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Marine pests testing methods:contributing to establishment of an Australian Testing Centre for Marine Pests

Deveney, M.R., Giblot-Ducray, D. and Wiltshire, K.H.

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SARDI Research Report Series No. 963

SARDI Aquatics Sciences
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EXECUTIVE SUMMARY

Shipping is a major vector for marine pest introductions, and port areas are therefore at relatively high risk of new marine pest introductions. Knowledge of pest occurrence around ports is also required for management, including to ensure compliance with ballast water regulations. As part of Australia's *National System for the Prevention and Management of Marine Pest Incursions* (the National System), a national monitoring strategy was devised, which called for biannual surveys to be conducted at 18 specified locations as a minimum requirement. A monitoring manual and guidelines were published, and a Monitoring Design excel Template (MDeT) produced to facilitate this monitoring strategy. Surveys for introduced marine species (IMS) at most ports around Australia, however, have rarely been conducted, with the high cost of implementing the surveys described in the manual recognised as a major impediment. Molecular techniques for marine pest surveillance offer cost and time savings over traditional techniques, and so are receiving increasing attention.

This project was designed to validate sampling and processing methods and quantitative polymerase chain reaction (qPCR) analytical approaches for detecting marine pests in plankton samples. Samples were collected from six ports around Australia (Darwin, Cairns, Sydney, Melbourne, Hobart, and Adelaide) to assess preservation, post-collection processing, DNA extraction and quality assurance protocols with samples from various geographical origins. Preservation methods tested included the use of sulfate buffer, filtering and freezing samples promptly after collection, or keeping samples cold but not frozen after collection. Post-collection, samples were freeze-dried with or without being filtered. Methods used for DNA extraction were sand processing with extraction from a subsample and direct extraction from the entire sample. Several experiments were also completed to further assess aspects of sampling, processing and sensitivity of detection, primarily using samples collected in Adelaide.

The survey results showed that all the pests expected to be at each of the locations were detected. A new record of *Crassostrea gigas* was detected in Adelaide in samples from March 2016. The assay for *Corbula gibba* provided detections in Darwin, despite the species only being found in temperate waters, probably due to detection of DNA from related native species. This assay is not sufficiently specific and requires re-design. The assay for *Arcuatula senhousia* provided one detection in Darwin and one in Cairns, where it has not been recorded, and requires further assessment for specificity where native tropical relatives occur.

The best preservation was achieved by adding sulfate preservative to each sample at collection. Unpreserved samples displayed higher inhibition and low recovery of the *Artemia* sample control, suggesting that sample degradation contributes to inhibition.

Some samples from all locations showed signs of PCR inhibition, which decreases sensitivity. Inhibition is often associated with chemicals in the sample which are co-extracted with DNA. Severe inhibition was observed in all unfiltered samples where DNA was extracted from whole samples which contained residual sea salt. Some unfiltered samples where DNA was extracted from a partial subsample and diluted (sand processed samples) also showed severe inhibition. Samples filtered to minimise salt inclusion and processed to extract all DNA from the sample (direct extraction) displayed the least inhibition on average, although there were some samples processed by this method that had higher inhibition than sand processed samples.

Samples processed to extract all DNA generally contained higher concentrations of pest DNA than sand processed samples, suggesting greater sensitivity than material that was subsampled, although the effect of method on probability of detection was not significant overall. Filtering and extracting all DNA from samples was therefore used exclusively in later experiments. Across all sample processing methods, higher inhibition was associated with lower likelihood of PCR detection, although there was no effect of inhibition on probability of detection in filtered samples.

Analysis of sampling methods showed no advantage to increasing plankton net tow length, because net efficiency declined with tow length due to clogging. There was no clear pattern of net mesh size on chance of detection, but target DNA concentrations were higher for some pests in samples taken with a 50 µm mesh net than with a 150 µm mesh net.

The assays are sufficiently sensitive to detect fewer than 10 larvae in plankton samples, but gametes at similar concentrations were not reliably detected. Spiking methodology and enumeration, and quantitative expression of diagnostic sensitivity, require further assessment. We also provide in the implementation section, an approach to designing and conducting surveys which are robust and which will provide data suitable for comparison to traditional survey techniques. These surveys will also provide information on the pest status of the ports concerned and data that will assist in developing future monitoring systems.

The system we have developed is robust and can be applied to existing surveillance systems. Ongoing refinement will, however, be applied to the approach. These methods contribute substantially to SARDI's capacity to provide robust, reliable molecular testing for marine pests.

1. INTRODUCTION

1.1. Background

Marine pest incursions can cause significant ongoing damage to aquaculture, biodiversity, fisheries habitat, infrastructure, social amenity and economic burden. There is relatively little surveillance of marine pests in the majority of countries worldwide. Accurate and rapid identification of marine pests is central to early detection and management. The high cost of surveys is recognised as a major impediment to implementation (Arthur *et al.* 2015), which has motivated progress towards the development of molecular diagnostic tools. Technical advances have provided a platform for the development of practical, specific, sensitive and rapid diagnosis and surveillance tools for marine pests (Bott *et al.* 2010b).

Arthur *et al.* (2015) noted that a primary purpose for marine pest survey data in Australia was to inform port status for an Australian domestic ballast water management system, which under the National System for the Prevention and Management of Marine Pest Incursions focuses on seven indicator species that are established in Australia. Molecular survey methods, using plankton tow samples tested with qPCR assays for ten marine pest species, including the seven indicator species, have been successfully applied in Port Adelaide (Bott and Giblot-Ducray 2011; Wiltshire and Deveney 2011). These surveys detected all assayed pests known to occur in the area, including one not detected by traditional methods (Wiltshire and Deveney 2011). That study used standard molecular approaches, and while it was successful in detecting known pest species, no attempt was made to optimise sampling or processing techniques. It was also unclear if the methods and assays were suitable for application in other port areas with different species pools and different potential contaminants. Inconsistency of capture using plankton tows of fixed length, variability in preservation efficiency, and variable DNA yield have also all proved problematic in this type of survey (Bott *et al.* 2012). This project was designed to develop systems to address these barriers to implementation.

We designed this project in a way that would complement existing approaches. The National System includes tools to undertake traditional surveys including the *Marine Pest Monitoring Manual* (the Manual) (National System for the Prevention and Management of Marine Pest Incursions 2010b), the *Marine Pest Monitoring Guidelines* and a survey design tool, the Monitoring Design excel Template (MDeT) (National System for the Prevention and Management of Marine Pest Incursions 2010a). These outline approaches, governance arrangements and design parameters for traditional surveys under the National System and these informed the approach or were used throughout this study.

1.2. Objectives

- Assess and optimise sampling methodology, methods for preserving and transporting plankton samples, and DNA extraction protocols for analysis by qPCR, for the marine pest species which are indicators for the proposed domestic ballast water system:
    - Northern Pacific Seastar (*Asterias amurensis*)
    - Asian Date Mussel (*Arcuatula senhousia*, formerly *Musculista senhousia*)
    - European Green Crab (*Carcinus maenas*)
    - Wakame (*Undaria pinnatifida*)
    - European Fan Worm (*Sabella spallanzanii*)
    - Pacific Oyster (*Crassostrea gigas*)
    - Basket Shell Clam (*Corbula gibba*)
- Assess if the assays were likely to detect non-target organisms in a range of environments.
- Provide advice on implementation including defining sampling and field methods.

2. METHODS

2.1. Field methods

Samples were collected using a 50 or 150 µm mesh conical plankton net, dimensions: 50 cm diameter, 2.5 m long (Sea-gear corporation) wide, fitted with a Sea-gear flow meter and towed behind a vessel at a speed of ~1 kt. The length of tow was calculated based on GPS coordinates, with 100 m tows being used as standard. Effective tow length was calculated by comparing flow meter readings with GPS distance as a measure of sampling efficiency. Plankton was coarse filtered after collection using 2 mm mesh and the <2 mm fraction retained. If this resulted in more than 40 mL volume being retained, the sample was re-filtered through mesh of the same size as the sample collection (50 or 150 µm) using the plankton net cod end to <40 ml. Samples were transferred to 120 mL sample tubes, sealed and placed on ice. The site name/code, GPS waypoint identifiers (start and finish), flow meter readings (start and finish) and any other relevant notes were recorded. Between field sites, plankton nets and all sampling equipment exposed to seawater were cleaned in 60°C freshwater containing 200 mg/L active hypochlorite.

In Adelaide, sampling took place within the Port of Adelaide in areas where some of the target species have been detected, and multiple sub-locations were sampled throughout the experiments, but for some experiments, multiple samples were collected within a small area to limit sample variability. Sampling in locations other than Adelaide usually covered a wider area, focusing on wharves, marinas and other areas where target pests, if present, would be likely to occur.

2.2. General methods

Plankton samples of ~40 ml volume were collected with a towed plankton net (see field methods), placed in 120 ml sample tubes and returned to the laboratory. To assess preservation methods, some samples were added to 80 ml sulfate based preservation buffer (similar to Stanford University 2015) at collection. A sample quality assurance (SQA) control (see Section 2.3) was used in all preserved samples (except as noted below) and in a subset of other samples, and was added to tubes prior to sampling. Where the SQA control was used in an un-preserved sample, it was added in an aliquot of preservative. After collection, samples were kept cold (on ice or in a refrigerator at <4°C) until processing. In the laboratory, filtered samples were filtered using a manifold following Giblot-Ducray and Bott (2013), filter papers transferred to 50 ml centrifuge tubes and then frozen at -20°C, while unfiltered samples were frozen at -20°C in the 120 ml tubes without removing the seawater. All samples were freeze dried until completely dehydrated prior to DNA extraction. Tubes were covered with gauze during the freeze-drying process. Two

processing methods were trialed: sand processing and direct extraction. Sand processed samples were weighed and made up to 10 g by adding acid washed, oven-dried paving sand, and then homogenized, with DNA extracted from a 2 g subsample. Direct extraction involved DNA extraction from the entire freeze-dried sample without additional pre-processing.

Two series of field experiments were carried out, the first aiming to assess the use of sulfate preservative and of processing methods, and the second to assess sampling methods and alternative preservation methods. Sampling locations are shown in Figure 1.

2.3. Sampling and quality assurance controls

A pilot trial was conducted to assess methods for addition of the *Artemia salina* (hereafter *Artemia*) SQA control and the quantity of sand for sand processing (see laboratory methods). Artemia is an invasive species (Amat *et al*. 2005) but is not recorded from South Australian waters. Two sources of *Artemia* nauplii were used: lab-hatched (using Ocean NutritionTM brine shrimp eggs), and commercial (using Ocean NutritionTM Instant Baby Brine Shrimp), with each type added two ways: by volume from a slurry or by weight after freeze-drying. In addition, lab-hatched nauplii were added by counting 5 or approximately 50 individual nauplii. Samples were made up to 10 g or 50 g with sand. *Artemia* DNA yield was determined using the *Artemia* qPCR assay (Mackie and Geller 2010), with the average yield and variation between samples assessed using five replicates per method. The coefficient of variation was low for addition by either volume or weight (0.10 – 0.13), but high for addition by counts (0.51 – 0.98). DNA yield was low for samples using counted *Artemia*, and within each method was lower for samples with 50 g compared with 10 g sand. Samples made up to 50 g with sand had a lower coefficient of variation than those made up to 10 g, although the difference was small compared with the difference between *Artemia* addition methods. DNA yields were greater for the commercial than lab-hatched *Artemia*, although acceptable in both cases. Addition by volume from a slurry of commercial nauplii was selected as the preferred method due to it being more rapid and simpler than the other acceptable alternatives. This product is specified free of contamination and previous testing of this *Artemia* using the pest qPCR assays did not detect any other target DNA.

2.4. Laboratory methods

After freeze-drying and sand processing (where applied), 20 mL of DNA extraction buffer containing an internal control (exogenous organism added to each sample at a standardised amount) (Ophel-Keller *et al.* 2008) was added to each sample before physical disruption. DNA was extracted from samples using the method developed by SARDI Molecular Diagnostics (Ophel-Keller *et al.* 2008). Final elution of the DNA was done in 160 µl elution buffer. Each DNA

extract was then tested in singleplex quantitative polymerase chain reaction (qPCR) using SARDI developed assays for the marine pests *Asterias amurensis*, *Carcinus maenas*, *Undaria pinnatifida* and *Ciona intestinalis*, *Arcuatula senhousia*, *Corbula gibba* and *Perna canaliculus*, *Crassostrea gigas* and *Mytilopsis sallei* (see Table 1). Assays are referred to hereafter by the genus name of the target. Testing included negative controls and standardised positive controls.

Table 1. Target pest species, assay gene target, reference for assay and stage of validation.

Species	Gene target	Reference	Stage of validation (July 2016)
*Arcuatula senhousia**	28S rDNA	Bott and Giblot-Ducray (2011)	Lab validated
*Asterias amurensis**	Cox1	Bott *et al.* (2010a)	Lab validated
*Carcinus maenas**	Cox1	Bott *et al.* (2010a)	Lab validated
Ciona intestinalis	ITS-2 rDNA	Bott *et al.* (2010a)	Partially lab validated
*Crassostrea gigas**	Cox1	Bott and Giblot-Ducray (2012)	Lab validated
Mytilopsis sallei	Cox1	Bott *et al.* (2012)	Lab validated
*Sabella spallanzanii**	28S rDNA	Ophel-Keller *et al.* (2007)	Partially lab validated
Perna canaliculus	IGS	Bott and Giblot-Ducray (2011)	Lab validated
*Undaria pinnatifida**	Cox1	Bott *et al.* (2010a)	Lab validated
*Corbula gibba**	28S rDNA	Bott and Giblot-Ducray (2011)	Lab validated

* Australian Ballast Water Management system indicator species

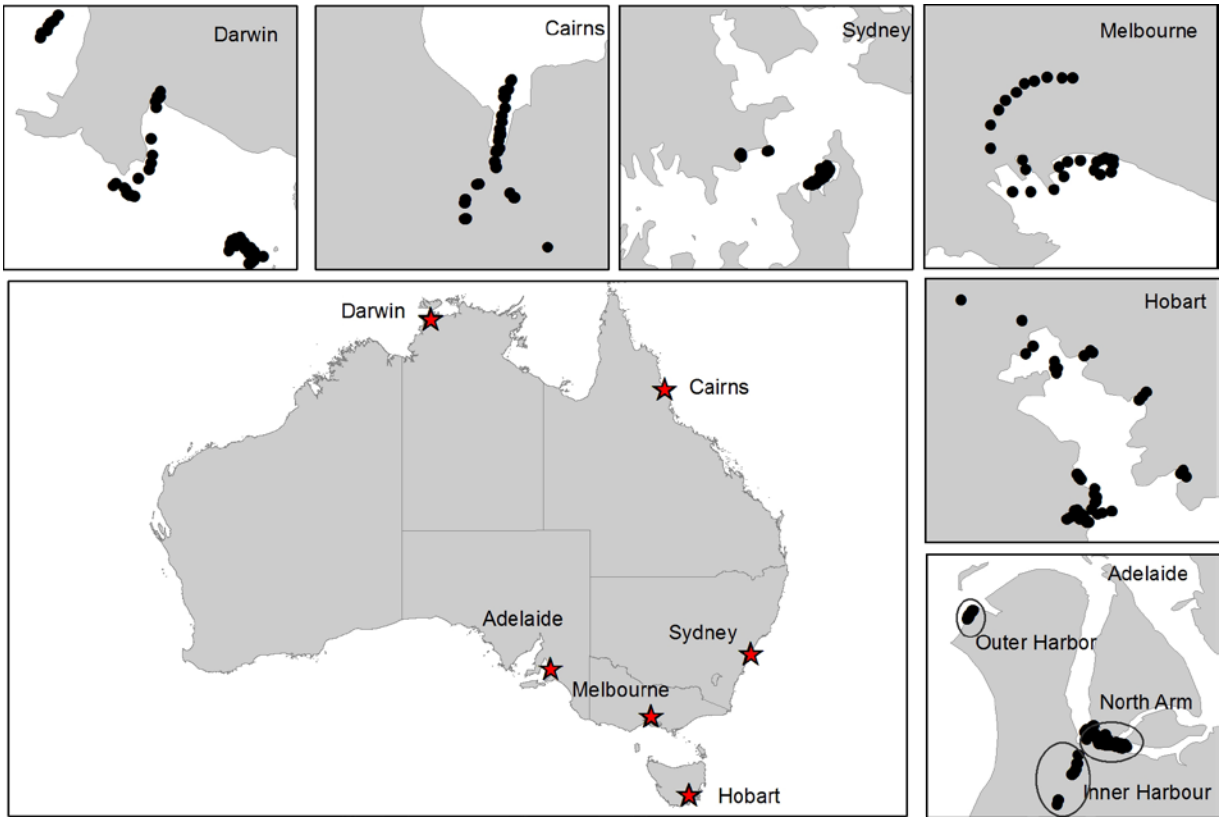

Figure 1. Plankton sampling locations. Index maps for each location are shown at 1:350 000 except the detail map showing Port Adelaide sub-locations (1:150 000). Detailed maps of sampling sets are shown in the results.

