Marine Environment and Ecology

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Molecular tools for detection of marine pests: Development of putative diagnostic PCR assays for the detection of significant marine pests: *Asterias amurensis, Carcinus maenas, Undaria pinnatifida* and *Ciona intestinalis*.

Nathan J. Bott, Danièle Giblot-Ducray and Marty R. Deveney

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South Australian Research and Development Institute
SARDI Aquatic Sciences
2 Hamra Avenue
West Beach SA 5064

Telephone: (08) 8207 5400
Facsimile: (08) 8207 5406
http://www.sardi.gov.au

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Author(s): Nathan J. Bott, Danièle Giblot-Ducray and Marty R. Deveney

Reviewer(s): Herdina and Kathryn Wiltshire

Approved by: Dr Jason Tanner
Principal Scientist – Marine Environment & Ecology

Signed: 

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Executive Summary

The *National System for the Detection 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 larval stages) from environmental samples facilitate rapid, low-cost surveillance to be undertaken and effective control strategies to be implemented where marine pest incursions are detected. The use of quantitative PCR (qPCR) techniques will be suitable for this purpose. The polymerase chain reaction (PCR) is an enzymatic technique used for the amplification of nucleic acids (e.g. DNA), and quantitative PCR (qPCR) is a PCR technique monitored in real-time through changes in fluorescence.

The use of qPCR offers the ability to conduct testing of very large numbers of samples to rapidly identify the genetic material of the targeted organisms (referred to as high-throughput screening). The successful development of these methods will allow for the collection and testing of plankton samples to rapidly verify the presence of potential pest species in marine waters. Currently detection of marine pest is based primarily on dive surveys.

In this study we have tested the performance of a qPCR assay previously developed for the Northern Pacific Seastar, *Asterias amurensis* (developed by CSIRO Marine Research) and developed putative qPCR assays for the European Green Shore Crab, *Carcinus maenas*, the Vase Tunicate, *Ciona intestinalis* and Japanese Seaweed, *Undaria pinnatifida.*

The *A. amurensis* assay can reliably detect 2 fg/µl of target DNA, and is quantifiable over 6 orders of magnitude, making it a potentially powerful tool for quantifying biomass of *A. amurensis* from environmental samples (water and sediments). The three qPCR assays newly developed as part of this project will require ongoing development in order for the assays to be suitable for conducting analyses of environmental samples. Following further development of these three qPCR assays; their use in conjunction with the *A. amurensis* and other qPCR assays will enable opportunities to provide a comprehensive marine pest testing service that will be able to quantify target biomass. This service will enable high-throughput testing that will complement traditional taxonomic expertise.

Specific diagnosis of marine pests is central to rapidly establishing the prevalence and distribution of marine pest species, monitoring changes in marine pest distribution spatially and temporally, and conducting targeted eradication and control programmes where economics and logistics permit.

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Introduction

The *National System for the Detection 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 larval stages) from environmental samples facilitate rapid, low-cost surveillance, and inform effective control strategies where marine pest incursions are detected.

Marine pests have the potential to cause significant harm to endemic biodiversity and habitats (Galil, 2007; Wallentinus and Nyberg, 2007). Marine pests can be translocated and introduced by numerous vectors including ship ballast, hull fouling, floating debris and man-made structures such as drilling platforms and canals (Bax et al., 2003). Marine pest introductions continue to occur and threaten the marine environment and associated industries (Hayes and Sliwa, 2003). With increasing globalisation comes faster and more frequent shipping and air transport of live seafood. Propagule pressure is only likely to increase unless effective strategies are employed for early detection, prevention and control. Central to such strategies is the ability to rapidly identify the presence of a particular pest species.

The development and implementation of rapid, sensitive and accurate diagnostic techniques for the identification and surveillance of marine pests from environmental samples (e.g. sea water, sediments, and ship's ballast), particularly in areas that are currently pest free, is an essential step in early detection and control of marine pests.

Current marine pest diagnostics research at SARDI includes the development and refinement of specific, sensitive, quantitative PCR assays for the detection of a number of marine pest species. Polymerase Chain Reaction (PCR) is an enzymatic technique used for the amplification of nucleic acids (e.g. DNA). Through consultation between SARDI Aquatic Sciences, Biosecurity SA, and the Adelaide and Mt Lofty Ranges Natural Resources Management Board, it was decided to undertake a study to implement and/or develop quantitative polymerase chain reaction (qPCR) assays for four marine pest species of significance to Australia, three of which: Northern Pacific Seastar*, Asterias amurensis*; European Green Shore Crab*, Carcinus maenas*; and Japanese Seaweed, *Undaria pinnatifida* are part of the Consultative Committee on Introduced Marine Pest Emergencies (CCIMPE) Trigger List, which is endorsed by the National Introduced Marine Pest Coordinating Group (NIMPCG). The fourth is the Vase Tunicate*, Ciona intestinalis,* commonly found in Port Adelaide, and was chosen because of its highly invasive nature and potential for negative impacts on aquaculture and man-made structure (see Therriault and Herborg, 2008).

