Marine Environment & Ecology

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

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Contents

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 larval stages) from environmental samples facilitate rapid, low-cost surveillance and effective control strategies to be implemented.

Detection of marine pests is currently based on dive surveys, dredges, traps and plankton tows, with organisms identified by traditional taxonomy. This process is slow and expensive. The use of quantitative PCR (qPCR) techniques is suitable for marine pest surveillance because it can test very large numbers of samples and rapidly identify the genetic material of the targeted organisms (referred to as high-throughput screening). 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 development of assays for marine pests using this method will facilitate testing plankton samples to verify the presence of a potential pest species in marine waters. This surveillance method is faster and cheaper than traditional surveys.

In this study we have continued the work of Bott et al. (2010a). We have further evaluated the specificity of the European green shore crab, *Carcinus maenas,* and Japanese seaweed, *Undaria pinnatifida,* qPCR assays; testing the *C. maenas* assay with other portunid crustaceans and the *U. pinnatifida* assay with Laminariales including *Ecklonia* and *Sargassum.* We have completely re-designed the vase tunicate, *Ciona intestinalis,* assay and further tested the specificity of this assay with a wider range of tunicate genera. These three assays now appear to be specific based on the available DNA controls in our collection. We tested DNA extracted from plankton samples from Port Adelaide using the qPCR assays for *Carcinus maenas*, *Ciona intestinalis* and *Undaria pinnatifida,* and the previously developed *Asterias amurensis* qPCR assay (see Bott et al., 2010a). *Ciona intestinalis*, commonly found in Port Adelaide, was positive and in high prevalence in both the November/December 2010 and May 2011 surveys, while one sample was positive for *C. maenas* in the November/December 2010 survey.

These four assays (*A. amurensis, C. maenas, C. intestinalis* and *U. pinnatifida*), in conjunction with other previously developed marine pest assays and plankton sampling methodologies, provide a basis for ongoing routine surveillance of marine pests. There is still, however, a need to validate these methods in a wider variety of geographical locations to provide improved confidence in the approach.

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Introduction

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 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). With increasing globalisation comes faster and more frequent shipping, and air transport of live seafood and aquarium fish. Propagule pressure is likely to increase unless effective strategies are employed for prevention, early detection and control. Central to such strategies is the ability to rapidly identify the presence of a pest species.

Eradication of marine pests once they are established is virtually impossible. The early detection of newly arrived pests is essential to allow for any response to minimise spread. Australian federal, 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 and minimise the spread and impact of pests already established in Australia.

In developing the Regional Natural Resources Management Plan (as required under the Natural Resources Management Act 2004), the Adelaide and Mount Lofty Ranges Natural Resources Management Board identified the need to support local initiatives to implement the National System. This project was supported by the AMLRNMR Board to facilitate collaborative actions to help safeguard regional marine waters from marine pest invasions.

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 larval stages) from environmental samples facilitate rapid, low-cost surveillance, and inform effective control strategies where marine pest incursions are detected.

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). Quantitative PCR (qPCR) is a PCR technique that enables monitoring in real-time through changes in fluorescence. Through consultation between SARDI Aquatic Sciences, Biosecurity SA, and the Adelaide and Mt Lofty Ranges Natural Resources Management Board, it was decided 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), now formally known as the Marine Pest Sectoral Committee (MPSC). The fourth species is the vase tunicate*, Ciona intestinalis,* commonly found in Port Adelaide, which was chosen because of its invasiveness and potential for negative impacts on aquaculture and man-made structures (see Therriault and Herborg, 2008). This project continues the work undertaken by Bott et al. (2010a). This report documents the continued development of the *C. maenas, C. intestinalis* and *U. pinnatifida* qPCR assays as well as the results of molecular surveys of plankton collected from Port Adelaide for these species and *A. 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 (Aquenal, 2008a). While there are no current economic impacts attributed to *C. maenas* in Australia, it has the potential to cause serious impacts on bivalve aquaculture and inshore crustacean fisheries. *Carcinus maenas* has demonstrated substantial environmental impacts in its 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 Aquenel 2008a).*Carcinus maenas* has previously occurred around Port Adelaide, along the Adelaide coast, in western Gulf St Vincent and the Coorong, but has rarely been found in recent years aside from one sighting in West Lakes in 2009 (Wiltshire et al., 2010).