Each DNA extract was also tested using a qPCR assay specific to the internal control (Ophel-Keller *et al.* 2008) to assess the efficiency of the extraction process and check the quality of the DNA extracted. A reference sample of 2 µg mL^{-1} of a pure culture of the control organism in MilliQ™ (Merck Millipore, Billerica, MA, USA) water was also extracted and tested by qPCR to use as a reference to determine if PCR inhibition occurred during analysis. A scaling factor was calculated for each sample by comparing the amount of internal control DNA detected in each sample to that detected in the reference sample. For each set of samples, preservation efficiency was estimated by comparing the amount of *Artemia* DNA detected in samples with the SQA control between preservation methods. *Artemia* detection was also used to assess sample cross-contamination.

The cycle threshold (Ct) results from qPCR give a relative measure of the concentration of target DNA present in the sample, with lower Ct values corresponding to exponentially higher DNA concentration. Standard curves are available for the *Artemia* assay and all pest assays except *Ciona* and *Sabella*, allowing the concentration of target DNA to be determined from the Ct value of a positive detection. Where stated, DNA yields are given in picograms per µL (pg $µL^{-1}$) of the DNA extract without correction for plankton mass, water volume sampled or scaling. Correction using the calculated scaling factors is likely to be correct up to a scale factor of 1.6, but the relationship between inhibition and the test used to assess it is not well understood for marine samples. Plankton sample wet and dry weights were recorded for experiments comparing different sampling methodologies, but are likely to include some residual salt and/or preservative solution. Relative mass for these samples is comparable, but actual mass of plankton is uncertain, as is the proportion of inorganic content. Sampled water volume can be derived from the flow meter distance and plankton net diameter, but is not available for some samples due to the flow meter occasionally catching on the net edge or being knocked, resulting in incorrect readings.

2.5. Geographic sampling 1: post-collection processing assessment

The first series of geographic sampling was conducted to assess preservative use and methods for post-collection processing. All samples for this series were collected using 100 m tows of a 150 µm mesh net. An initial experiment was conducted in Port Adelaide in March 2015. In this experiment 36 samples were collected, comprising 12 in preservative (all filtered) and 24 without preservative, of which 12 were filtered and the other 12 processed without filtering. *Artemia* was used in 6 of the 12 samples for each treatment, with 3 of each of the 6 replicates sand processed and 3 processed by direct extraction (Appendix 3i). All samples with preservative were filtered, as the preservative is known to cause inhibition (Giblot-Ducray and Bott 2013). Following this experiment, samples were collected in Cairns, Darwin, Hobart, Sydney and Melbourne between

March and July 2015 (see Table 2 for dates). For these experiments, 30 samples were collected, comprising 6 in preservative with *Artemia* (all filtered), 12 with *Artemia* but no preservative (6 filtered, 6 unfiltered), and 12 with no *Artemia* and no preservative (6 filtered, 6 unfiltered). Three of the 6 replicates for each treatment were sand processed prior to DNA extraction, while the other 3 used direct extraction (Appendix 3ii). Filtering was conducted at SARDI in Adelaide, with samples collected elsewhere being transported by air in an insulated container with gel ice packs.

Table 2. Dates and locations for first series of geographical sampling, conducted using 150 µm mesh net, testing filtered vs unfiltered, sand vs direct extraction, and use of preservative vs cold transport.

Location	No. samples	Field Date
Port Adelaide	36	March 2015
Cairns	30	March 2015
Darwin	30	April 2015
Hobart	30	May 2015
Melbourne	30	July 2015
Sydney	30	October 2015

2.6. Geographic sampling 2: sampling methods and preservation assessment

Two experiments were conducted in the Port of Adelaide, in January and June 2015, to assess sampling methods. For the first experiment, a plankton pump based on Nayar *et al.* (2002) was used to collect samples of 80 L volume through 150 µm mesh from a wharf, while a 150 µm plankton net was pulled along the wharf to filter an equivalent volume. 36 samples were collected, 18 with each sampling method, 6 of which were processed unfiltered with sand, 6 filtered with sand and 6 filtered with direct extraction. Three replicates of each treatment contained *Artemia* (Appendix 3iii). In the second experiment, plankton tows were conducted over three distances: 20, 100 and 300 m, using both 50 and 150 µm mesh nets. Samples were also collected with the plankton pump onto the equivalent mesh sizes, with pumping occurring simultaneously with a net tow so that plankton was collected over an equivalent travel distance and time. Tow length is not directly related to the volume sampled by the pump, but tows of shorter distance were also of shorter duration, therefore limiting the volume sampled by the pump. 45 samples were collected, comprising 3 replicates of each treatment. All were filtered and processed by direct extraction. No *Artemia* or preservative was used (Appendix 3iv).

A second series of geographic sampling was conducted to assess sampling methods across locations. Based on results of the first series of experiments, filtering with direct extraction was used for processing of these samples. Sampling was conducted in Cairns in November 2015, with

30 samples collected, comprising 10 with preservative and *Artemia*, 10 with *Artemia* and no preservative, and 10 with no *Artemia* and no preservative. Five of each of the 10 samples were collected with a 50 µm mesh plankton net and 5 with a 150 µm net (Appendix 3v). Further trials in this series additionally aimed to test the suitability of freezing as an alternative method for preservation. Samples were collected in Darwin, Hobart and Sydney in January and February 2016. In these experiments, 18 samples were collected, 6 in preservative with *Artemia*, and 12 with no preservative (6 with *Artemia*, 6 without). Three of each of the 6 replicates were collected with a 50 µm mesh plankton net and 3 with a 150 µm net (Appendix 3vi). During field sampling for these experiments, 6 samples of filtered seawater were also collected. 40 ml seawater was filtered for each sample through a 0.22 µm Millipore filter using a syringe. Samples without preservative were filtered in a local laboratory on the day of collection, frozen at -20°C and transported back to Adelaide frozen on dry ice. Preserved samples were transported cold and filtered at SARDI as per the first series.

During sampling in Darwin, Hobart and Sydney, and also in Port Adelaide (see Table 3), 4 'bulk' plankton samples were collected, each comprising material from up to 4 x 100 m tows with the 50 µm net. Material was combined and placed in a 375 ml jar with preservative. In the laboratory, these samples were homogenised by mixing on a magnetic stirrer and divided into 4 subsamples prior to filtration. One subsample per bulk sample was processed with direct extraction. The remaining three subsamples were retained for future processing using alternative extraction protocols for comparison.

2.7. Cross-contamination

The geographical sampling 1 series showed evidence of cross-contamination of *Artemia* from spiked to non-spiked samples, despite some being tested separately and assay negative controls not showing signs of cross-contamination. This indicated that cross-contamination was occurring during the process up to and including DNA extraction. All handling was undertaken using disposable plasticware, only one sample was open at a time, and the robot and PCR system had been validated for the SARDI Root Disease Testing System. Freeze-drying and handling during extraction were therefore suspected to be the sources of cross-contamination. Samples from Hobart, where several target pests are known to occur, were freeze-dried with samples from Darwin, where environmental conditions are outside the range for any of the known pests present in Hobart, and then processed separately to assess if the freeze-dryer was a source of cross-contamination.

To assess if cross-contamination was occurring during DNA extraction or qPCR analysis, samples from Sydney with and without *Artemia* were freeze-dried separately and then processed together.

Sample tubes from the second *Asterias* spiking experiment (see Table 4) were freeze-dried covered with a doubled layer of Kimwipes™ rather than gauze to determine if this would prevent transfer between samples.

Table 3. Experiments used to assess sampling methods in Port Adelaide, second geographical series testing sampling and preservation methods, and cross-contamination. Except where otherwise noted, samples were filtered and processed by direct extraction.

Location	No. samples	Date	Parameters tested
Port Adelaide	36	January 2015	Net vs pump (150 µm mesh), filtered vs unfiltered, sand vs direct extraction
Port Adelaide	36	June 2015	Tow length (20, 100, 300m) x mesh sizes (50 & 150 µm mesh) x net vs pump
Cairns	30	November 2015	Net mesh sizes (50 & 150 µm), preservative vs cold transport, *Ciona* spiking
Darwin	18 + 4 bulk + 6 filtered water	January 2016	
Hobart	18 + 4 bulk + 6 filtered water	January 2016	
Sydney	18 + 4 bulk + 6 filtered water	February 2016	Net mesh sizes (50 & 150 µm), freezing vs preservative, *Artemia*/non-*Artemia* samples freeze-dried separately

2.8. Limit of detection experiments

Pest species were added to some samples in the laboratory to assess effective limits of detection. Pest spikes used were *Crassostrea* D-stage larvae, *Ciona* embryos and *Asterias* sperm. The effects of processing method on detection were assessed in an initial trial using laboratory-prepared samples without plankton. Dilutions of ~10, ~100, ~1000 D-stage *Crassostrea* larvae, 50µl *Artemia* or no spike were added to 40 mL seawater or 40 mL phosphate buffered saline. Eight replicates were made of each treatment, with 4 of these filtered and 4 unfiltered. Two of each of these 4 were processed using sand and 2 by direct extraction. The samples were then tested using the internal control, *Artemia* and *Crassostrea* assays. To assess detection in field samples, D-stage *Crassostrea* larvae were added to two sets of plankton samples collected in the Port of Adelaide. The first set of 96 samples was collected in March 2015 and dilutions of ~10, ~100, ~1000 D-stage *Crassostrea* larvae were added to 24 samples each prior to processing, with 24 samples having no *Crassostrea*. 12 of each of the 24 samples also contained *Artemia*, 6 of each of these 12 were filtered, and 3 replicates of each treatment processed using sand and 3 by direct extraction (Appendix 3vii). For the second experiment, 4 L of plankton was collected in

September 2015 using a 50 µm mesh net and then subsampled in the laboratory to give 24 plankton samples of each of three volumes: 5 mL, 25 mL, 125 mL. Samples were filtered in the laboratory and *Crassostrea* larvae of each dilution were added at filtration to 6 samples of each volume with 6 samples having no *Crassostrea*, and *Artemia* added to 3 replicates of each treatment (Appendix 3viii). *Asterias* sperm were added to two sets of samples from Port Adelaide collected in September 2015 and March 2016. Each set comprised 24 samples, 12 with *Artemia* and 12 without. Three dilutions of *Asterias* sperm were added to 3 replicates of each sample type during filtration, with remaining samples having no *Asterias*. Dilutions used for the first trial were 1:10 000, 1:100 000 and 1: 1 000 000, dilutions for the second trial were 1:10, 1:100 and 1:1000 (Appendix 3ix). During field sampling for the second *Asterias* spiking experiment, 6 samples of filtered seawater were also collected and added to jars containing *Artemia*, 3 with and 3 without preservative. *Ciona* embryos were added to samples collected in Cairns in January 2016 after filtration. Between 1 and 5 embryos were added to 18 of the 30 samples, with the number of embryos present recorded prior to addition.

Table 4. Dates locations and details of sampling for limit of detection experiments. Except where otherwise noted, samples were filtered and processed by direct extraction.

Location	No. samples	Date	Parameters tested
Port Adelaide	96	March 2015	*Crassostrea* detection x filtered/unfiltered, *Artemia/*no *Artemia,* sand/direct extraction
Port Adelaide	72	September 2015	*Crassostrea* detection x sample size (5, 20, 125 ml plankton), *Artemia/*no *Artemia,*
Port Adelaide	24	September 2015	*Asterias* detection, *Artemia/*no *Artemia*
Port Adelaide	24	March 2016	*Asterias* detection, *Artemia/*no *Artemia,* preservative/no preservative, kimwipes cover during freeze-drying

2.9. Statistical methods

Scale factor, as assessed by recovery of the internal control in each sample, is a measure of PCR inhibition. Where inhibition is high, detection of target species DNA may be compromised. We were therefore interested in whether scaling was influenced by filtering and processing method, and if so, whether scaling had a significant effect on likelihood of detection within the range of scale factors observed. Performing this analysis in two stages allowed us to assess both of these aspects. A method with generally lower scaling should be preferred even if a significant decline in detection did not result, since high scaling may still inhibit likelihood of detection in samples with low target DNA present. First, the effect of filtering (filtered/unfiltered) and processing method (sand/direct extraction) on scale factor was assessed by 2-factor Analysis of Variance (ANOVA)

using the experiments where all methods were applied, i.e. those shown in Table 2, plus the first *Crassostrea* limit of detection experiment from Port Adelaide conducted in March 2015 (Table 4). We used a subset of these data set for testing the effect of likelihood of detection. For this analysis, we excluded experiments with no detections, since these were performed in ports where none of the pests were known to occur, and therefore lack of detection was likely due to the absence of target DNA, not to scale factor. Generalised linear modelling (GLM) with the binomial family and log link was used to test the effect of scale factor on likelihood of detection, using likelihood ratio tests to compare full and reduced models. Testing likelihood of detection by processing method across all methods would have been confounded by differences in inhibition, therefore, likelihood of detection was compared between processing methods only for filtered samples using binomial GLM. The effect of processing method on pest DNA recovery (or Ct for *Ciona*) was tested by ANOVA for pests with a minimum of 30 total detections using the subset of samples where each pest was detected, and considering only filtered samples.