Northern Pacific Seastar*, Asterias amurensis*

It is widely recognised that incursions of *A. amurensis* cause impacts through predation to scallop and mussel fisheries and aquaculture in Tasmania (http://marinepests.gov.au/__data/assets/pdf_file/0010/952489/Asterias-ncp-08.pdf). In 2006, 25 tonnes of *A. amurensis* were caught as by-catch by Tasmanian commercial scallop fishermen, as well as reports of scallop spat collector bags and suspended 'grow-out' cages containing very large numbers of *A. amurensis* (ABARE, 2007; Lister, 2007). *A. amurensis* has significant potential to also affect abalone fisheries through predation (McManus & Proctor, 2001). There are no confirmed reports of *A. amurensis* in South Australian waters (Wiltshire et al., 2010).

Figure 1: Northern Pacific Seastar, *Asterias amurensis*

European Green Shore Crab*, Carcinus maenas*

Carcinus maenas, the European Green Shore Crab, has had significant impacts on commercially important bivalve species in North America through predation (see http://marinepests.gov.au/__data/assets/pdf_file/0003/952509/carcinus-ncp-08.pdf). While there

are no current economic impacts attributable to *C. maenas* in Australian waters, it has the potential to cause serious impacts to aquaculture, in particular bivalve aquaculture. *Carcinus maenas* has demonstrated significant environmental impacts in both its native and introduced range; predation effects due to *C. maenas* have the potential to influence the distribution and abundance of a range of marine taxa. In Tasmania there have been demonstrated significant impacts to native bivalve and crab populations due to *C. maenas* invasion (see http://marinepests.gov.au/__data/assets/pdf_file/0003/952509/carcinus-ncp-08.pdf). *Carcinus maenas* has previously occurred around Port Adelaide, along the Adelaide coast, in western Gulf St Vincent and the Coorong, but has been rarely found in recent years aside from one sighting in West Lakes in 2009 (Wiltshire et al., 2010).

Figure 2: European Green Shore Crab, *Carcinus maenas* Source: Anthony Fisher

Vase Tunicate, *Ciona intestinalis*

Ciona intestinalis is a solitary tunicate with a cylindrical, gelatinous body, up to 14 cm long. *Ciona intestinalis* is considered to be an invasive species and grows in dense aggregations on any floating or submerged substrate, particularly artificial structures like pilings, aquaculture gear, floats and boat hulls, in the lower intertidal to sub-tidal zones. Peterson and Riisgard (1992) have shown that *C. intestinalis* may have an important phytoplankton grazing impact; this may have the

potential to have negative impacts on native phyto-planktivores. *Ciona intestinalis* has been identified at a wide range of geographical locations and has exhibited negative impacts on shellfish aquaculture (see Therriault and Herborg, 2008). *Ciona intestinalis* is common in Port Adelaide, North Haven and West Lakes, with further occurrences in Port Lincoln, Wallaroo and American River (Kangaroo Island) (Wiltshire et al., 2010).

Figure 3: Vase Tunicate, *Ciona intestinalis* Source: http://blogs.dickinson.edu/sciencenews/

Japanese Seaweed, *Undaria pinnatifida*

Undaria pinnatifida, the Japanese Seaweed or Wakame, competes with native seaweeds outside of its native range. Disturbance plays an important role in the invasion ecology of *U. pinnatifida*. This alga is a very prolific and hardy species, with a growth rate measured at 1-2 cm per day, and a maximum length of 3 m (see http://marinepests.gov.au/__data/assets/pdf_file/0009/952569/undaria-ncp-08.pdf). *Undaria pinnatifida* has not been recorded from South Australian waters to date, although it has been recently reported from Apollo Bay, western Victoria (http://www.dse.vic.gov.au/CA256F310024B628/0/86F73266B27503DECA257700001F5315/$File/Coastline+Update+Autumn10.pdf).

Figure 4: Japanese Seaweed, *Undaria pinnatifida* Source: NOAA

Molecular Testing Methods for marine pests

Development of rapid testing methods for marine pests has recently focussed on molecular techniques. A broad range of these techniques have been developed for marine pests (see Bott et al., 2010 and references therein). The use of qPCR offers the ability for high-throughput screening of assays for numerous pest species. Polymerase Chain Reaction (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 highly 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. qPCR allows the amplification of a target DNA to be monitored in real-time as amplification occurs. qPCR offers a relatively rapid analysis (< 2 hours), the potential for high-throughput applications, allows linear quantification over a wide dynamic range (>6 orders of magnitude) and 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). Recent advances have seen a number of studies utilising qPCR-based techniques for the identification of marine pests (see Galluzi et al., 2004; Pan et al., 2007).