Figure 1: European Green Shore Crab, *Carcinus maenas* Source: Anthony Fisher

Vase Tunicate, *Ciona intestinalis*

Ciona intestinalis is an invasive solitary tunicate with a cylindrical, gelatinous body up to 14 cm long, which grows in dense aggregations on any floating or submerged substrate, particularly artificial structures such as pilings, aquaculture gear, floats and boat hulls, in the lower intertidal to sub-tidal zones. Peterson and Riisgard (1992) demonstrated that *C. intestinalis* has an important phytoplankton grazing impact; this may 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 2: Vase Tunicate, *Ciona intestinalis* Source: http://blogs.dickinson.edu/sciencenews/

Japanese Seaweed, *Undaria pinnatifida*

Undaria pinnatifida, the Japanese seaweed or wakame, is one of the world's worst 100 invasive species (Global Invasive Species Database, http://www.issg.org/database/species/search.asp?st=100ss&fr=1&str=&lang=EN), and invasive populations compete with native seaweeds. Disturbance plays an important role in the invasion ecology of *U. pinnatifida*. This alga is very prolific and hardy, with a growth rate of 1-2 cm per day, and a maximum length of 3 m (seeAquenel 2008b). *Undaria pinnatifida* has not been recorded from South Australian waters (Wiltshire et al., 2010), but has been reported recently from Apollo Bay, western Victoria (http://www.dse.vic.gov.au/CA256F310024B628/0/86F73266B27503DECA257700001F5315/$File/Coastline+Update+Autumn10.pdf).

Figure 3: 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., 2010b and references therein). The use of qPCR offers the ability for high-throughput screening of assays for numerous pest species. 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. 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 species closely related to the target organism and testing environmental samples containing unknown taxa is required. 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, due to the inherent high diversity present in these types of samples.

Many 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 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, 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 are particularly useful as 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 continued development of qPCR assays for the specific detection of European green shore crab, *Carcinus maenas,* vase tunicate, *Ciona intestinalis* and Japanese seaweed, *Undaria pinnatifida*, and the results of Port Adelaide plankton surveys using these qPCR assays and the *Asterias amurensis* qPCR assay.

Methods

Quantitative PCR (qPCR) Assay Design

All assays at SARDI Diagnostics (see Figure 4: **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 Bioedit, a sequence manipulation software program (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 of that marker to be able to delineate the target from heterologous species and also the availability of marine species sequences from public databases. The National Centre for Biotechnology Information (NCBI), a division of the United States of America's 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, one of these databases, is an annotated collection of all publicly available nucleotide and amino acid sequences. A range of DNA sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) to allow the identification of a suitable target region for assay development.

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 in all individuals of the target taxa but with sufficient differences to distinguish the target from related and other 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 (the specific components in qPCR assays) that display suitable thermodynamic properties and nucleotide content for efficient amplification of target genomic DNA (gDNA).

Figure 4: SARDI Diagnostics Laboratory

Samples

Samples were collected, and immediately stored frozen, or preserved in ethanol, for 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 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). 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).

Plankton sampling

Plankton samples were collected from Port Adelaide using the methodology developed by SARDI . Two freeze dried brine shrimp, *Artemia* sp. were added to the sample at collection, and a brine shrimp qPCR assay was used as a control to monitor that the sample had been maintained appropriately and quality had not been significantly affected by handling. Briefly, plankton was collected in plankton nets, the contents transferred to a specimen container containing RNAlater ,and brine shrimp added. Thesample was later filtered through a 48mm qualitative paper filter (Filtech) in the laboratory andDNA extracted. Extracted plankton DNA was then tested using the *A. amurensis, C. maenas, C. intestinalis* and *U. pinnatifida* qPCR assays. The complete method is outlined in a commercial-in-confidence report to Biosecurity SA .