Tests of the effects of sampling method were performed using data from experiments where both net mesh sizes were used, i.e. those shown in Table 3, but excluding the first sample method experiment in Adelaide, where tows were taken by hand from a wharf. Only data from 100 m tows from the second Port Adelaide sampling method assessment were included in the analyses. The effect of mesh size on sampling efficiency and on relative sample mass (dry weight) was assessed using ANOVAs. An ANOVA with mesh size and pest DNA/Ct as factors was performed for pests that had a minimum of 30 total detections using the subset of samples where each pest was detected to further assess the effect of mesh size on detection.

Artemia DNA concentrations from experiments that had at least some preserved samples were used to assess sample preservation. Only samples spiked with *Artemia* were included in the analyses. The experiments shown in Table 2, the second samples from Cairns (Table 3) and the March 2016 limit of detection experiment in Port Adelaide (Table 4), were used to compare preserved vs chilled samples. Since all preserved samples were filtered, these data were analysed to test the effect of three treatments (preserved/filtered, unpreserved/filtered and unpreserved/unfiltered) on *Artemia* DNA recovery. Being concentrations, these data did not fulfil the assumptions of the linear model, and so were analysed using gamma GLMs, and comparing full and reduced models with likelihood ratio tests. Where significant interactions were found between treatment and DNA recovery, GLM was repeated within relevant levels of the interacting factors. Three experiments from the second series of geographic sampling (Table 3) were used to compare prompt filtering and freezing with frozen transport to the use of preservative on

Artemia DNA recovery. These samples were filtered and processed by direct extraction, so only preservation method (preservative/frozen) and experiment were included as factors in the GLM.

Experiment was also included as a factor in all analyses where the data sets used included samples from multiple experiments. Location was not included separately as a factor, since in nearly all analyses the experiments included were all from different locations. In a few analyses there was more than one experiment from Port Adelaide included, but no more than one from any other location. We did not perform any post hoc assessment where significant effects of experiment were found. In all cases, assumptions of the analyses were checked by observation of residuals vs fitted, scale-location and Q-Q plots. Analyses were carried out in R (R Core team 2015).

3. RESULTS

3.1. Pest detections by location

Detections by location across all experiments are shown in Figure 2 to Figure 9, with detections and expected target pest presence for each location shown in Table 5 to Table 10. Results of methods assessments made over these experiments are presented in the subsequent sections of the results. Detections of spiked pest DNA are not shown in these tables or figures but are discussed in the section on limit of detection experiments. In the tables summarising detections, expected target pest occurrence is shown as "+" expected to be present, "-" not expected to be present, or "?" uncertain presence, for species previously but not recently recorded, or which are known from the wider area, but not recorded from the specific locality sampled. Where detections occurred, the number of samples in which a target was detected is shown. Detections consistent with the expected presence of target species are shown in green. Inconsistent results are highlighted, with yellow for detection where a pest was not expected and is unlikely to occur (conditions at the sampled port are outside known environmental tolerances), orange where the pest was not recorded but could occur, and blue where there was no detection of a pest expected to be present. Failure to detect an uncertain occurrence is not highlighted.

3.2. Cairns

None of the target species are expected to occur in Cairns. One detection was recorded, for *Arcuatula*, in a single sample from the second (November 2015) sampling set (Table 5, Figure 2).

Table 5. Summary of PCR assay results for Cairns. See Figure 2 for map.

Target	**No. samples**	***Arcuatula***	***Asterias***	***Crassostrea***	***Carcinus***	***Mytilopsis***	***Perna***	***Undaria***	***Corbula***	***Ciona***	***Sabella***
Expected		-	-	-	-	-	-	-	-	-	-
Detected - March 2015	30										
November 2015	30	1									

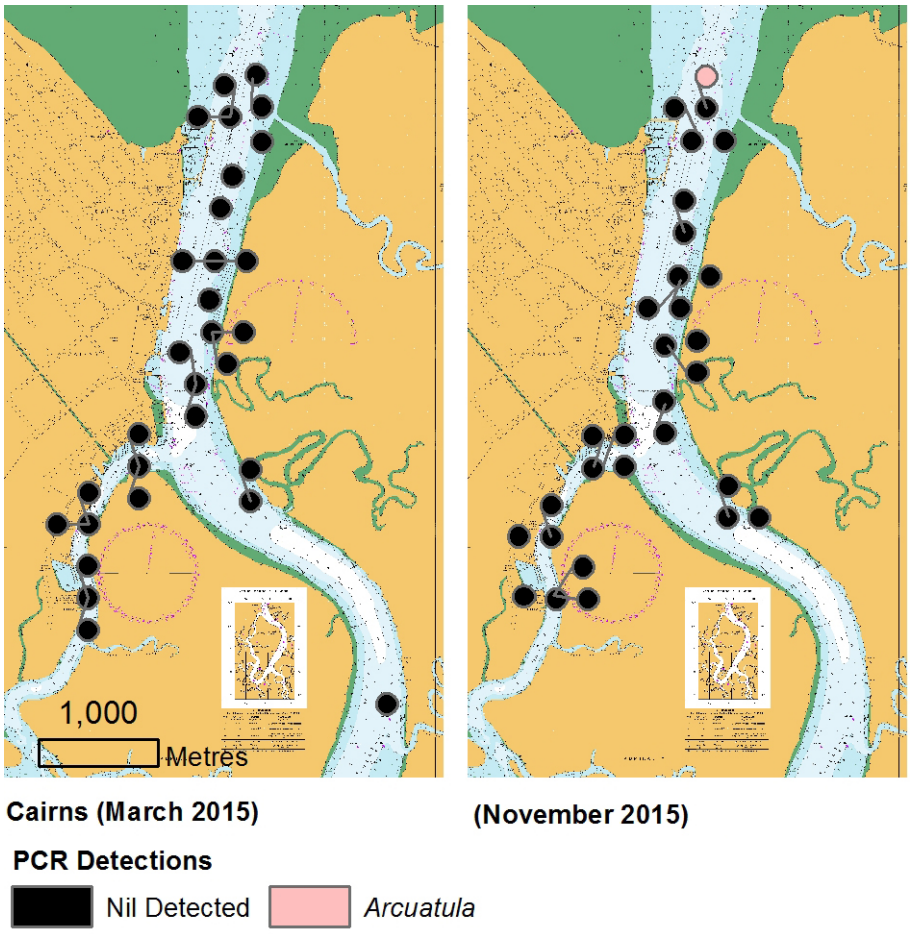

Figure 2. Target pest qPCR detections for Cairns in March (left) and November 2015 (right). To avoid symbol overlap, some samples are shown offset with a leader line to their actual position. Position shown is the midpoint of each tow.

3.3. Darwin

None of the target species are expected to occur in Darwin, but *Corbula* was widely detected across both sampling times (Table 6, Figure 3). One detection was recorded for *Arcuatula*, in a single sample from the second (January 2016) sampling set. The January 2016 samples were freeze-dried together with samples from Hobart as part of a series of trials to assess cross-contamination. *Crassostrea*, which was commonly detected in Hobart (Table 7, Figure 4), was detected in three of these samples. These detections are shown here to demonstrate that the occurrences were not from a consistent location. Full results of trials into cross-contamination are presented in Section 3.10.

Table 6. Summary of PCR assay results for Darwin. See Figure 3 for map.

Target	No. samples	*Arcuatula*	*Asterias*	*Crassostrea*	*Carcinus*	*Mytilopsis*	*Perna*	*Undaria*	*Corbula*	*Ciona*	*Sabella*
Expected		-	-	-	-	?	-	-	-	-	-
Detected - April 2015	30								14		
January 2016	22	1		3					21		

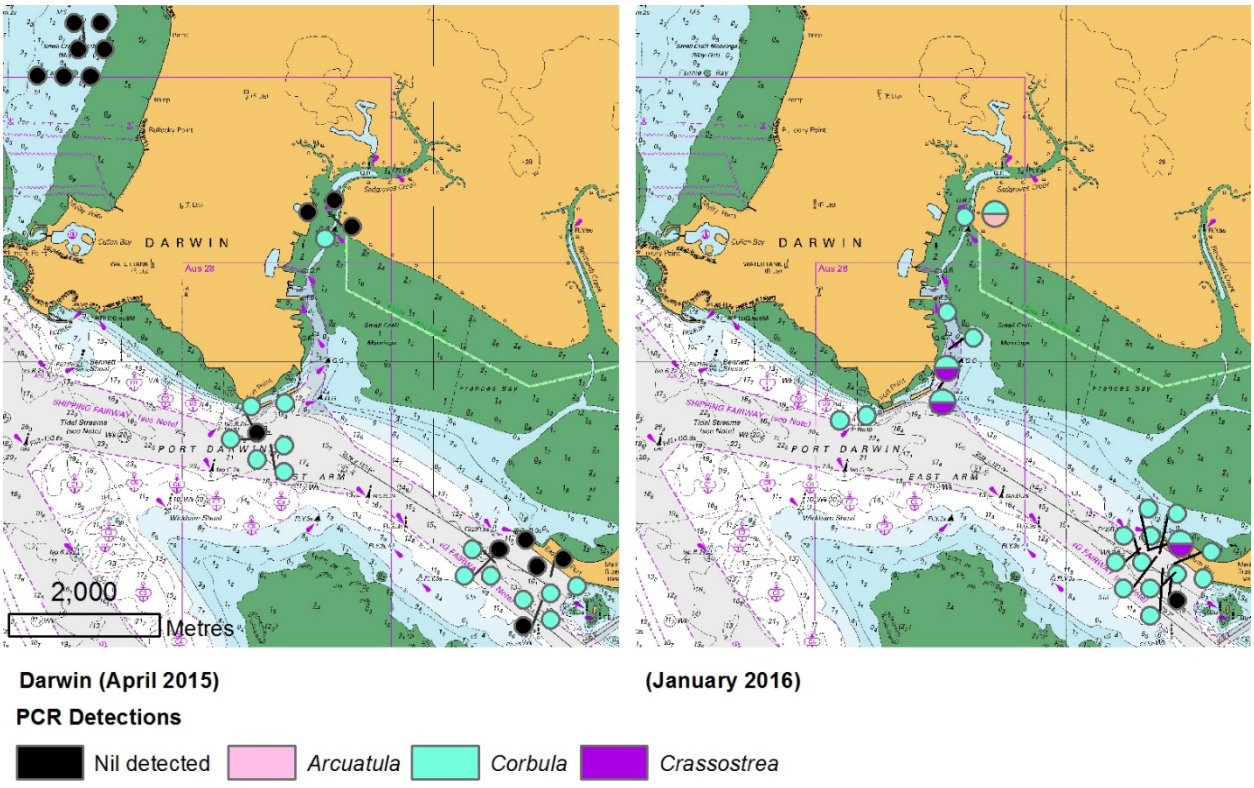

Figure 3. Target pest qPCR detections for Darwin in April 2015 (left) and January 2016 (right). Symbols are shown scaled to the number of species detected. To avoid symbol overlap, some samples are shown offset with a leader line to their actual position. Position shown is the midpoint of each tow. Note that *Crassostrea* detections are the result of sample cross-contamination during trials to investigate this issue but are shown to demonstrate that they did not occur in a single location.

3.4. Hobart

Six of the target species occur in the vicinity of Hobart. Four were detected in the first sampling and all six in the second sampling (Table 7, Figure 4). *Undaria* was regarded as an uncertain occurrence in Hobart since its nearest known population, near the entrance to the Derwent estuary, is approximately 15 km from the location sampled (Whitehead 2008).

Table 7. Summary of PCR assay results for Hobart. See Figure 4 for map.

Target	No. samples	*Arcuatula*	*Asterias*	*Crassostrea*	*Carcinus*	*Mytilopsis*	*Perna*	*Undaria*	*Corbula*	*Ciona*	*Sabella*
Expected		-	+	+	+	-	-	?	+	+	-
Detected - May 2015	30		2	1					10	13	
January 2016	22		6	22	15			4	13	10	

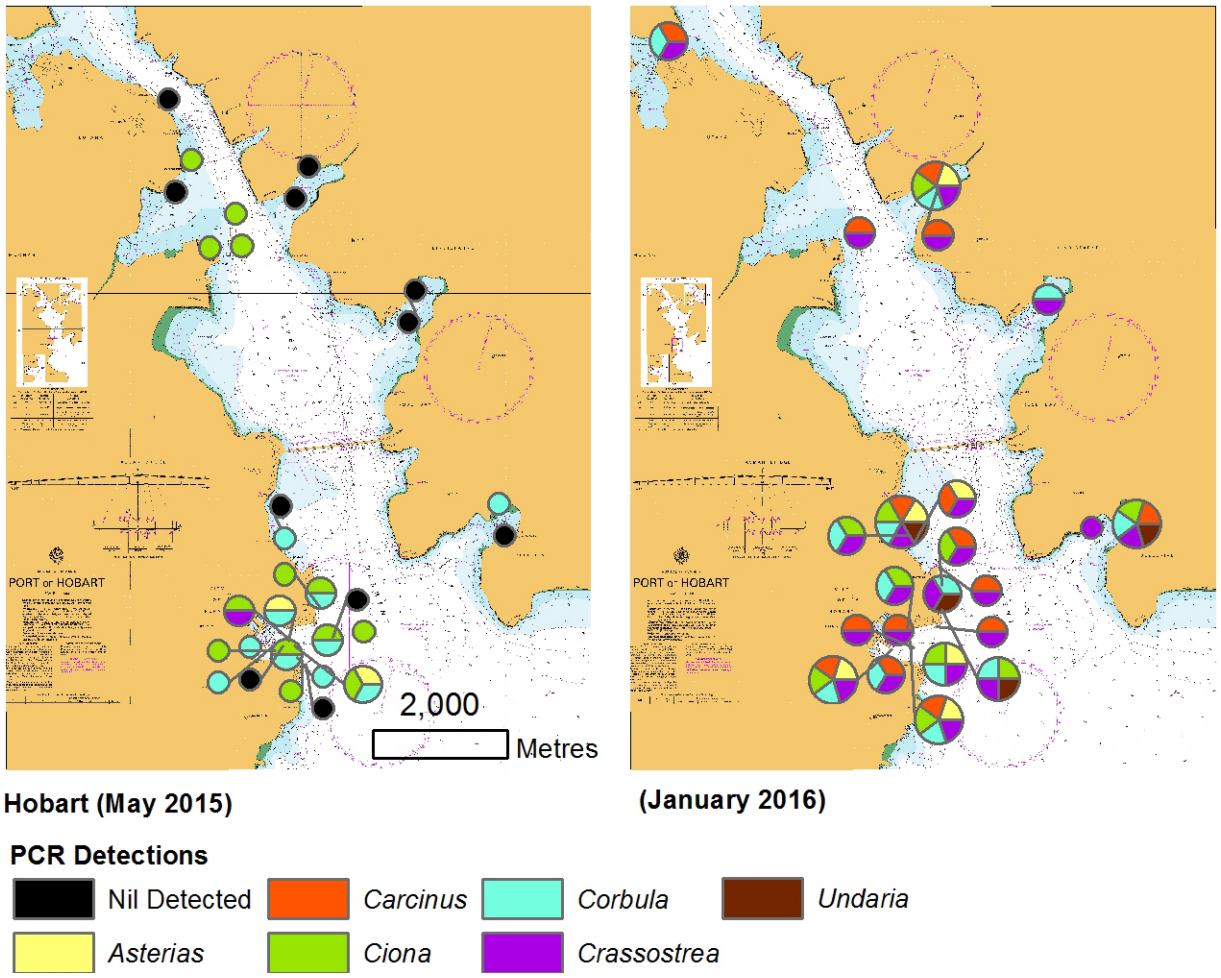

Figure 4. Target pest qPCR detections for Hobart. Symbols are shown scaled to the number of detections. To avoid symbol overlap, some samples are shown offset with a leader line to their actual position. Position shown is the midpoint of each tow.