The development of these tests requires that the target organism is taxonomically unambiguous. Testing of a number of species closely related to the target organism is required as well as testing of environmental samples containing unknown taxa. Most test development achieves the first

criterion but for implementation, it is important to validate tests on samples exhibiting higher complexity such as water and sediment samples.

Many PCR-based tests are developed based on nuclear ribosomal and mitochondrial gene sequences. Genes evolve at different rates and a suitable DNA region should vary in sequence sufficiently to allow the identification of an individual 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.

In nuclear genes and spacers, there is typically little variation amongst individuals of a species within and between populations (Larsen et al., 2005; Livi et al., 2006). The ribosomal DNA (rDNA) genes, Internal Transcribed Spacers (ITS) and Intergenic Spacer (IGS)/ Non-transcribed spacer (NTS) regions have been shown to be particularly useful in defining species specific markers for marine pest assay development. 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 (e.g. Blair et al., 2006, Kamikawa et al., 2008).

In this report we detail the adoption of the qPCR assay for the detection of Northern Pacific Seastar, *Asterias amurensis* (developed by CSIRO Marine Research), and the development of putative qPCR assays for the specific detection of European Green Shore Crab, *Carcinus maenas,* Vase Tunicate, *Ciona intestinalis* and Japanese Seaweed, *Undaria pinnatifida*.

Methods

Quantitative PCR (qPCR) Assay Design

All assays at SARDI Diagnostics (see **Figure 5: SARDI Diagnostics Laboratory** 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 a sequence manipulation software program Bioedit (available from http://www.mbio.ncsu.edu/RNaseP/info/programs/BIOEDIT/bioedit.html.), and aligned using Clustal W. The genetic marker of choice is defined by the ability for that marker to be able to delineate the target from heterologous species and also the availability of sequences from publicly available databases. A range of DNA sequences were obtained from the public domain database GenBank (http://www.ncbi.nlm.nih.gov/genbank/). 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 is one of these databases and is an annotated collection of all publicly available nucleotide and amino acid sequences.

Sequences of target and related taxa were aligned to infer sequence regions that appeared to be useful diagnostic regions. DNA sequences were identified which are common to the target taxa but where there are enough differences to distinguish target from related taxa. Specific PCR primers and TaqMan MGB probes were developed for target taxa using the assay design software Primer Express v2.0 (Applied Biosystems), an application that designs primers and TaqMan MGB probes that display suitable thermodynamic properties and nucleotide content for efficient amplification.

Figure 5: SARDI Diagnostics Laboratory

Samples

Samples were collected, and immediately stored frozen, or preserved in ethanol, for genomic DNA (gDNA) extraction. Algal samples (for testing the *U. pinnatifida* qPCR assays) were sourced as pre-extracted gDNA from the Gurgel laboratory, University of Adelaide.

DNA extractions

Genomic DNA was extracted from target and non-target samples by one of two methods. The first method being 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). gDNA for qPCR specificity experiments was typically diluted to 200 pg/µl.

Quantitative PCR

qPCR reactions were carried out in 384 well plates for analysis on an ABI HT 7900 sequence detection system (Applied Biosystems, Foster City, CA) using QuantiTect™ QPCR mastermix

(QIAGEN). Each PCR assay is run with plate controls (no DNA control and a positive control for each assay) and is analysed with ABI SDS 2.3 software (Applied Biosystems). The PCR cycling conditions were: 15 minutes at 95°C (activation), 40 cycles of 15 secs at 95°C (denaturation) & 60°C at 1 minute (annealing).

Results

Primers and Probes

We designed a range of potential qPCR assays for the detection and enumeration of *Carcinus maenas, Ciona intestinalis* and *Undaria pinnatifida.* Table 1 shows the primers and TaqMan MGB probes that have exhibited the highest level of specificity to date, other primer and probe combinations that did not offer appropriate specificity or amplification efficiency were not considered further (data available on request). Table 1 lists the genetic marker that the qPCR assay hybridises to, the nucleotide content of the primers and probes, and the melting temperature (Tm) of the primers and probes which is important for determining the reaction conditions of qPCR experiments.