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 exhibited the highest specificity. 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 markers to which the qPCR assay hybridises, the nucleotide content of the primers and probes, and the melting temperature (Tm) of the primers and probes, which are 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)
Carcinus maenas	Cox1	ATGAACAGTCTATCCTCCTTTAG	59	GAAAGAACGCATATTGATAATAGTTG	60	6FAM-AGTTGATTTAGGGATTTTC-MGB	69.8
Ciona intestinalis	Cox1	AAAGTAGATTCTCAGGTTCAGCT	59	CAACTCTAGTATTAGAGTGTCTGATA	61	6FAM- ATAAAGGAGGGTAAACTGT -MGB	68
Undaria pinnatifida	Cox1	CTTTAATTACAGCGTTTTTATTGTTGT	59	AGTAGTATTAAAATTACGATCTGTTAGT	60	6FAM-CGGTTTTAGCAGGTGCT-MGB	69

Key: Cox1-Cytochrome c oxidase 1 gene of mitochondrial 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 were 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 (ΔRn) (Figures 6 – 10). The lower the Ct value, the more target DNA detected.

European Green Shore Crab, *Carcinus maenas*

Bott et al. (2010a) designed primers and probes for two assays (*C. maenas* qPCR assays No. 1 and No. 2) from the cytochrome c oxidase 1 (*cox1*) mitochondrial gene, and tested these assays against a range of crustacea collected from Gulf St Vincent and other marine invertebrate DNA in our collection. Further specificity testing made it apparent that *C. maenas* assay No. 1 was the most suitable for development for routine use due to better specificity. We tested the specificity of the *C. maenas* assay with a wider range of portunid crabs, and as Bott et al. (2010a) determined, the specificity of *C. maenas* assay No. 1 is appropriate (

Table 2). Figure 5 illustrates the real-time amplification curve for *C. maenas* assays; the only gDNA amplified (i.e. above the green horizontal line) is *C. maenas.*

Table 2: Results of specificity testing for *C. maenas* qPCR assay

Phylum	Class	Genus	Species	DNA (pg/ul)	Ct Values
Crustacea	Decapoda	*Carcinus*	*maenas*	200	21
		Charybdis	*helleri*	200	UD
		Nectocarcinus	*sp.*	200	UD
		Squila	*mantis*	200	UD
		Portunus	*armatus*	200	UD
		P.	*pelagicus*	200	UD
		P.	*rugosa*	200	UD
		Thalamita	sp.	200	UD
		Leptomithrax	*gaimardii*	200	UD
		Actaea	*calculosa*	200	UD
		Lamarckdromia	*globulosa*	200	UD
		Pilumnus	*tomentosus*	200	UD
		Belosquilla	*laevis*	200	UD
		Alpheus	*villosus*	200	UD
		Austrodromidia	*octodentata*	200	UD
		Melicertus	*latisulcatus*	200	UD
	Siphonostomatidea	*Caligus*	sp. 1	200	UD
		C.	sp. 2	200	UD
		C.	sp. 3	200	UD
		C.	sp. 4	200	UD
Echinodermata		*Asterias*	*amurensis*	200	UD
Chordata		*Asciediella*	*aspersa*sp.	200	UD
		Ciona	*intestinalis*	200	UD
Mollusca	Bivalvia	*Limnoperna*	*securis*	200	UD
		Musculus	*miranda*	200	UD
		M.	*cummingianus*	200	UD
		Modiolus	*micropterus*	200	UD
		Trichomya	*hirsutus*	200	UD
		Musculista	*senhousia*	200	UD
		Perna	*canaliculus*	200	UD
		P.	*viridis*	200	UD
	N/A	NTC			UD