3.5. Sydney

Two of the target species are known from Sydney Harbour, but *Ciona* was considered uncertain as this species has not been recorded recently. One detection was recorded for *Ciona* in Watson's Bay (Table 8, Figure 5). *Crassostrea* was not detected in the first (October 2015) sampling, but was detected in all samples from the second (February 2016) sampling.

Table 8. Summary of PCR assay results for Sydney. See Figure 5 for map.

Target	No. samples	*Arcuatula*	*Asterias*	*Crassostrea*	*Carcinus*	*Mytilopsis*	*Perna*	*Undaria*	*Corbula*	*Ciona*	*Sabella*
Expected		-	-	+	-	-	-	-	-	?	-
Detected - October 2015	30									1	
February 2016	22			22							

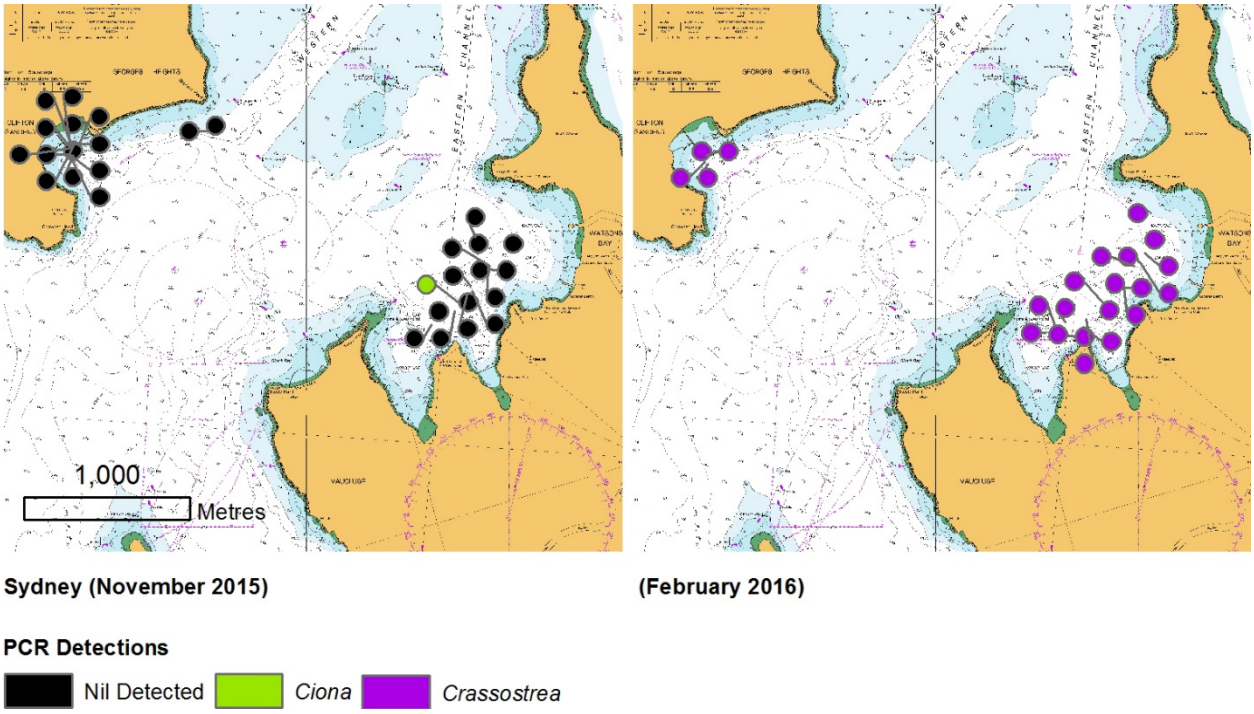

Figure 5. Target pest qPCR detections for Sydney. Symbols are shown scaled to the number of detections. To avoid symbol overlap, some samples are shown offset with a leader line to their actual position. Position shown is the midpoint of each tow.

3.6. Adelaide

All of the three target species that occur in the Port of Adelaide were detected across the six sets of field samples (including those for methods assessments and limit of detection experiments). Detections are shown in Table 9 and Figure 6 - Figure 9. Detections from the January 2015 samples are not mapped as these were collected from a wharf in North Arm rather than by vessel, and no GPS coordinates were recorded. The location for the January 2015 sampling is adjacent to the Torrens Island market shown in Figure 6. Plankton used to construct samples of varying mass for limit of detection experiments was collected during the September 2015 sampling in Inner Harbor, in the area shown in Figure 8. *Ciona* was detected in all sets of samples, with *Carcinus* detected in 5 sets and *Sabella* in four sets (Table 9). *Arcuatula* is recorded in Port

Adelaide, but has not been recently found so was regarded as uncertain and was not detected. *Crassostrea*, which is not known to occur in Port Adelaide, was detected in 3 samples in March 2016.

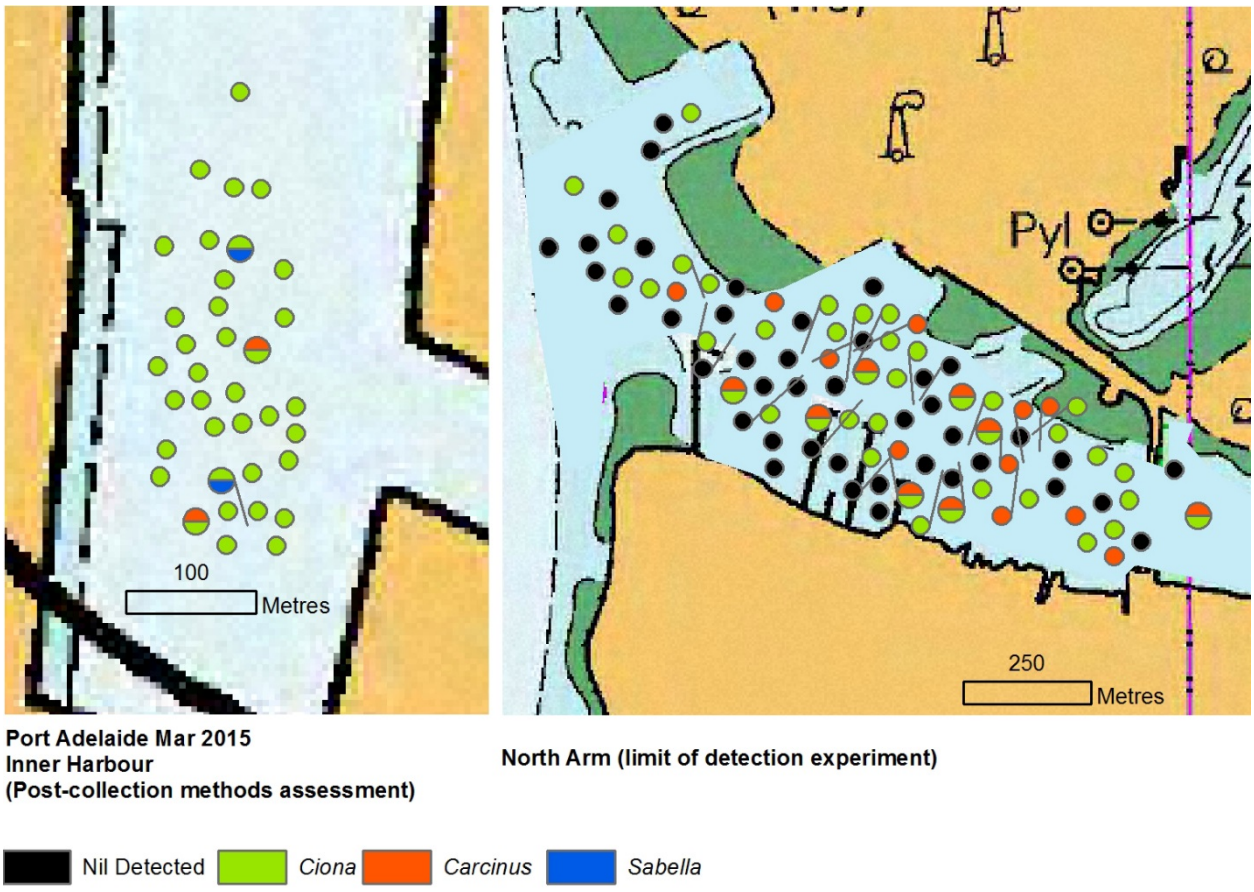

Figure 6. Target pest qPCR detections for March 2015 sampling in Adelaide for assessment of post collection methods (Inner Harbour, left) and limit of detection experiments (North Arm, right).Symbols are shown scaled to the number of detections. To avoid symbol overlap, some samples are shown offset with

a leader line to their actual position. Position shown is the midpoint of each tow.

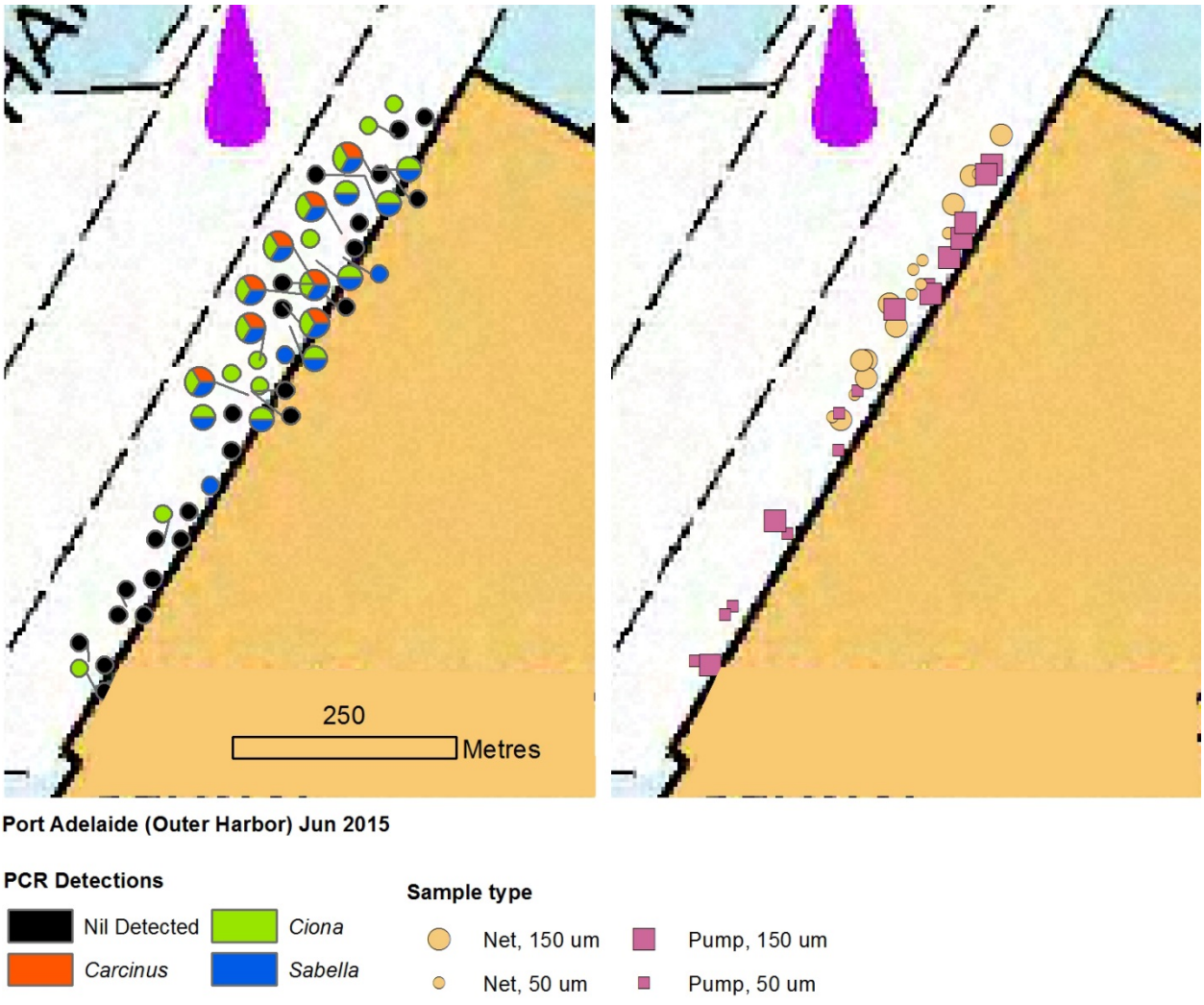

Figure 7. Left: Target pest qPCR detections for June 2015 sampling in Adelaide (Outer Harbor) for sampling methods assessment.Symbols are shown scaled to the number of detections. To avoid symbol overlap, some samples are shown offset with a leader line to their actual position. Right: map of sampling by gear type and mesh size. For both maps the position shown is the midpoint of each tow.

Table 9. Summary of PCR assay results for samples from Port Adelaide. See Figure 6-Figure 8 for maps.

