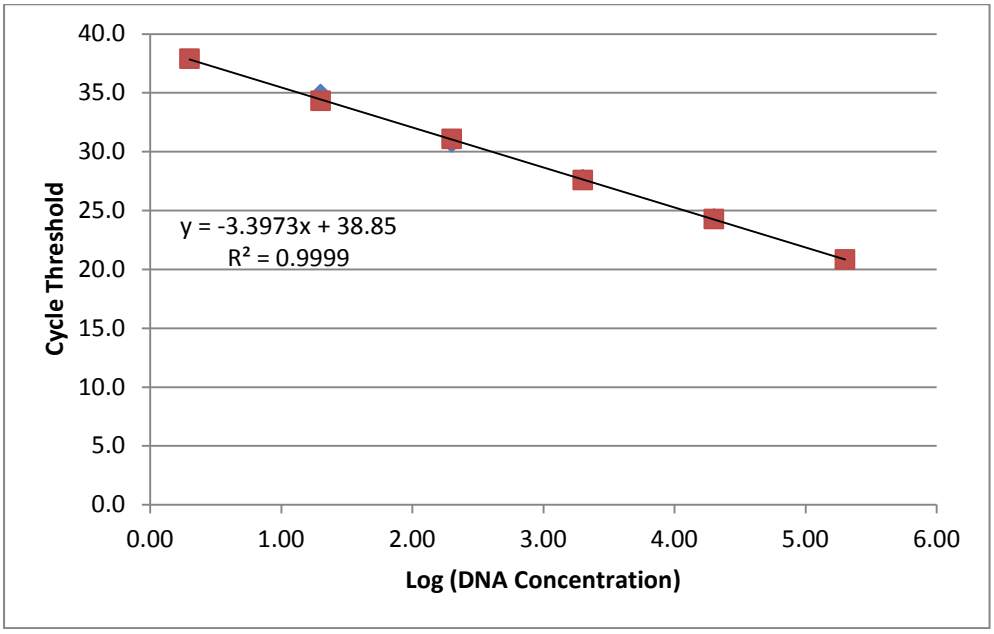

Figure 5: Standard curve of serial dilutions of *M. sallei* gDNA

(200,000 to 2 fg/µl)

Plankton samples

All plankton samples from Port Hedland (WA) and Christmas Island analysed with the *M. sallei* qPCR assay were negative (below detectable limits).

Spiking experiment

The results of the qPCR on selected plankton DNA samples previously determined to be negative for *M. sallei* and subsequently spiked with a serial dilution of *M. sallei* DNA (2000 fg/µl to 0.2 fg/µl) are shown in Table 3. DNA in elution buffer (last column of Table 3) could be detected down to 2 fg/µl, supporting the results of the sensitivity assessment. The sensitivity of the qPCR assay was reduced when *M. sallei* DNA was diluted in plankton DNA from Adelaide (Nov. 2010), Christmas Island, Fremantle, Dampier and Kangaroo Island, but not in plankton DNA from Adelaide (May 2011), Swan River or Western Port. The results show that while the sensitivity of the *M. sallei* qPCR assay in plankton samples is reduced somewhat, it could still reliably detect 20 fg/µl of *M. sallei* DNA, and in some instances 2 fg/µl.

Table 3: Results of spiking experiment

	qPCR results (Ct) for *M. sallei* gDNA in plankton DNA								
Mytilopsis sallei spiked DNA (fg/µl)	Adelaide Nov 10	Adelaide May 11	Christmas Island	Swan River	Fremantle	Dampier	Kangaroo Island	Western Port	Elution buffer
2000	27.9	27.9	27.9	27.8	28.0	27.9	27.9	28.1	27.7
200	31.3	31.2	31.4	31.2	30.7	30.9	31.2	31.6	31.4
20	34.9	34.6	33.9	34.2	34.4	34.4	34.2	35.5	35.4
2	UD	36.7	UD	35.7	UD	UD	UD	36.7	37.0
0.2	UD	UD	UD	UD	UD	UD	UD	38.1	UD
NTC	UD	UD	UD	UD	UD	UD	UD	UD	UD