Table 1: Primers and TaqMan MGB probes

Assay	Genetic Marker	Forward Primer (5'-3')	Tm (°C)	Reverse Primer (5'-3')	Tm (°C)	Taqman MGB probe	Tm (°C)
*Asterias amurensis**	Cox1	GCACAACCGGGATCTTTACTTC	59	AGTCATTTACCAAATCCTCCTATCATAATA	58	6FAM- TCATGCTCTTGTAATGATAT- MGB	68
Carcinus maenas No. 1	Cox1	ATGAACAGTCTATCCTCCTTTAG	59	GAAAGAACGCATATTGATAATAGTTG	60	6FAM-AGTTGATTTAGGGATTTTC-MGB	69.8
C. maenas No. 2	Cox1	GAACAGTCTATCCTCCTTTAGCA	61	GCCGAAAGAACGCATATTGATAATA	61	6FAM-AGTTGATTTAGGGATTTTC-MGB	69.8
Ciona intestinalis	ITS-2 rDNA	TATTAACTATCGCCGCTTCGG	60	GACCGCTCCACTGATTTTCG	60	6FAM-CGCGCTTGCGGTC-MGB	69.3
Undaria pinnatifida No. 1	Cox1	CTTTAATTACAGCGTTTTTATTGTTGT	59	AGTAGTATTAAAATTACGATCTGTTAGT	60	6FAM-CGGTTTTAGCAGGTGCT-MGB	69
U. pinnatifida No. 2	Cox1	TACAGCAATGTCTGTTTTTATCC	58	ACATTATACAACTGATGATTTCCC	58	6FAM-ATTGCAATTAGCTAGCCCTG-MGB	69.8

Key: *- Assay developed by CSIRO. Cox1-Cytochrome c oxidase 1 gene of mitochondrial DNA; ITS-2 rDNA-Second internal transcribed spacer of ribosomal DNA. Tm-Melting temperature of primer/probe. 6FAM- 6 Carboxyfluorescein (fluorophore), MGB-Minor Groove Binder non fluorescent quencher

Specificity of qPCR assays

All qPCR assays were tested on a range of non-target taxa to demonstrate that the assays are specific and did not detect non-target species. These experiments included a range of related and unrelated taxa (listed below in Tables 2 - 5). qPCR results are given as cycle threshold (Ct) values. The Ct value represents the PCR cycle number at which the fluorescence signal passes a fixed threshold, displayed as a horizontal green line in plots showing number of qPCR cycles vs magnitude of the fluorescence signal intensity (∆Rn) (see Figures 6 - 10). The lower the Ct value, the more target DNA detected.

Asterias amurensis

A TaqMan MGB qPCR assay was designed by CSIRO Marine Research for *A. amurensis* (Bax et al., 2006); the authors tested this assay with a wide range of heterologous echinoderm DNA from species endemic to Australia and found the assay to be specific to *A. amurensis*. We further tested the *A. amurensis* qPCR assay with a range of heterologous DNA to further assess the specificity (see Table 2). We did not observe any issues with the specificity of this assay.

Table 2: Results of specificity testing for *A. amurensis* qPCR assay

Phylum	Class	Genus	Species	DNA. (pg/ul)	Ct values
Echinodermata		*Asterias*	*amurensis*	200	17.4
Chordata		*Ciona*	*intestinalis*	200	UD
		Ascidiella	*sp.*	200	UD
Mollusca	Bivalivia	*Perna*	*viridis*	200	UD
		Limnoperna	*securis*	200	UD
		Musculus	*miranda*	200	UD
		Musculus	*cummingianus*	200	UD
		Perna	*viridis*	200	UD
		Modiolus	*micropterus*	200	UD
		Trichomya	*hirsutus*	200	UD
		Musculista	*senhousia*	200	UD
		Perna	*canaliculus*	200	UD
		Perna	*viridis*	200	UD
		Undaria	*pinnatifida*	200	UD
Crustacea	Decapoda	*Carcinus*	*maenas*	200	UD
N/A	N/A	NTC		UD	UD

Key: NTC- No Template Control, UD-undetected

Carcinus maenas

While CSIRO Marine Research began the development of a PCR assay for *Carcinus maenas*, we were unable to access their data and so designed our own putative qPCR assay. We designed primers and probes from the mitochondrial gene, cytochrome c oxidase 1 (*cox1*), and tested the assay against a range of crustacea collected from Gulf St Vincent and other marine invertebrate DNA in our collection. So far we have no issues with the specificity of this assay (Table 3). Figure 6 and Figure 7 illustrate the real-time amplification curves for *C. maenas* assays No. 1 and No.2 respectively; the only gDNA amplified (i.e. above the green horizontal line) in both figures is *C. maenas.*

Table 3: Results of specificity testing for *C. maenas* qPCR assays

Phylum	Class	Genus	Species	DNA (pg/ul)	Ct values	
					C. maenas 1	*C. maenas 2*
Crustacea	Decapoda	*Carcinus*	*maenas*	200	21	21.2
		Squila	*mantis*	200	UD	UD
		Portunus	*pelagicus*	200	UD	UD
		Melicertus	*latisulcatus*	200	UD	UD
	Siphonostomatidea	*Caligus*	sp. 1	200	UD	UD
		Caligus	sp. 2	200	UD	UD
		Caligus	sp. 3	200	UD	UD
		Caligus	sp. 4	200	UD	UD
Echinodermata		*Asterias*	*amurensis*	200	UD	UD
Chordata		*Asciediella*	sp.	200	UD	UD
		Ciona	*intestinalis*	200	UD	UD
Mollusca	Bivalvia	*Limnoperna*	*securis*	200	UD	UD
		Musculus	*miranda*	200	UD	UD
		Musculus	*cummingianus*	200	UD	UD
		Modiolus	*micropterus*	200	UD	UD
		Trichomya	*hirsutus*	200	UD	UD
		Musculista	*senhousia*	200	UD	UD
		Perna	*canaliculus*	200	UD	UD
		Perna	*viridis*	200	UD	UD
N/A	N/A	NTC			UD	UD