Target	**No. samples**	***Arcuatula***	***Asterias***	***Crassostrea***	***Carcinus***	***Mytilopsis***	***Perna***	***Undaria***	***Corbula***	***Ciona***	***Sabella***
Expected		**?**	**-**	**-**	**+**	**-**	**-**	**-**	**?**	**+**	**+**
January 2015 (North Arm)	36									13	5
March 2015 (Inner Harbour)	36				2					36	2
March 2015 (North Arm)	96				18					39	
June 2015 (Outer Harbor)	36				8					18	16
September 2015 (Inner Harbour)	24				21					24	
September 2015 (Constructed samples)	72				40					74	

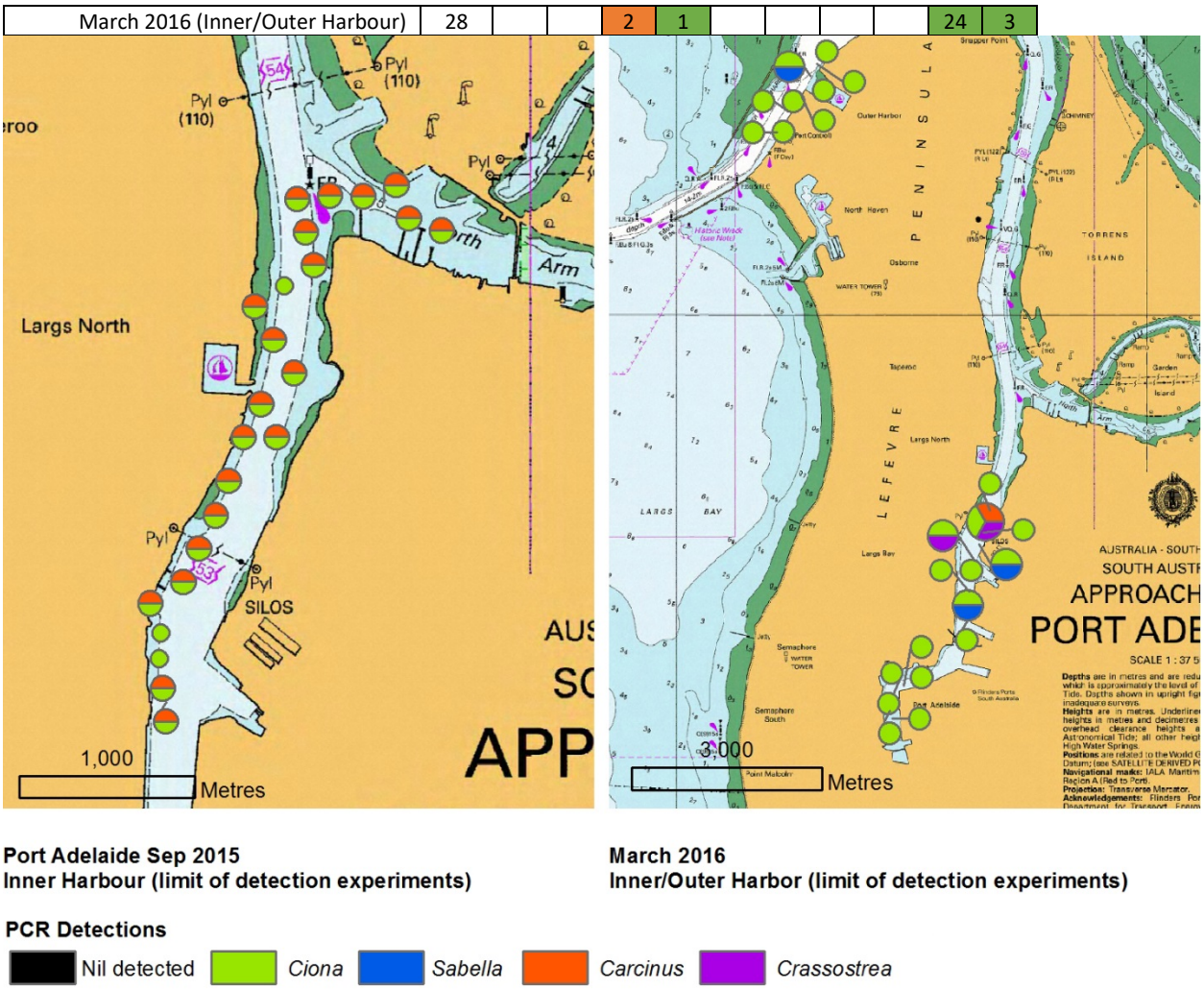

Figure 8. Target pest qPCR detections for sampling in Adelaide in September 2015 (left) and March 2016 (right). Symbols are shown scaled to the number of species detected. To avoid symbol overlap, some samples are shown offset with a leader line to their actual position. Position shown is the midpoint of each tow.

3.7. Melbourne

Six of the target species are known to occur around Melbourne, and all were detected in the single set of samples collected July 2015 (Table 10, Figure 9), with *Asterias* detected in all samples. *Undaria* was detected in 7 samples despite this sampling occurring outside the spawning season for this species. The sampling took place following a period of rough weather, which may have resulted in plant fragments becoming suspended in the water.

Table 10. Summary of PCR assay results for Melbourne. See Figure 9 for map.

Target	No. samples	*Arcuatula*	*Asterias*	*Carcinus*	*Ciona*	*Crassostrea*	*Mytilopsis*	*Perna*	*Sabella*	*Undaria*	*Corbula*
Expected		-	+	+	+	-	-	-	+	+	+
Detected - July 2015	30		30	6	7				4	7	2

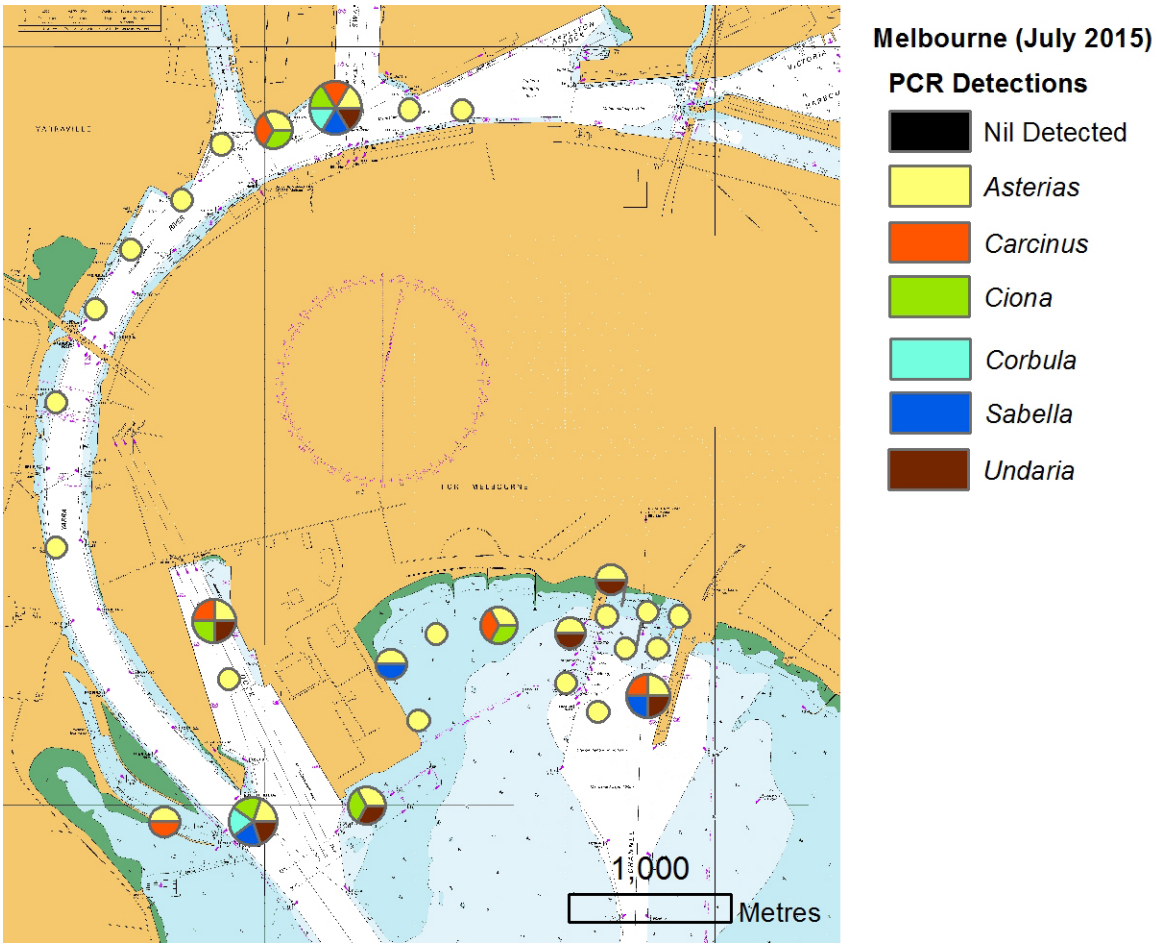

Figure 9. Target pest qPCR detections for July 2015 sampling in Melbourne.Symbols are shown scaled to the number of species detected. Position shown is the midpoint of each tow.

3.8. Post-collection processing assessment

Scale factor, as a measure of inhibition, was compared between processing methods (filtered/unfiltered and sand/direct extraction) using data from experiments where all methods were employed, i.e. those experiments shown in Table 2, with the exception of, plus the first *Crassostrea* limit of detection experiment from Port Adelaide conducted in March 2015 (Table 4). The impact of inhibition on detection was also assessed using this data set, but excluding Cairns, where no pests were detected The percentage of samples with at least one pest detection is

shown in Table 11. There was a clear difference in scale factor between methods (Figure 10), with unfiltered samples having much high scale factors (greater inhibition) than unfiltered, especially when processed by direct extraction. This was confirmed by ANOVA, which showed that there was a significant interaction of filtration x process on scale factor ($F_{1,228}$=12.37, p<0.001), and also a significant effect of experiment ($F_{5,228}$=15.49, p<0.001). The binomial GLM showed a significant effect of scale factor on likelihood of detection (X^2_1=6.971 p=0.008), with fewer detections at high scaling. Testing likelihood of detection by method would therefore have been confounded by differences in inhibition. Due to the high inhibition observed in unfiltered samples, it was decided to filter subsequent samples. When binomial GLM was performed considering only filtered samples, there was no significant effect of scale factor on detections (X^2_1=0.718, p=0.397). The effect of processing method on likelihood of detection was therefore assessed for only filtered samples, and was found not to be significant (binomial GLM, X^2_1=1.21, p=0.272). As can be seen in Table 11, there were similar levels of detection between methods within filtered samples, although detections were slightly higher for direct extraction.

Table 11. Summary of pest detections by method from the first series of geographic sampling and the limits of detection experiments. No detections were recorded in Cairns (data not shown).

Location (Date)	Total Samples	Total samples with Detections	Filtered		Unfiltered	
			10 g Sand	Direct extraction	10 g Sand	Direct extraction
Pt Adelaide (Mar 2015)	36	36	100%	100%	100%	100%
Pt Adelaide (Mar 2015)	96	54	58%	67%	63%	38%
Darwin (Apr 2015)	30	14	44%	67%	33%	33%
Hobart (May 2015)	30	19	44%	67%	100%	50%
Melbourne (Jul 2015)	30	30	100%	100%	100%	100%
Sydney (Oct 2015)	30	1	11%	0%	0%	0%
		Overall:	61%	68%	65%	48%

Pest DNA concentrations were generally higher for direct extraction than sand, as would be expected, due to the inherent dilution of the sample by sand processing, although differences were marginally non-significant in most cases as assessed by ANOVAs for each pest with >30 detections ($F_{1,25}$=3.94,p=0.055 for *Asterias* in Melbourne, $F_{1,21}$=3.62, p=0.071 for *Carcinus* in Adelaide + Melbourne, $F_{1,16}$=8.90, p=0.357 for *Corbula* in Darwin + Hobart). There was a significant 3-way interaction of Experiment x Filtering x Processing Method for *Ciona* Ct values

from Adelaide and Hobart (ANOVA $F_{2,76}$=6.00, p=0.004). ANOVAs performed on the subset of filtered samples from each experiment showed that *Ciona* Ct values were significantly lower in direct extraction than sand processed samples from Adelaide ($F_{1,50}$=7.51, p=0.008), but not from Hobart ($F_{1,4}$=0.001, p=0.972).

Overall likelihood of detection was similar over this series of experiments. Direct extraction filtered samples had an average scale factor which was lower than sand processing, although a small number of samples showed higher scaling than observed with sand processing (Figure 10). These indicate that direct extraction from filtered samples is the most appropriate processing method. Direct extraction also removes a processing step which streamlines processing.

3.9. Sampling method assessment

Data from experiments where multiple sampling methods were applied were used for this assessment. This includes all experiments shown in Table 3.

In the first Port Adelaide trial using the plankton pump and net simultaneously, *Ciona* was detected in 13 and *Sabella* in 5 of 18 net samples, but there were no detections in the pump samples. *Ciona*, *Carcinus* and *Sabella* were all detected in the second sampling method trial, these detections are shown in Table 12 and Figure 7. The rate of detection was much higher in net than pump samples, with only 3 detections, all for *Sabella*, in the pump samples. Within net samples, there was no clear pattern of detection likelihood with tow length, but a greater number of detections of *Sabella* and *Carcinus* occurred in the 50 µm than 150 µm net samples. *Ciona* was detected in all net samples. The mass of plankton captured by either net (50 µm or 150 µm) was greater than by the pump, and increased with tow length (Figure 11). The total amount of plankton captured by 300 m tows was less than double that collected by 100 m tows, indicating a decline in efficiency. This is reflected by the calculated net efficiency (effective length/GPS length), which declined with increasing tow length (Figure 12). The 50 µm net collected more material than the 150 µm net for the equivalent tow length, but with slightly lower efficiency. This trial showed little benefit to having tow lengths >100 m for sampling, but suggested that the 50 µm net may be more suitable. Both 50 and 150 µm nets were used for the second series of geographic sampling to further assess effects of mesh size.

Net efficiency varied widely between sites and sampling seasons, but was generally lower in the tropical locations (Cairns and Darwin) than other sites (Figure 13). ANOVA using data from trials where both nets were applied (Table 3) plus the second sampling method trial from Port Adelaide (100 m tows only for comparison) showed that there was a significant interaction of mesh size and experiment on efficiency ($F_{4,95}$=3.58, p=0.009). Although efficiency was low at the tropical

sites, tows at these locations captured a similar amount of material to tows in Sydney and Port Adelaide, as shown by the dry weight of plankton (Figure 14). Neither mesh size captured more material consistently, and the effect of mesh on mass of sample captured was not statistically significant (ANOVA $F_{1,95}$=0.811, p=0.368).

In Cairns, there was only one detection, which occurred in a 50 µm mesh net sample. In other experiments in this set, pest detections occurred in all samples except for one, therefore, the effect of net type on overall chance of detection could not be tested. The effect of net type on pest DNA concentration or Ct value was tested for *Carcinus* and *Ciona*, respectively, in both cases using results from two experiments (Hobart and Adelaide), as these species had the most detections in this series of experiments. From these samples, DNA of *Carcinus* was higher in the 50 µm mesh net (ANOVA $F_{1,19}$=5.77, p=0.027), while there was a significant interaction of net mesh and experiment on *Ciona* Ct value (ANOVA $F_{1,24}$=6.45, p=0.018). Examining the two experiments separately showed that there was no effect of mesh size on *Ciona* Ct in Hobart ($F_{1,8}$=0.513, p=0.494), but the Ct value in Adelaide was significantly lower for the 50 than 150 µm mesh net ($F_{1,16}$=10.87, p=0.004). These results suggest that sensitivity is likely to be greater for the 50 µm mesh net.

Table 12. Pest detections by sampling method from three replicate samples for Port Adelaide (June 2015).