Key: NTC- No Template Control, UD-undetected

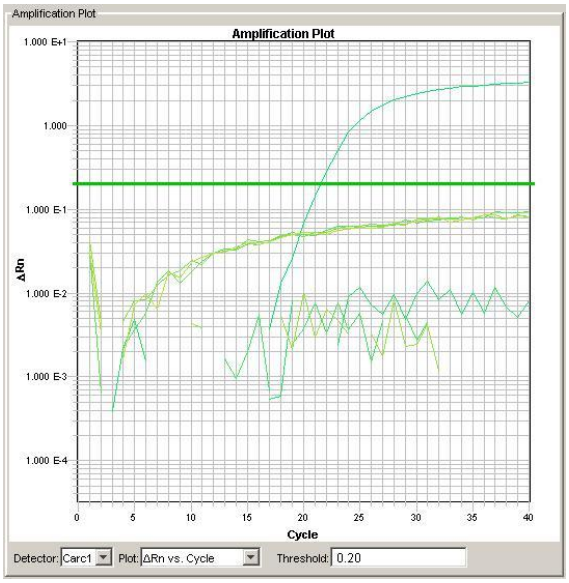

Figure 5: Amplification plot for *C. maenas* qPCR assay. Amplified product is *C. maenas* gDNA.

Vase Tunicate, *Ciona intestinalis*

We experienced cross-reactivity when the original *C. intestinalis* qPCR assay was applied to the tunicate *Ascidiella aspersa* (see Bott et al., 2010a). We therefore redesigned the qPCR assay from the mitochondrial cytochrome C oxidase 1 (cox1) marker, and obtained further tunicate specimens from a wider variety of genera for specificity testing (Table 3). Figure 6 illustrates the real-time amplification curve for the *C. intestinalis* assay; the only gDNA amplified (i.e. above the green horizontal line) is *C. intestinalis.* The cox1 qPCR assay displayed improved sensitivity compared to the previous assay. With the cox1 qPCR we have observed cross-reaction in several specimens of both *A. aspersa* and *Styela clava*, but have not observed it in other individuals of these same two species (data not shown), and thus we believe it is due to contamination with *C. intestinalis* gDNA. The high density of *C. intestinalis* in many ports and harbours means that it is likely to be difficult to obtain *A. aspersa* samples from these areas without *C. intestinalis* contamination. The most closely related *Ciona* species*, Ciona savigni,* collected from New Zealand, does not cross-react, supporting the assertion that the cross-reactions observed are likely to be contaminations.

Table 3: Results of specificity testing for *C. intestinalis* qPCR assay.

Phylum	Class	Genus	Species	DNA (pg/ul)	Ct values
Chordata		*Ciona*	*intestinalis*	200	18.2
		C.	*savigni*	200	UD
		Ascidiella	*aspersa*	200	UD
		Corella	sp.	200	UD
		Styela	*clava*	200	UD
		S.	*plicata*	200	UD
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
		P.	*viridis*	200	UD
		NTC			UD

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

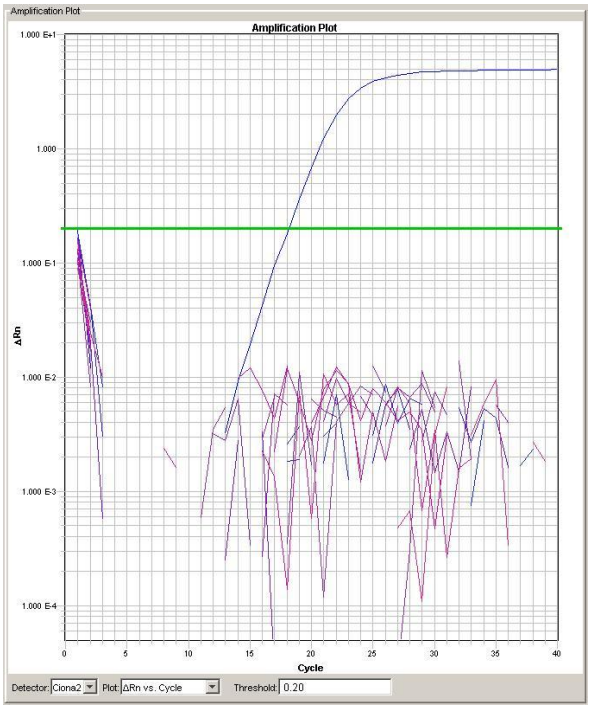

Figure 6: Amplification plot for *C. intestinalis* qPCR assay. Amplified product is *C. intestinalis* gDNA.