Key: NTC- No Template Control, UD-undetected

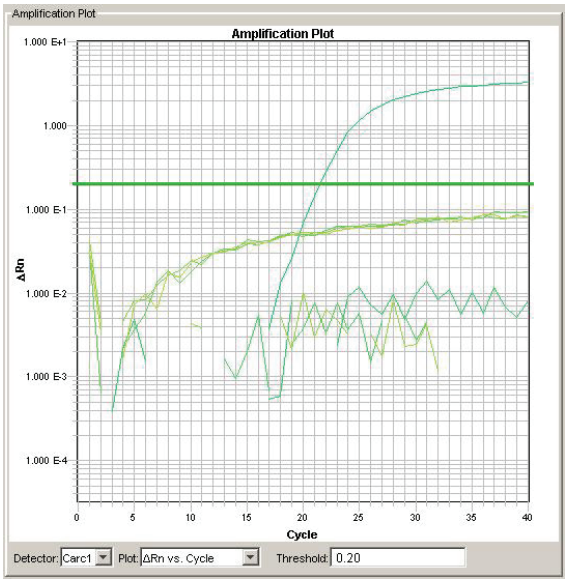


Figure 6: Amplification plot for *C. maenas* qPCR assay No. 1. Amplified product is *C. maenas* gDNA.

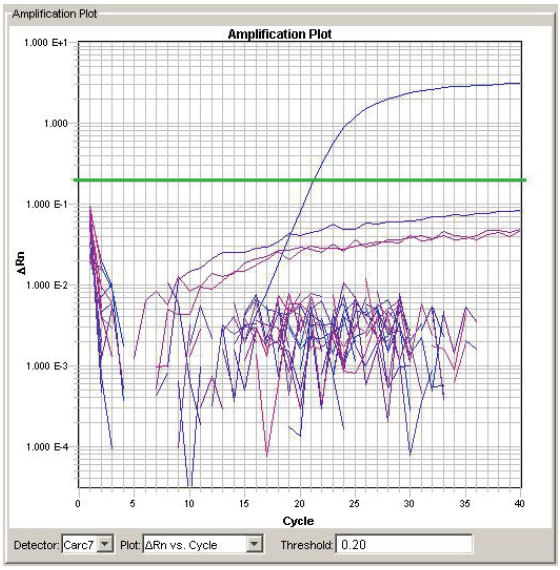


Figure 7: Amplification plot for *C. maenas* qPCR assay No. 2. Amplified product is *C. maenas* gDNA.

Ciona intestinalis

We have designed a putative qPCR assay for *C. intestinalis*, based on the second internal transcribed spacer of ribosomal DNA (ITS-2 rDNA). We have tested this qPCR assay on ascidian and other invertebrate species (see Table 4). Figure 8 illustrates the amplification curve for the *C. intestinalis* qPCR assay. Two gDNA samples are amplified, the product on the left is *C. intestinalis* while the product on the right (which denotes late amplification) is an ascidian, *Ascidiella* sp. from the Port River. This issue will be assessed, and steps will be taken to ensure that this assay achieves the level of specificity required for routine use.

Table 4: Results of specificity testing for *C. intestinalis* qPCR assay.

Phylum	Class	Genus	Species	DNA (pg/ul)	Ct values
Chordata		*Ciona*	*intestinalis*	200	24.65
		Ascidiella	sp.	200	36.09
Mollusca	Bivalvia	*Perna*	*viridis*	200	UD
		Limnoperna	*securis*	200	UD
		Musculus	*cummingianus*	200	UD
		Musculus	*miranda*	200	UD
		Perna	*viridis*	200	UD
		Dentimodiolus	*setiger*	64*	UD
		Modiolus	*micropterus*	200	UD
		Trichomya	*hirsutus*	200	UD
		Musculista	*senhousia*	200	UD
		Perna	*canaliculus*	200	UD
		Perna	*viridis*	200	UD
		NTC			UD

Key: NTC- No Template Control, UD-undetected, *-sample with low gDNA yield.