Method	Mesh (µm)	Tow length (m)	*Ciona*	*Sabella*	*Carcinus*
Net	50	20	3	3	1
		100	3	3	3
		300	3	3	2
	150	20	3	2	-
		100	3	2	1
		300	3	2	1
Pump	50	20	-	-	-
		100	-	-	-
		300	-	-	-
	150	20	-	1	-
		100	-	2	-
		300	-	-	-

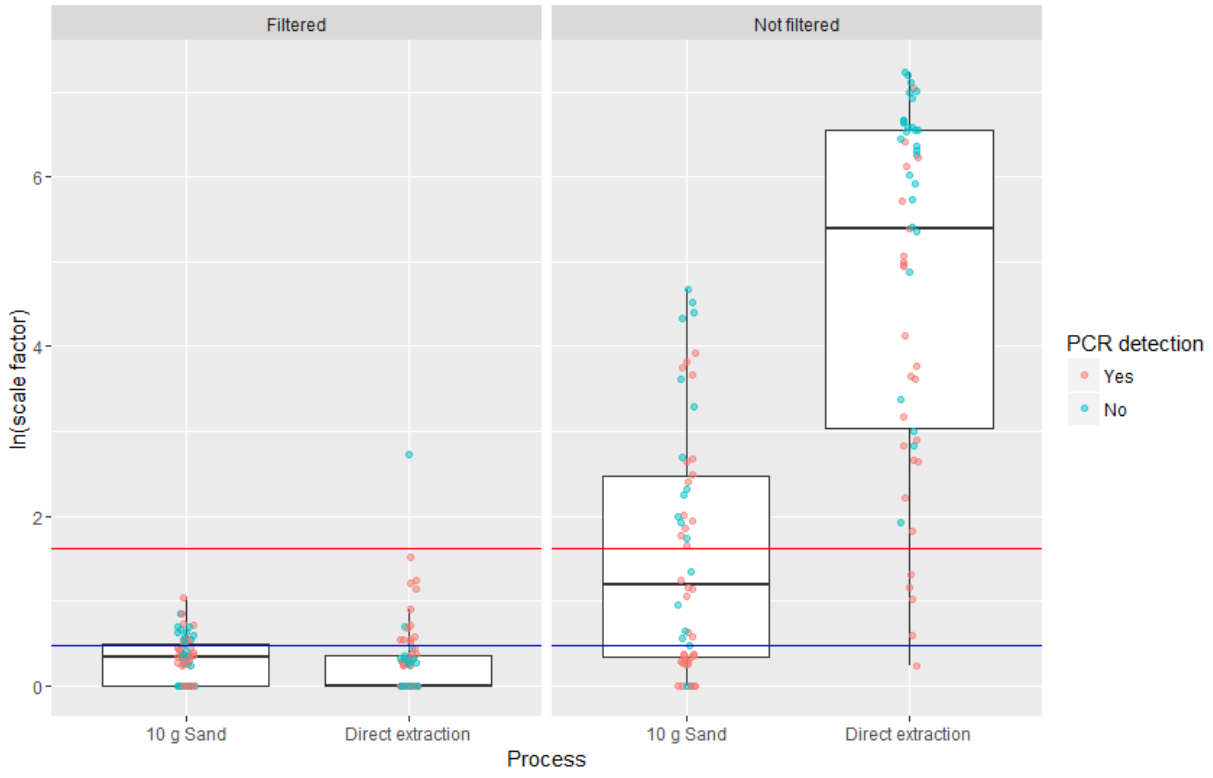

Figure 10. Box plots of natural logarithm of scale factor by method. The centre line of each box is the median value, with box top and bottom showing quartiles (75th and 25th percentile, respectively) and the whiskers showing 95% confidence intervals. A log scale is used to better visualise differences between processes. The blue line represents a scale factor of 1.6 (high scaling) and the red line a scale factor of 5 (very high scaling).

3.10. Sample preservation assessment

The experiments used to assess sample preservation were all those that had at least some preserved samples. These are those shown in Table 2 and Table 3 except for the trials in Port Adelaide that did not use preservative, and the March 2016 limit of detection Port Adelaide trial in Table 4. The first series of geographic sampling plus the second sampling for Cairns were used to assess whether preservative was required where samples were transported cold by comparing *Artemia* DNA concentrations in preserved and unpreserved samples. *Artemia* DNA results from these experiments are shown in Figure 15. *Artemia* DNA recovery was highly variable but consistently higher in preserved than unpreserved samples irrespective of processing. All preserved samples were filtered, therefore these data were analysed to test the effect of three treatments (preserved/filtered, unpreserved/filtered and unpreserved/unfiltered) on *Artemia* DNA. ANOVA showed there was a significant interaction of treatment x processing method x experiment on *Artemia* DNA concentration (gamma GLM, X^2_{10}=33.6, p<0.001).

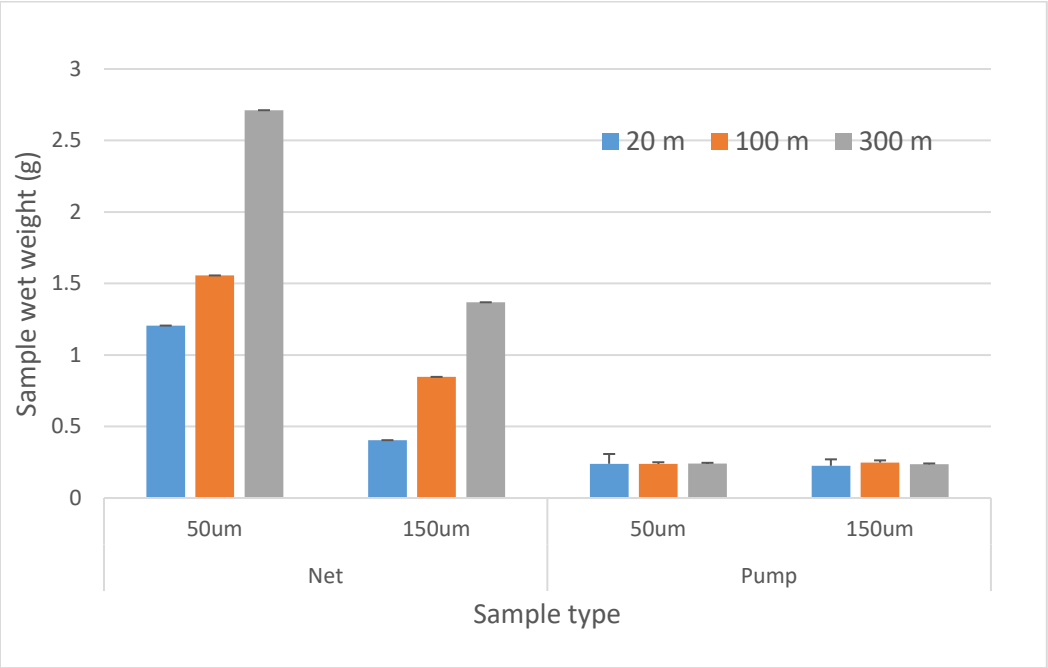

Figure 11. Sample wet weight from net tows and plankton pump for two mesh sizes and three tow lengths. Error bars show standard error (n=3). Samples were collected at Outer Harbor, Port Adelaide.

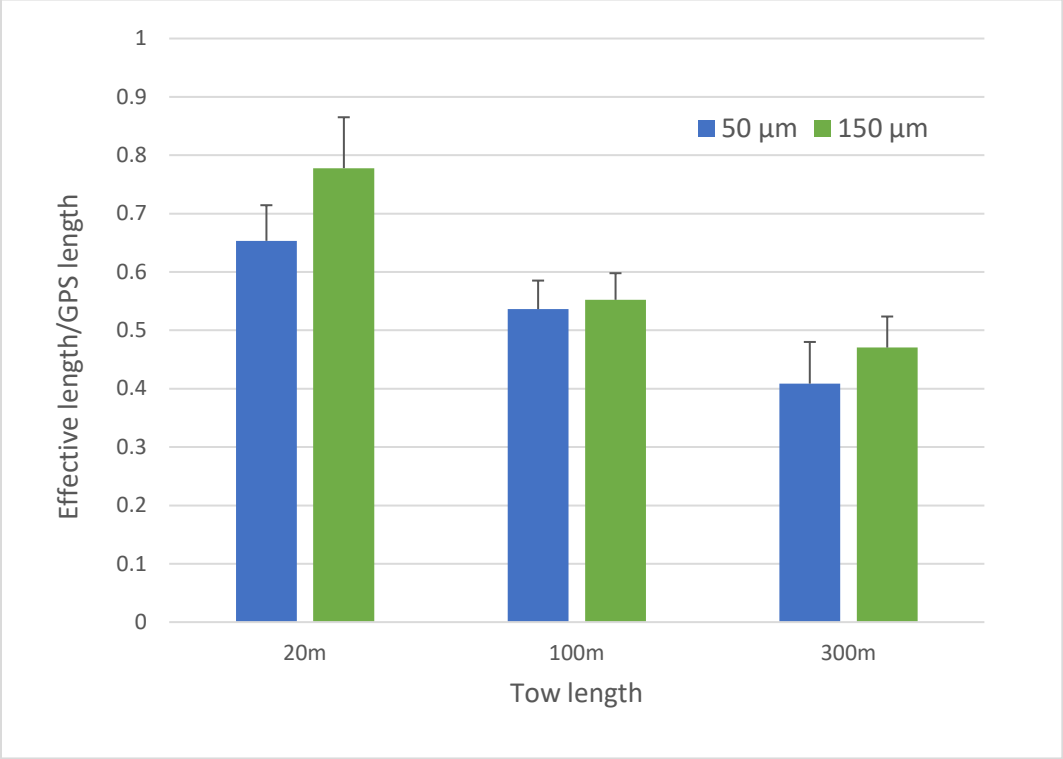

Figure 12. Net efficiency for two mesh sizes and three tow lengths from sampling in June 2015 at Outer Harbor, Port Adelaide. Error bars show standard error (n=3).

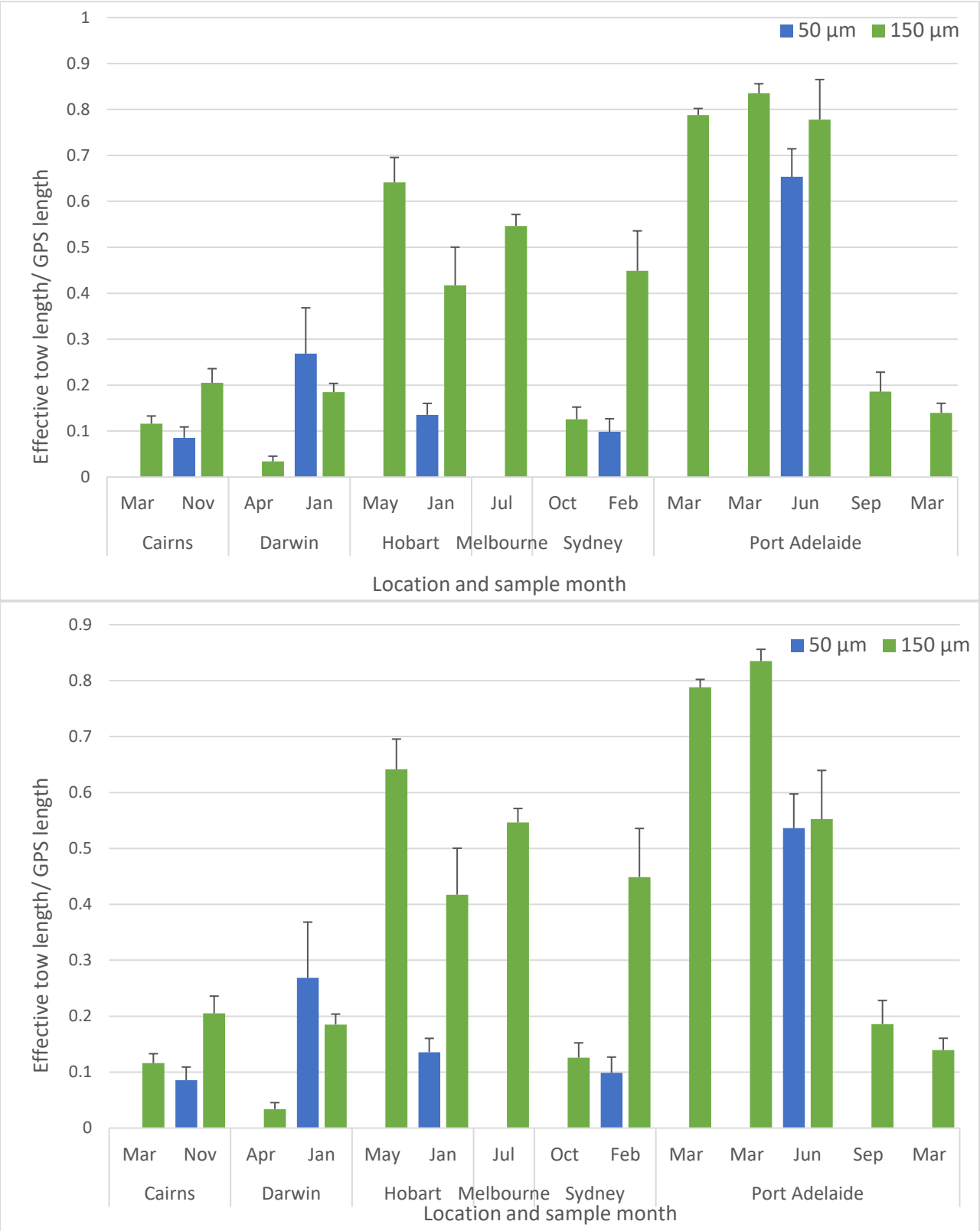

Figure 13. Net efficiency from 100 m tows for two mesh sizes over all sample sets. Error bars show standard error (n=number of samples as per Tables 1-3).