454 pyrosequencing

The 454 GS Junior sequencing experiment produced 70,388 sequences at a mean length of 202 bp. Using Geneious 5.3.6, the sequence data were split according to the different plankton DNA origins and *Mytilopsis sallei* DNA concentrations via the "separate by barcode" option. What we unexpectedly found was using the Chariton et al. (2010) primers, we amplified the SARDI PCR inhibition control (used to monitor the presence of PCR inhibitors), and this sequence was present in high numbers. *Mytilopsis sallei* was, however, detected from the Western Port plankton DNA spiked with 2000 fg/µl of *M. sallei* DNA. There were also a range of other sequences generated from a diverse range of phyla, the most dominant being crustacea and diatoms.

Discussion

We have developed and laboratory validated a qPCR assay for the detection of the black-striped mussel, *Mytilopsis sallei.* The assay has a limit of detection between 2 and 20 fg/µl depending on the sample, and quantification is linear over six orders of magnitude. The specificity testing conducted using SARDI's range of heterologous controls showed that this assay satisfies the requirements for a species-specific qPCR assay. The late amplification of exotic *Dreissenia rostriformis bugensis* DNA could be due to either a weak cross reaction or contamination. Given that only one of four *D. rostriformis bugensis* gDNA samples exhibited late amplification, it is most likely that this result was caused by sample contamination. A small aerosol droplet of undiluted *M. sallei* gDNA entering a sample may cause contamination strong enough to be a PCR positive given the high sensitivity of qPCR. It should be noted, however, that *D. rostriformis bugensis* is a freshwater pest species that is exotic to Australia so it is unlikely to occur in Australian marine plankton samples - if it were found, it would be of significant concern. Australia has no endemic dreissenid species. If this cross-reaction is real, it indicates that any positive result will indicate the presence of an exotic dreissenid, but it may preclude the use of this qPCR assay for the specific detection of *M. sallei* in countries where *D. rostriformis bugensis* is established or endemic. It would be advantageous to obtain fresh *D. rostriformis bugensis* specimens to explore this issue further.

The detection limit of the qPCR assay when testing spiked plankton samples was within the range reported (2 to 20 fg/µl) for *M. sallei* DNA in buffer (i.e. no other DNA present). This demonstrates that the *M. sallei* qPCR assay can detect minute amounts of *M. sallei* DNA from environmental samples, provided that the sample does not significantly inhibit PCR. It would be advantageous to obtain planktonic stages of *M. sallei* to accurately determine the limit of biological detection of the *M. sallei* qPCR assay.

Further work to develop sampling regimes and methodologies is needed to ensure that molecular surveys for marine pests using these assays are robust, statistically relevant and comparable to traditional surveys. All marine pest qPCR assays require rigorous field proofing of specificity by being applied to field samples from a wide variety of localities with different endemic fauna and flora. While we have tested some plankton samples from localities that fall within the expected invasive range of *M. sallei,* we note that the number of samples tested are likely insufficient and further field validation is required. This is needed

to further assess the assays' specificity against a wider range of DNA from non-target species in plankton samples. Correlating positive qPCR results with introductions and/or discovery of pest species detected by traditional means is important for building confidence in qPCR assay utility and validating the system for broader use.

454 pyrosequencing ecogenomics

The sequencing of the SARDI PCR inhibition control greatly affected the ability of the pyrosequencer to detect spiked *M. sallei* sequences in very low sequence numbers. While the sample we were able to detect *M.* sallei from was spiked with 2000 fg/µl *M. sallei* gDNA, it should be noted that this is actually a small amount of DNA. It would be advantageous to obtain larval stages of *M. sallei* to determine the amount of DNA per larva, and the detection limit of pyrosequencing in terms of larvae per sample. Ophel-Keller et al. (2007) estimated that the total amount of DNA from five *Corbula gibba* (a bivalve marine pest) larvae was 1,738,000 fg (347,600 fg/ larva), and thus we believe 2000 fg/µl of target DNA would represent considerably less than 1 whole *M. sallei* larva. This trial is a step forward in developing a new surveillance tool to complement the existing qPCR assays, and more research will enable us to work with samples prepared for qPCR analysis (i.e. including quality controls) and maximise the likelihood of detecting target species. The key step towards achieving this is the re-design of sequencing primers that do not amplify inhibition control DNA material, once this is resolved it will allow us to work with DNA extracted for qPCR analyses.