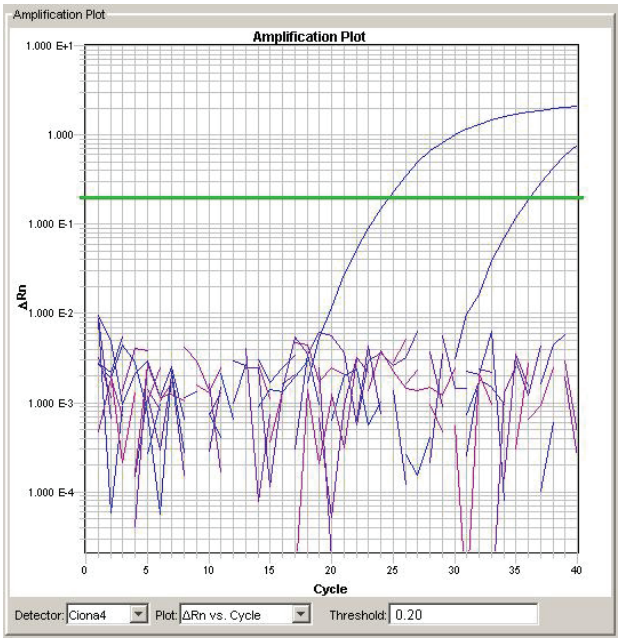


Figure 8: Amplification plot for *C. intestinalis* qPCR assay. Amplified product on left is *C. intestinalis* gDNA while amplified product on right is *Ascidiella* sp. gDNA .

Undaria pinnatifida

CSIRO Marine Research began the development of a PCR assay for *Undaria pinnatifida*, we were unable to access their data so we designed our own putative qPCR assay. We have designed two putative qPCR assays for *U. pinnatifida* from the *cox1* gene. We have tested these assays against a range of algal and seagrass species (see Table 5) and so far have not experienced any issues with specificity. Figure 9 and Figure 10 illustrate the real-time amplification curve for *U. pinnatifida* assay No. 1 and No.2 respectively; the only gDNA amplified (i.e. above the green horizontal line) in both figures is *U. pinnatifida.*

Table 5: Results of specificity testing for *U. pinnatifida* qPCR assays.

Phylum	Class	Genus	Species	DNA (pg/ul)	Ct values	
					U. pinnatifida 1	*U. pinnatifida* 2
Heterokontophyta	Phaeophyceae	*Undaria*	*pinnatifida*	200	19.9	19.9
Chlorophycophyta	Bryopsidophyceae	*Caulerpa*	*remotifolia*	200	UD	UD
		Caulerpa	*racemosa* var. *cylindracea*	200	UD	UD
		Caulerpa	*flexilis*	200	UD	UD
		Caulerpa	*racemosa* var. *cylindracea*	200	UD	UD
		Caulerpa	*trifaria*	200	UD	UD
		Caulerpa	*obscura*	200	UD	UD
		Caulerpa	*taxifolia*	200	UD	UD
Magnoliophyta	Monocots	*Amphibolis*	*antarctica*	200	UD	UD
		Posidonia	*angustifolia*	200	UD	UD
Rhodophycophyta	Florideophyceae	*Ceramium*	cf. *flaccidum*	200	UD	UD
		Laurencia	*implicata*	200	UD	UD
		Gelidiopsis	*scopania*	200	UD	UD
		Peyssonnelia	sp.	200	UD	UD
		Chondria	sp.	200	UD	UD
		Pradaea	sp.	200	UD	UD
		Dudresnaya	sp.	200	UD	UD
		Ganonema	*pinnatum*	200	UD	UD
		Balliella	sp.	200	UD	UD
		Haloplegma	*dupreyri*	200	UD	UD
		Kallymenia	sp.	200	UD	UD
		Gibsmithia	*hawaiiensis*	200	UD	UD
		Hypnea	sp.	200	UD	UD
		Martensia	*parvula*	200	UD	UD
		Dudresnaya	sp.	200	UD	UD
		Balliella	sp.	200	UD	UD
		Spyridia	sp.	200	UD	UD
		Chondrophycus	sp.	200	UD	UD
		Gracilaria	sp.	200	UD	UD
		Red	1	200	UD	UD
		Red	2	200	UD	UD
?	?	Mystery	red	200	UD	UD
N/A	N/A	NTC	NTC		UD	UD