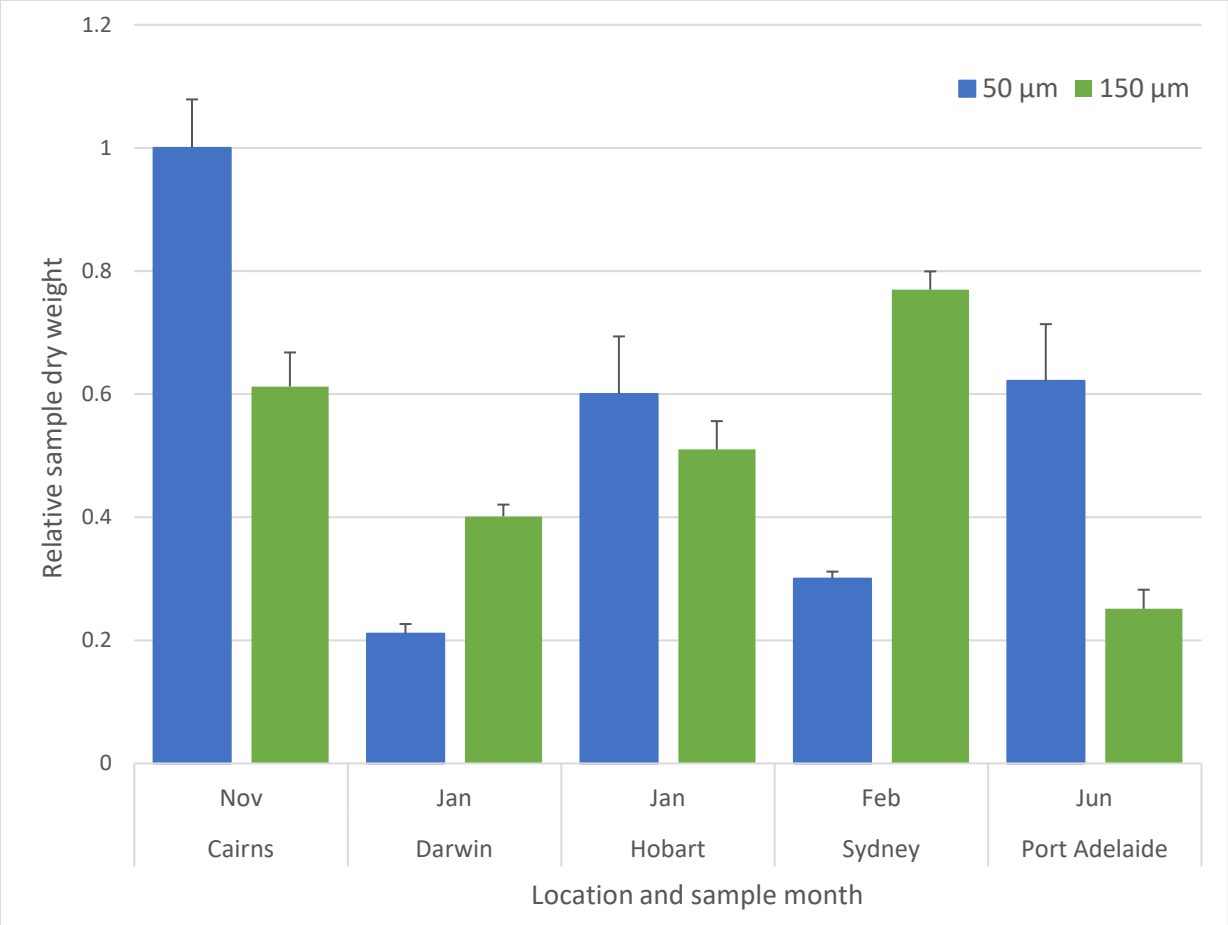

Figure 14. Relative sample dry weight from net tows with two mesh sizes from the second set of geographic sampling and sample method refinement trials (data from 100 m tows only for comparison). Error bars show standard error (n = number of samples as per Table 3).

Direct extraction and sand processed samples are shown separately in Figure 15 as the expected *Artemia* yield from sand processed samples is 20% of that expected from direct extraction. Note that sand processing was only used for initial experiments, and all samples from Cairns in November 2015 and Port Adelaide in March 2016 were filtered. When the GLM was repeated using the subset of filtered samples processed by direct extraction (the only method used in later trials), there was a significant interaction of experiment and preservative on *Artemia* DNA (X^2_5=59.6, p<0.001), driven by the much larger discrepancy in *Artemia* DNA between preserved/unpreserved samples in some experiments than others. It is clear from Figure 15 that *Artemia* DNA concentration was always highest in preserved samples.

Three experiments from the second series of geographic sampling (Table 3) were used to compare prompt filtering and freezing with frozen transport to the use of preservative. *Artemia* recovery was again consistently higher in preserved samples, particularly in samples from Darwin

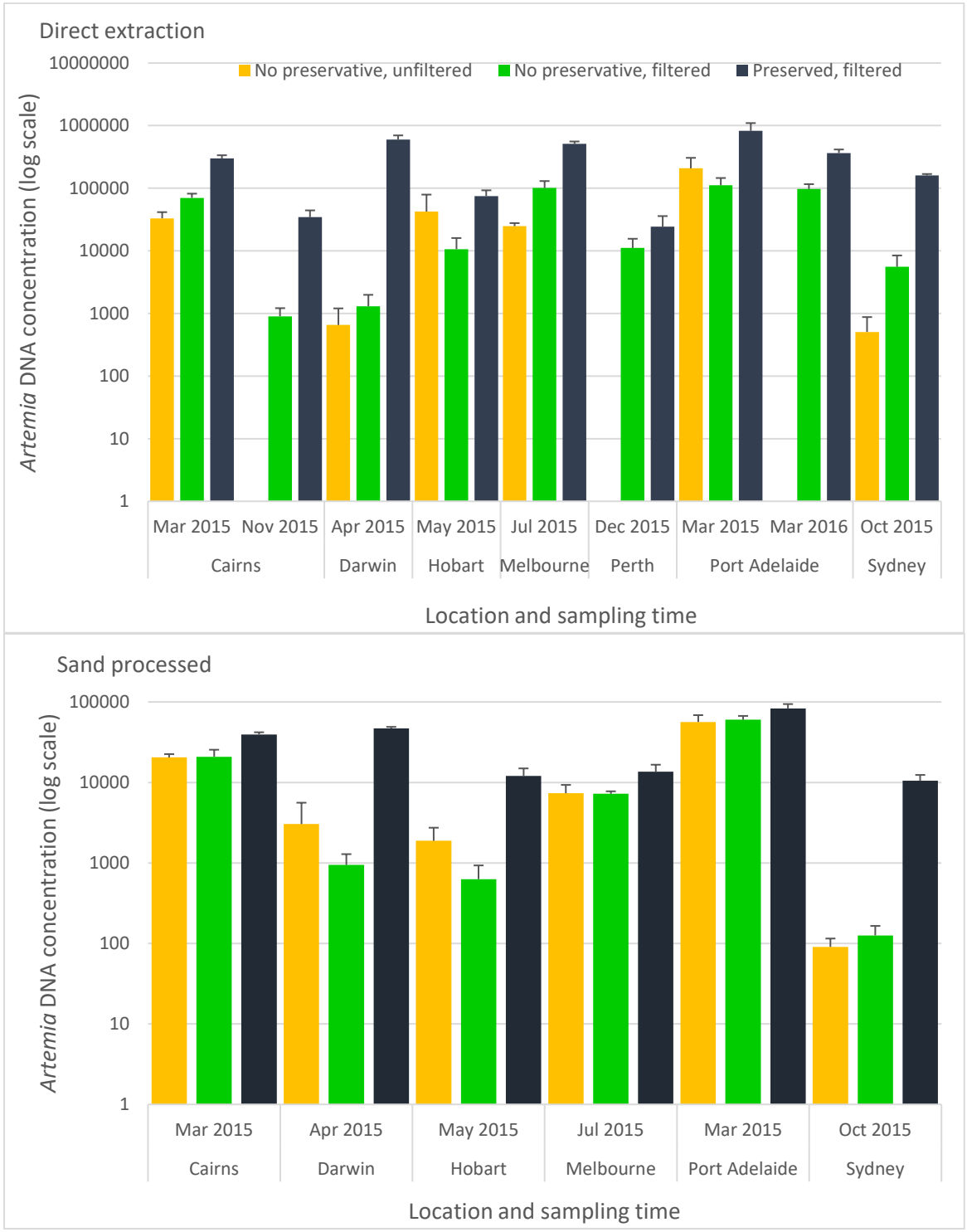

Figure 15. Mean *Artemia* DNA concentration (pg μL^{-1}) by preservation and processing: filtering for direct extraction (top) and sand processed samples (below) from experiments comparing preservation to cold transport. A log scale is used to better allow differences between processes to be visualised.

(Figure 15). ANOVA showed a significant interaction of experiment and preservation method on *Artemia* DNA from these experiments (X^2_2=19.9, p<0.001). This is driven by the larger difference between frozen and preserved samples from Darwin as compared to Hobart and Sydney.

High scale factors were generally associated with low levels of *Artemia* recovery, particularly in unfiltered samples, but this relationship is difficult to assess because measured *Artemia* DNA recovery is affected by scaling, and the linearity of the response of the assay at very high scaling has not been assessed. High scale factors rarely occurred in preserved samples, suggesting that sample degradation in unpreserved samples may be one factor contributing to high scaling.

3.11. Cross-contamination

All plate negative controls were negative, but *Artemia* was detected in at least some unspiked samples from most experiments, indicating that cross-contamination was occurring. High rates of cross-contamination were observed in initial experiments (Table 13) with possible sources of contamination identified as being poor laboratory practice during sample or sample tube preparation (for laboratory prepared and field samples, respectively), or transfer during filtering, freeze-drying or subsequent processing for PCR analysis. The use of disposable plasticware was implemented, along with greater attention to good laboratory practice, but some cross-contamination continued to occur, though levels of *Artemia* DNA in contaminated samples were low (Table 13). Freeze-drying was identified as the most likely step for this cross-contamination to be occurring, as this required sample tubes to be open and in close proximity. The freeze-dried plankton was observed to be fine and powdery in consistency, leading to the possibility that the vacuum applied during freeze-drying could draw fine particles out of tubes. Samples from different experiments were freeze-dried separately, so it was unclear whether cross-contamination was limited to *Artemia*, which was present in samples at a much higher concentration than detected pests, or whether transfer of other DNA could occur. Samples from Hobart and Darwin were used to test this, and *Crassostrea* DNA was recorded in 4 samples from Darwin, including one filtered water sample. The likelihood of *Crassostrea* occurring in Darwin is remote, because water temperatures in Darwin are above the likely thermal limits of this species. The detection across multiple samples from two sub-locations (Figure 3), and in a filtered water sample, also make it unlikely that these are field detections. Mean *Crassostrea* DNA concentration (± s.d.) in Hobart samples was 117 526 ± 39 961 pg μL^{-1}, while in Darwin samples was only 21 ± 5 pg μL^{-1}, further suggesting cross-contamination. The source of cross-contamination was further confirmed to be during freeze-drying from the February 2016 Sydney samples, in which *Artemia* control and non-*Artemia* samples were freeze-dried separately but otherwise processed together, resulting in no cross-contamination. Samples from Port Adelaide from March 2016 were freeze-dried together

using Kimwipes™ rather than gauze as covering to check whether this would prevent cross-contamination, but this was not successful. Processing of samples subsequent to this project has shown that freeze-drying samples individually eliminates cross-contamination.

3.12. Limit of detection experiments

Crassostrea was consistently detected in the laboratory-prepared samples, including in samples spiked with ~10 larvae (15/16). Detections from unfiltered samples were lower (15/24) than filtered samples (22/24). In unfiltered samples, *Crassostrea* was not detected in samples spiked with ~10 *C. gigas* larvae.

In the first set of Port Adelaide samples spiked with *Crassostrea,* we detected two pests targeted by our assays which are known to occur in Port Adelaide (*Ciona* and *Carcinus*) (Figure 7 right). *Crassostrea* was detected in 6 of 96 samples (72 of which had been spiked with *Crassostrea* larvae), comprising 2 in samples spiked with ~100 larvae and 4 in samples spiked with ~1000 larvae. *Crassostrea* DNA concentrations were highly variable but similar between the two detected spiking levels, with mean DNA concentration (± s.d.) of 77 ± 43 pg μL^{-1} for samples spiked with ~1000 and 116 ± 43 pg μL^{-1} for samples spiked with ~100 larvae. There were no *Crassostrea* detections in the samples spiked with ~10 larvae, or the 24 unspiked samples. Unfiltered samples showed substantial PCR inhibition, which resulted in scaling factors >10 in 31/44 unfiltered samples.

In the second set of spiked plankton samples, *Crassostrea* was detected in 50/54 spiked samples (total samples = 72), and we again detected *Ciona* and *Carcinus*. These samples were constructed from bulk plankton collected in Inner Harbor, Port Adelaide, so cannot be mapped precisely, but collection of plankton occurred around the area shown in Figure 8 (left). The four samples where *Crassostrea* was not detected were all spiked with ~10 larvae. Given the high rate of detection, there was no pattern evident for any effect of sample volume or *Artemia* presence/absence on detection of *Crassostrea*. *Crassostrea* DNA concentrations (mean ± s.d.) for ~10, ~100 and ~1000 larvae were 53 ± 31, 273 ± 103, and 2955 ± 1676 pg μL^{-1} respectively. The scaling factor for all samples was 1.0, indicating that there was no PCR inhibition.

For the first set of Port Adelaide samples spiked with *Asterias* sperm, there were no detections of *Asterias*. *Ciona* and *Carcinus* were detected (Figure 8 left). Higher *Asterias* concentrations were therefore used in the second set, and *Asterias* was detected in 14/18 spiked samples, including all samples spiked with the two higher levels, and 2/6 spiked with the most dilute sperm suspension. *Asterias* DNA concentrations (mean ± s.d.) for the three dilutions were (from lowest

to highest concentration): 1.9 ± 2.2, 222 ± 372 and 578 ± 414 pg μL^{-1}. *Ciona*, *Carcinus*, *Sabella* and *Crassostrea* were detected (Figure 8 right).

Ciona was detected in 11 of 18 samples from Cairns spiked with *Ciona* embryos.

Table 13. Number of samples showing *Artemia* cross-contamination, and *Artemia* DNA concentration in spiked and unspiked (contaminated) samples. Experiments shown in chronological order.

Experiment	Number of non-spiked samples with *Artemia* (total unspiked samples)	*Artemia* DNA in spiked samples (mean ± s.d.)	*Artemia* DNA in unspiked samples (mean ± s.d.)
***Artemia* pilot trial**	7 (10)	116 885 ± 23 693	719 ± 30
Port Adelaide (Jan 2015)	10 (18)	121 517 ± 37 438	3 316 ± 199
Port Adelaide (Mar 2015)	12 (18)	223 698 ± 77 988	2 191 ± 72
Port Adelaide (Mar 2015)	19 (48)	111 234 ± 15 827	10 212 ± 307
***Crassostrea* spiking**	40 (56)	194 282 ± 51 840	19 200 ± 294
Cairns (Mar 2015)	7 (12)	80 609 ± 2 4735	225 ± 9
Darwin (Apr 2015)	8 (12)	10 8225 ± 55 002	163 ± 5
Hobart (May 2015)	3 (12)	23 715 ± 8 689	91 ± 12
Melbourne (Jul 2015)	1 (12)	110 730 ± 44 771	6
Port Adelaide (Sep 2015)	3 (12)	5 303 ± 2 033	22 ± 1
Port Adelaide (Sep 2015)	13 (36)	229 502 ± 13 325	447 ± 9
Sydney (Oct 2015)	4 (12)	29 571 ± 14 296	26 ± 2
Cairns (Nov 2015)	2 (10)	18 292 ± 5 467	11
Hobart (Jan 2016)	1 (6)	121 742 ± 45 578	12
Darwin (Jan 2016)	3 (6)	219 224 ± 100 244	1 335 ± 312
Sydney (Feb 2016)	0 (6)	142 579 ± 25 104	-
Port Adelaide (Mar 2016)	8 (16)	133 731 ± 26 594	46 ± 1

4. DISCUSSION

Environmental diagnostics is a field in its infancy. The approaches available for marine pest surveillance specifically, and environmental molecular detection generally, are less well defined and understood than those used in veterinary or medical diagnostics. Many areas remain superficially understood or with few data to address knowledge gaps. This project aimed to establish an efficient plankton sampling and preservation protocol for reliable detection of marine pests using qPCR.