454-based technology will assist us to understand and identify problems with assay specificity, particularly with DNA of species that are not available on Genbank and were not used during laboratory validation. This technology offers the ability for future projects to pre-screen samples from various localities to better understand the sequence diversity in the environment. This will ultimately aid in future qPCR test development, sampling design and understanding of baseline aquatic biodiversity. qPCR is still preferred to 454 pyrosequencing for environmental surveillance of marine pests as it offers high-throughput capability, and is a highly rapid method of targeted surveillance.

Conclusions and future approaches

It is vital to have access to marine pest larvae and gametes to more accurately validate marine pest qPCR assays, given that this is the life stage that these assays target. A better understanding of biological processes (including timing of, and *in situ* cues for, spawning of

marine pests in their invasive range) is required to optimise surveillance using molecular methods. The Marine Pest Monitoring Manual (DAFF, 2010) plankton sampling guidelines are designed to primarily detect listed phytoplankton species, not the larvae of benthic or pelagic pest species. The Monitoring Manual needs to be amended to take into account the spawning seasons of non-planktonic pest species. Future marine pest projects must improve our understanding of invasion biology, catalogue marine pest DNA sequence data to aid understanding phylogenetic relationships, and validate existing assays and sampling strategies in a wide range of localities.

This qPCR assay for *M. sallei*, in conjunction with assays developed for other marine pest species, is a step toward implementation of a comprehensive, rapid, cost-effective surveillance system for marine pests in Australia. With continued development, a surveillance system for monitoring marine pests in plankton using these assays has the potential to become an important platform to inform managers about the status of marine pests in Australian waters.

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Glossary of Terms

CCIMPE- Consultative Committee on Introduced Marine Pest Emergencies

Cox1- Cytochrome c oxidase 1 gene of mitochondrial DNA, an informative diagnostic region

Ct-Cycle threshold: qPCR cycle where fluorescence is observed above a threshold level i.e. indicates a positive result.

DNA- Deoxyribonucleic Acid: genetic information responsible for the development and function of all organisms, with the exception of some viruses.

Femtogram (fg): A unit of mass equal to 1×10^{-15} grams. PCR assays often can detect DNA mass to fg/µl.

gDNA-genomic Deoxyribonucleic Acid: the total DNA of an organism, or the genome of an organism.

ITS-2: second internal transcribed spacer; a region of ribosomal DNA that does not code for any genes

mtDNA- mitochondrial DNA: the genome of the intracellular organelles called mitochondria. Considered an informative diagnostic region.

NIMPCG- National Introduced Marine Pest Coordinating Group

NTC-No Template Control, a PCR reaction with no DNA template added, is used to ensure that PCR is not previously contaminated i.e. NTC should not be a positive result.

Nucleotide: Molecules, that when joined together make up the functional units of DNA.

MPSC- Marine Pest Sectoral Committee

PCR- Polymerase Chain Reaction: Enzymatic technique used for the amplification of nucleic acids (e.g. DNA)

qPCR-Quantitative Polymerase Chain Reaction-PCR reaction whereby amplification is monitored in real time through the use of fluorescent dyes or probe based chemistry.

TaqMan MGB-TaqMan Minor Groove Binder probe-hybridises to specific fragment of DNA, and emits fluorescence; used to quantify target DNA in a sample.

rDNA- ribosomal Deoxyribonucleic Acid: codes for vital cellular components in Eukaryotes; an informative diagnostic marker.

RDTS- Root Disease Testing Service; a commercial diagnostic service at SARDI