Key: NTC- No Template Control, UD-undetected

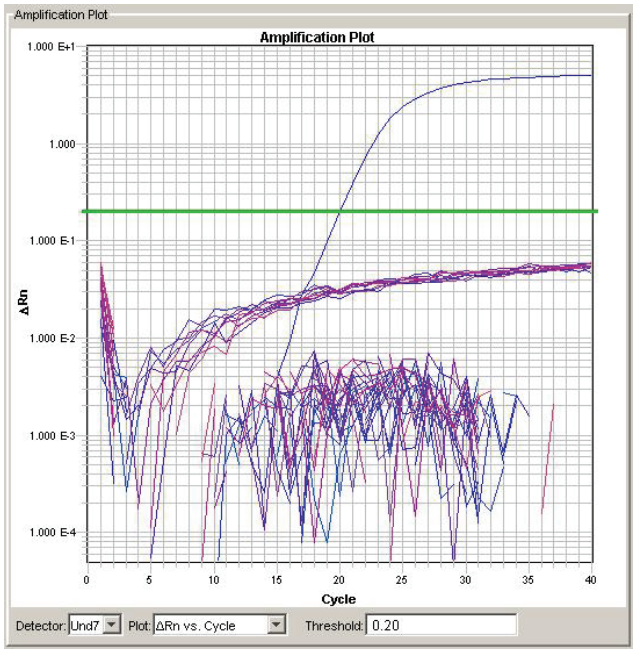


Figure 9: Amplification plot for *U. pinnatifida* qPCR assay No. 1. Amplified product is *U. pinnatifida* gDNA.

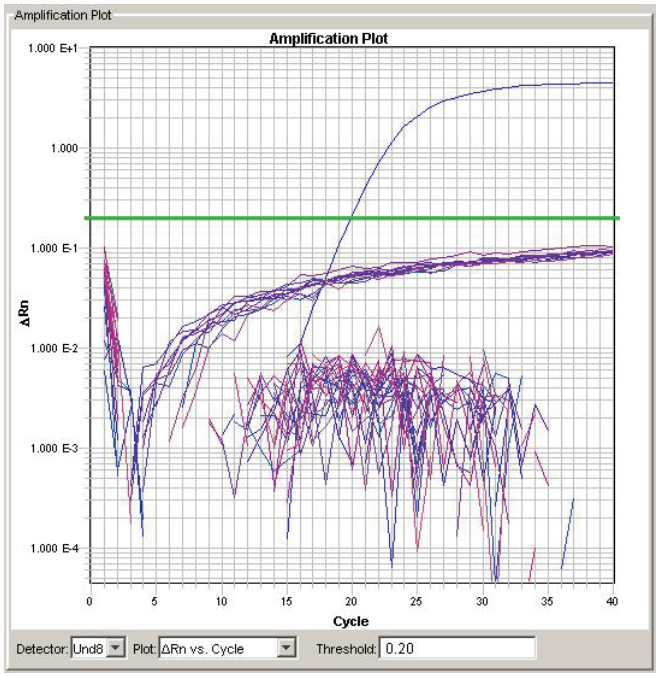
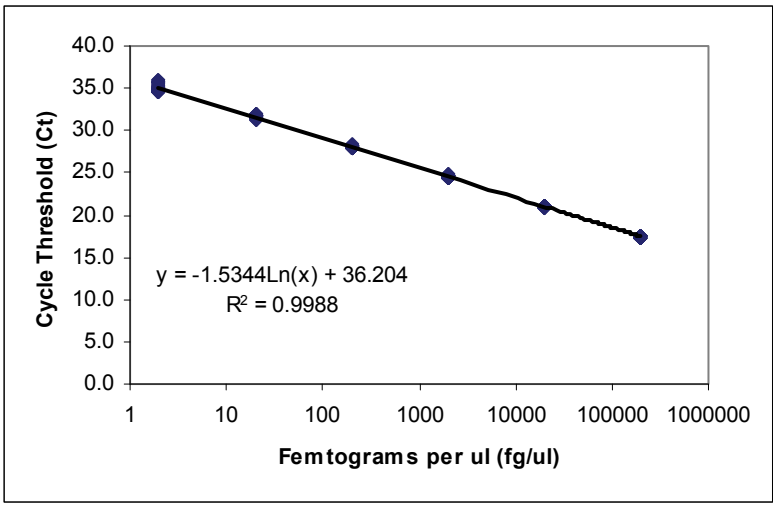


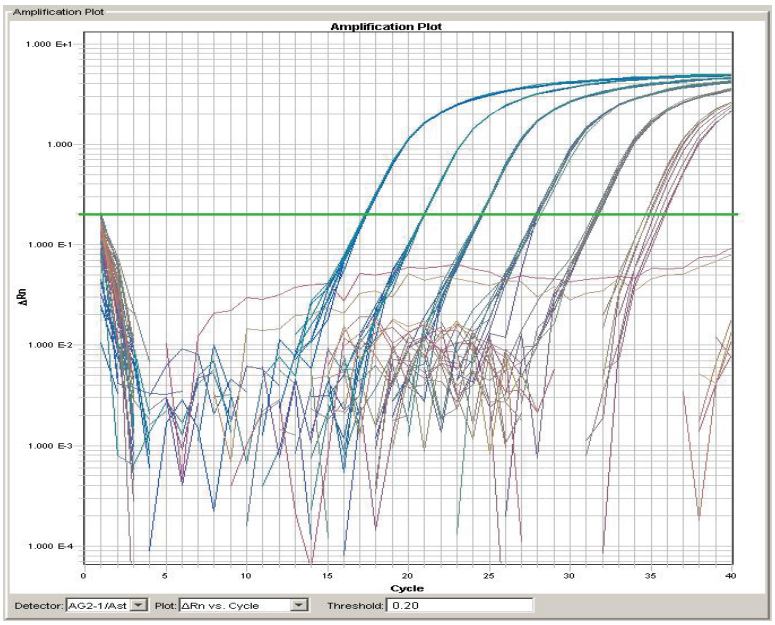
Figure 10: Amplification plot for *U. pinnatifida* qPCR assay No. 2. Amplified product is *U. pinnatifida* gDNA.