Japanese Seaweed, *Undaria pinnatifida*

Bott et al. (2010a) began the development of a qPCR assay for *Undaria pinnatifida*, and designed putative qPCR assays from the *cox1* gene (*U. pinnatifida* qPCR assay No. 1 and No. 2). *Undaria pinnatifida* assay No. 1 was more suitable for routine use due to better reaction efficiencies and sensitivity. We tested this assay against a range of algal and seagrass species (see Table 4) including species of Laminariales and did not experience any specificity problems. Figure 7 illustrates the real-time amplification curve for the *U. pinnatifida* assay; the only gDNA amplified (i.e. above the green horizontal line) is *U. pinnatifida.*

Table 4: Results of specificity testing for *U. pinnatifida* qPCR assay.

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

Key: NTC- No Template Control, UD-undetected

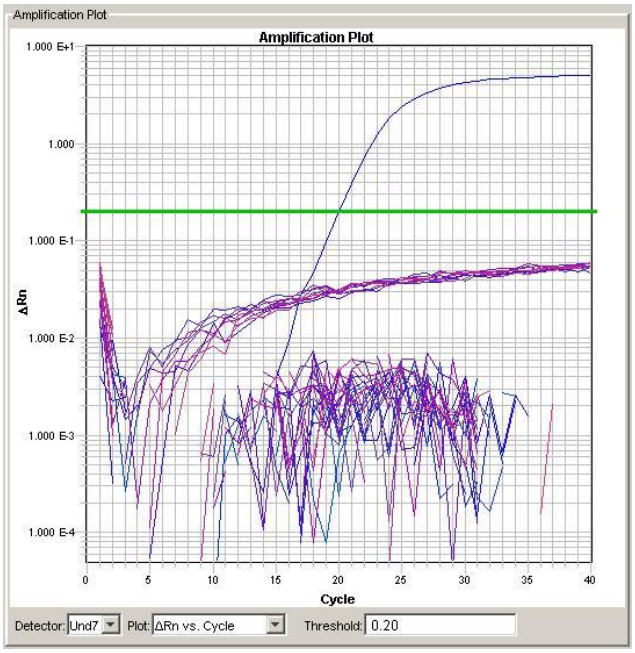

Figure 7: Amplification plot for *U. pinnatifida* qPCR assay. Amplified product is *U. pinnatifida* gDNA.

Plankton Sampling

Results of the plankton sampling from Port Adelaide for *A. amurensis, C. maenas, C. intestinalis* and *U. pinnatifida* are shown in Table 5. *Asterias amurensis* and *Undaria pinnatifida* were not detected in either the November/December 2010 or May 2011 samples. *Carcinus maenas* was detected in one sample from the November/December 2010 survey with a strong Ct value of 26, while *Ciona intestinalis* was present in both surveys at high prevalence (100% November/December 2010 and 93% May 2011).

Table 5: Results of qPCR surveys of plankton samples collected from Port Adelaide

	Number of positive qPCR samples			
	Asterias amurensis	***Carcinus maenas***	***Ciona intestinalis***	***Undaria pinnatifida***
November/December 2010	0/29	1/29 (3.4%, Ct: 26)	29/29 (100%, Ct: 17.1-35.3)	0/29
May 2011	0/29	0/29	27/29 (93%, Ct: 22.2-35.6)	0/29

Key: %= prevalence, Ct-range of cycle threshold for positives.

Discussion

Bott et al. (2010a) developed putative qPCR assays for *Carcinus maenas, Ciona intestinalis* and *Undaria pinnatifida.* This study continued the development of the qPCR assays for these three species. The *C. maenas* and *U. pinnatifida* assays showed acceptable specificity in the previous study, but testing with further closely related species was required for additional evaluation of the assays, to bring them to a comparable level of specificity testing to the other SARDI qPCR marine pest assays. The *C. intestinalis* qPCR assay exhibited some late level cross-amplification with *Ascidiella* sp., so the assay was re-designed. We also field tested these qPCR assays, and the *A. amurensis* assay, on plankton samples collected from Port Adelaide.