Sensitivity of Asterias amurensis *quantitative PCR assays*

The *A. amurensis* assay was tested for sensitivity on a dilution series of target DNA. The *Asterias amurensis* qPCR assay has a reliable DNA detection limit of 2 fg/µl. A total of 32 replicate DNA samples with 2 fg/µl of *A. amurensis* gDNA were reliably detected using the *A. amurensis* qPCR assay (Ct range: 34.5-36.0, SD=0.44). Figure 11 shows that serial dilutions of *A. amurensis* gDNA (200,000 to 2 fg/µl) when amplified with the *A. amurensis* qPCR are quantifiable over at least 6 orders of magnitude and in a log-linear range (R^2=0.9988), which indicates that this assay will be able to detect minute amounts (i.e. larvae) of *A. amurensis* from environmental samples.



a)



b)

Figure 11: a) Standard curve of serial dilutions of *A. amurensis* gDNA (200,000 to 2 fg/µl), and b) Amplification plot of *A. amurensis* gDNA serial dilutions

Discussion

Putative Quantitative PCR (qPCR) assays have been designed for *Carcinus maenas, Ciona intestinalis* and *Undaria pinnatifida* and a previously developed qPCR assay for *Asterias amurensis* has been adopted. These assays have been designed in TaqMan MGB format and we have tested the specificity of these assays against a wide range of target and heterologous taxa. Based on available specimens it appears that the *A. amurensis* qPCR assay is specific to *A. amurensis* gDNA and that the assay is able to amplify minute amounts (2 fg/µl) as well as being quantifiable over a wide log-linear range. The *A. amurensis* qPCR assay is a potentially powerful tool for

quantifying biomass of *A. amurensis* from environmental samples (water and sediments). Further specificity testing and validation studies will need to be carried out on the qPCR assays for *C. maenas, C. intestinalis* and *U. pinnatifida*, and re-designing of primers and/or probes may be required as qPCR assays are evaluated with a wider range of heterologous controls and field samples. The *C. maenas* qPCR assays require a wider diversity of crustaceans for testing to provide confidence in the assay specificity. The *C. intestinalis* qPCR assay requires further specificity testing with a range of ascidian species, including endemic species. The *U. pinnatifida* qPCR assays require further evaluation with brown algae (Phaeophyceae), particularly kelp species from the order Laminariales, such as *Ecklonia.* Once qPCR assays satisfy all requirements of the developmental testing they will require validation on a range of field samples from various locations (i.e. filtered plankton).

We have so far not experienced any specificity issues with the *C. maenas* and *U. pinnatifida* qPCR assays, while there is late amplification of an ascidian, *Ascidiella* sp. with the *C. intestinalis* qPCR assay. The cross-reaction with *Ascidiella* sp. occurs at a Ct of ~36, which is considered very late amplification, and may be background amplification. However to remove this cross-reaction and increase confidence in the *C. intestinalis* qPCR assay we may need to re-design aspects of this assay.

Conclusions and future approaches

With continued development of these qPCR assays, in conjunction with the continued development of assays for other significant marine pest species it is feasible that comprehensive surveillance for marine pests can be undertaken in South Australia. Molecular-based testing of environmental samples (water and sediments) offers the potential for more rapid and cost effective testing over traditional sampling methods, which are more resource and time intensive. The Australian Testing Centre for Marine Pests (ATCMP) is proposed for establishment in partnership with Biosecurity SA at SARDI's Diagnostic laboratories; qPCR assays developed for marine pests (including for this study) will be utilised for routine use at ATCMP.

In a parallel project (funded by Biosecurity SA); SARDI has been developing a plankton collection strategy, where samples are filtered and preserved for later molecular analyses. It is anticipated that this method can be used to detect a broad range of pest species for surveillance purposes.

Specific diagnosis of marine pests is central to: (a) rapidly establishing the prevalence and distribution of marine pest species in the environment in conjunction with traditional sampling techniques; (b) monitoring changes in marine pest distribution spatially and temporally; and (c) conducting targeted eradication and control programmes if economics and logistics permit.

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Glossary of Terms

ATCMP- Australian Testing Centre for Marine Pests

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.

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.

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