The further testing of the specificity of the *C. maenas, C. intestinalis* and *U. pinnatifida* qPCR assays described here shows that they satisfy all the requirements for a specific qPCR assay based on the DNA controls in our collection. It should be noted that these assays require rigorous field proofing of specificity through being applied to samples from ports across a wide variety of localities. This would further test these assays against a wider range of DNA from non-target species present in plankton samples. The ability to conduct plankton sampling and correlate positive qPCR results with known introductions and/or discovery of pest species (e.g. dive collection, scrapes, benthic grabs, trapping) is an important process to undertake because it builds confidence in assay utility and may identify problems with specificity from DNA of species the assays have not been tested against. SARDI's recent acquisition of a 454 GS Junior high-throughput pyrosequencer offers the ability for future projects to pre-screen samples from localities to better understand the sequence diversity in the environment, which will ultimately aid in test development, sampling design and understanding of baseline aquatic biodiversity. We anticipate 454-based technology will assist us to understand and identify problems with assay specificity. 454-based or similar technologies may also provide the basis of a new platform for marine pest surveillance.

Port Adelaide trial

The high level of detection of *C. intestinalis* is not surprising as it occurs in high densities in Port Adelaide (K. Wiltshire, pers. comm.). The detection of *C. maenas* (Ct: 26) in one sample of the November/December survey is interesting; while *C. maenas* has been reported from the Port River, the last report was from West Lakes in 2009 (Wiltshire et al., 2010). This finding suggests that a resident population of *C. maenas* is present in Port Adelaide, but below the threshold for detection by the conventional methods used by Wiltshire and Deveney (2011). Ideally, the

continued presence of *C. maenas* in the Port River should be confirmed by a targeted field survey. *Asterias amurensis* and *U. pinnatifida* have not been recorded from South Australian waters (Wiltshire et al., 2010). Regular surveillance for these and other marine pest species is vital to prevent incursions from establishing and to minimise impacts. The benefits of regular, cost effective surveillance methods will minimise the need for costly emergency response measures in future years.

Conclusions and future approaches

We have applied a new plankton sampling method (funded by Biosecurity SA). The success of utilising plankton sampling techniques for the detection of marine pests is contingent upon appropriate sampling strategies being implemented to maximise the chance of capturing and therefore detecting planktonic life history phases of marine pests. We cannot stress enough the importance of understanding biological processes, including spawning timing and cues, of marine pests in their invasive range to optimise surveillance strategies. Future marine pest projects must focus on improving understanding of invasion biology, cataloguing marine pest DNA sequence data to aid in the understanding phylogenetic relationships, and validation of existing assays and sampling strategies in a wide range of localities. It is vital to have access to marine pest larvae and gametes in order to more accurately validate marine pest qPCR assays, given that this is the life stage that these assays target. The availability of the new MISA Aquatic Biosecure facility at Roseworthy will help facilitate research on live marine pests, particularly those that are exotic to South Australia.

These qPCR assays, in conjunction with the continued development of assays for other significant marine pest species, make feasible a comprehensive, rapid, cost-effective surveillance system for marine pests in South Australia. The Australian Testing Centre for Marine Pests (ATCMP) is proposed for establishment in partnership with Biosecurity SA at SARDI's Diagnostic laboratories; and will routinely use qPCR assays developed for marine pests (including from this study) to inform managers of the presence of marine pests.

The development and field validation of these four marine pest qPCR assays (*A. amurensis, C. maenas, C. intestinalis* and *U, pinnatifida*) is central torapidly establishing the prevalence and distribution of thesespecies in the environment in conjunction with traditional sampling techniques. Once an incursion is detected, the assays can also be used to help rapidly and effectively monitor the spatial and temporal extent of its distribution, and assist with developing 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