Recorded pests were detected at all sites. Limited temporal spread of sampling across seasons may have influenced detections; *Sabella* was not always detected in Port Adelaide, where it is common. Proving absence is difficult (Wells *et al.* 2010) and building an information base over time is likely to be the only approach that is adequately robust to support management of marine pest vectors. In Hobart, *Undaria* and *Carcinus* were only detected in summer, and *Undaria* has been recorded only from sites several kilometres away from the sampled locations where it was detected (DAWR 2015, 2016a). *Crassostrea* was detected in many more summer samples in Hobart than in late autumn (May 2015) samples, and was only detected in summer samples in Sydney. This highlights the importance of seasonality and temporal spread of sampling, and suggests that appropriately timed sampling can provide good diagnostic sensitivity without precisely targeting pest distributions. The plankton survey method is suitable for detecting target pests in a general area, but does not give specific information about the location of pest populations, which may be some distance from detections in plankton samples (e.g. the detection of *Undaria* in Hobart).

Detections of *Crassostrea* in Adelaide likely represent a recent or emerging invasion; we did not detect this species in any of the other samples collected in Port Adelaide. Adelaide samples were used for limit of detection trials involving *Crassostrea* prior to the detected occurrence, but these trials all included some unspiked samples in which *Crassostrea* was never detected. *Crassostrea gigas* is farmed in South Australia and occurs at low abundance in many areas throughout the state (Olsen 1994, DAWR 2016b). Live oysters have been found in West Lakes, which adjoins the Port Adelaide River and removed (Wiltshire *et al.* 2010). It is likely those oysters were an intentional introduction and such releases are also recorded in other parts of South Australia (Wiltshire *et al.* 2010). After the detections in this study, reports were received of oysters suspected to be *Crassostrea* from Port Adelaide, Port Adelaide was investigated to assess if

Crassostrea had established there, and it was concluded that a recent establishment of *Crassostrea* has occurred in Port Adelaide.

Corbula detections indicate that the *Corbula* assay needs to be redesigned; the detections in Darwin occur in an environment outside the physiological tolerances for *V. gibba*, indicating the assay is detecting a non-target organism, probably a native tropical corbulid mussel with an identical 28s rDNA sequence. Native corbulid mussels have been collected as part of this project as a basis for redesigning this assay. Detections of *Arcuatula* in Darwin and Cairns are difficult to interpret. *Arcuatula* has been detected as biofouling on vessels entering Darwin Harbour (GHD 2009), and it is possible that this pest was present in these areas during sampling. Our *Arcuatula* assay, however, has been subject to limited testing for specificity with native tropical relatives, and limited material was available for assay development. Native relatives of this species are known to occur in tropical Australia. It is, further, unclear if the tropical *Arcuatula* used to develop our assay (the samples were from Singapore) are the same as temperate invasive *Arcuatula* (see Aquenal 2008).

The detection of *Crassostrea* in Darwin is almost certainly cross-contamination. Our measures to prevent cross-contamination were informed by that finding.

4.1. Sample collection

Two methods for sample collection were tested: towed plankton nets and a plankton pump. Towed plankton nets provided the best detection of target organisms but the efficiency of plankton net sampling varied between locations and requires further optimisation. Clogging of the mesh by fine particles is the most likely cause of decreased effective tow length. The efficiency was measured as a ratio of the tow distance (measured by GPS) and the tow length (measured by a flow meter at the mouth of the net). Relative movement of the vessel and water due to wind, tides and currents can affect the ratio between GPS distance and effective tow length, but Milroy (2015) noted that this method provides a reliable indicator of net efficiency across multiple tows. Consistently low sampling efficiency at several sites indicates that net clogging is more severe at these locations than others, and generally efficiency was lower for the finer (50 µm) net. There was no difference in overall rate of detection between mesh sizes of the nets, but samples collected with the finer net showed higher levels of pest DNA in some cases. More samples will need to be taken at sites where net efficiency is low than where it is high if survey sensitivity is to be the same between sites, particularly for detecting new incursions where pest populations may

be small and/or have a clumped distribution. Baselines for pest presence/absence, samples displaying inhibition and other relevant factors will need to be developed for monitored locations.

Samples collected by pump had lower detection of target pests. This may have been caused by the net sampling a different depth to the pump, the pump sampling a lesser volume than the net, or by the pump design facilitating avoidance (Singarajah 1975) or the pump pressure profile forcing a proportion of the plankton through the mesh (Miller and Judkins 1981). The pump approach has numerous potential advantages including precise measurement of filtered volume, sampling at specific depth, size fractionation of samples on multiple filters and lesser need for management during sampling (Miller and Judkins 1981), but due to the decreased efficacy we observed, we did not investigate pump sampling further in this project.

4.2. Sample quality assurance controls and preservation

We assessed the addition of different quantities of laboratory hatched and commercially prepared *Artemia* as a control spike. Commercial *Artemia* are more consistent and easier to use than laboratory grown *Artemia.* Addition of 50 µL of *Artemia* slurry in sulfate buffer proved to be a consistent and suitable control to assess sample stability. Extraction efficiency can be assessed using *Artemia* control results given an adequate number of samples to capture variability.

We assessed three methods of preservation: sulfate-based preservative, chilling with cold transport, and prompt filtering and freezing with frozen transport. Sulfate preservative consistently provided good preservation, resulting in generally high recovery of *Artemia* control DNA, while both chilling and freezing showed more variability and lower *Artemia* recovery. In all locations, more *Artemia* DNA was detected in preserved samples than in the samples that were stored chilled or filtered and frozen promptly. Non preserved:preserved sample *Artemia* DNA ratios were as high as 1:10 for several sites. *Artemia* recovery was particularly low in unpreserved samples from both sets of Darwin samples, suggesting that sample chilling in the field may be suboptimal for preservation in locations with high air and/or water temperature, although samples collected in Cairns did not show as much discrepancy between preserved and unpreserved. One advantage of the preservation buffer is that samples added to the buffer are immediately stabilised, while samples placed on ice may still take some time to chill.

4.3. Sample processing and handling

We trialed four sample pre-extraction processing approaches: samples were filtered or unfiltered and then DNA extracted from whole freeze-dried samples, or freeze-dried samples were made up to 10 g with autoclaved sand and DNA extracted from a subsampled portion of the sand.

Diluting samples with sand facilitates individual samples being extracted multiple times. The principal advantage of this approach is that if a sample fails to extract or the DNA is low quality, re-extraction can be attempted, using a different protocol if necessary. Inhibition was lower in unfiltered sand diluted than direct extraction samples, and no filtered sand processed samples had very high scaling, but inhibition was lowest overall in filtered direct extraction samples. Although differences in detection were not significant, sand processed samples generally showed lower DNA recovery of detected pests, as would be expected due to dilution. No sample extractions failed to provide DNA. Re-testing diluted DNA extracted from whole samples appears to be a more useful approach for high inhibition samples, and sand dilution was discontinued.

Extracting from samples that were freeze-dried including the seawater (and which therefore included the seawater salts in the dry sample) was proposed as an approach to minimise handling and the time for which sample tubes are open. Minimal handling and closed tube processing aids in preventing sample labelling and identification errors and cross-contamination. DNA extracted from all unfiltered samples displayed markedly increased PCR inhibition and high scaling factors, especially when direct extraction was applied. These results make it apparent that high concentrations of salts inhibit PCR, so samples should be filtered and freeze-dried before extraction.

4.4. Cross contamination

We identified freeze-drying as the stage at which sample cross-contamination was occurring in our system after eliminating other potential sources of cross-contamination. Covering sample tubes during freeze-drying did not prevent cross-contamination. Freeze-drying samples individually effectively prevents cross-contamination in our system.

4.5. Analysis

PCR inhibition was observed in a few samples processed using the optimised technique (preserved, filtered, whole sample extraction); inhibition was generally greater in unpreserved samples. Reliable detection was achieved irrespective of sample amount in the sample mass limit of detection experiment, indicating that the assays are reliable in samples with large mass and

high DNA yield, even where the DNA of the target species occurs in low concentration. The material used in the sample mass limit of detection experiment was collected from Port Adelaide during a diatom bloom. The absence of inhibition in all these samples suggests that the load of live biological material does not drive inhibition, and abiotic substances or dead and decaying organic material are more likely to be the source of inhibition observed at some sites. These experiments also supported that the most effective sample processing method is to filter and extract the whole sample, although samples with PCR inhibitors are likely to display greater inhibition for samples where the whole sample is extracted. Alternative DNA extraction methods should be further investigated to determine whether inhibition can be further reduced or eliminated.

Experiments using field samples spiked with *Crassostrea* larvae showed detection occurred even where the total mass of *Crassostrea* relative to the total sample mass was very low, and may indicate that the spiking methodology was poor. D-stage *Crassostrea* larvae are small (75 µm), which suggests that delivery to the samples was inconsistent despite using a very fine transfer pipette for low concentration spiking and checking the pipette to ensure larvae had been placed in the sample. The rate of detection was greater for the second experiment. Quantifying the concentration of *Asterias* sperm proved difficult, and it is possible that the doses added to the first spiked samples contained none or very few sperm. Using embryos or larvae would be more appropriate, but no eggs were available to facilitate this. *Ciona* embryos are larger, but detection was also variable, which may also be related to unreliable transfer of embryos to spiked samples. Analytical sensitivity (the smallest amount of substance in a sample that can accurately be measured by an assay) for our assays is described from experiments, diagnostic sensitivity (the proportion of samples which contain a pest which are identified by the assay as positive for the pest) is less well defined. More refined methods for adding pest larvae are required for better confidence about spiked experimental samples containing pest material prior to processing.

The system we have developed has well defined parameters (Figure 16): the volume of water filtered maximises the chance of sampling pests, sample preservation, processing, handling, extraction, analysis and interpretation of results are understood and amount of template and PCR chemistry used have been refined from results to improve sensitivity. This provides an understanding of the probability of catching a pest, how to minimise the chance of it being lost from a sample and to maximise the likelihood of detecting it using the assays we have developed. It is problematic for describing this diagnostic sensitivity statistically from our data that there is no “gold standard” test against which to compare qPCR, and that our methods have evolved

throughout this project. We are developing multiple high throughput sequencing assays for some target species to use for comparison to better understand diagnostic sensitivity.

Encounter rate remains the most poorly understood component for developing survey methodology and understanding diagnostic sensitivity for marine pests, and few data exist to improve this. We rejected taking static water samples as a sampling approach for marine pests early in development of this system because a 1 L sample would need to be replicated over 1,000 times to obtain the same theoretical likelihood of sampling pest larvae or DNA as a 100 m plankton tow. This project was not designed to develop specific monitoring plans based on reproductive patterns of individual species or the temporal distribution of life history stages in the water column, but work is under way to provide data to inform this issue. Development of an approach based on a model that predicts encounter rate and which is validated using field data is the most practical solution.

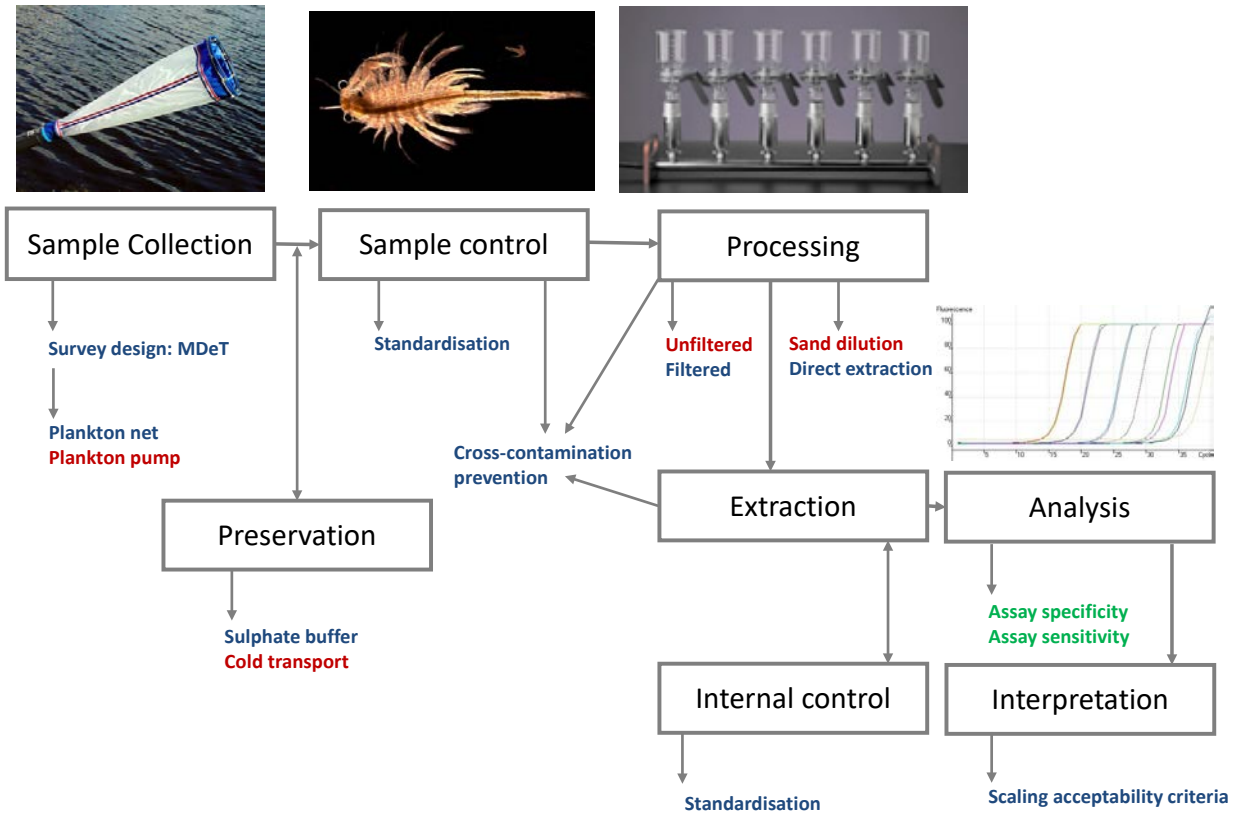

Figure 16. Pictorial representation of the proposed surveillance system and decisions made from this project to date. Red indicates options that have been considered and rejected. Blue indicates methods that have been identified but may require optimisation. Green indicates decisions that require analysis and ongoing review to incorporate them into a system for marine pest surveillance.

FURTHER WORK

Micromanipulation pipettes will be investigated to refine sample spiking methods and these samples will be used with qPCR and NGS assays to better define the diagnostic sensitivity of the approach. This sensitivity can be incorporated in a survey design tool to provide a stable basis for planning and implementing surveys using these molecular techniques. A better understanding of seasonality, encounter rate and the number of molecular samples required to achieve survey sensitivity equivalent to that of traditional surveys should be obtained by a validation process involving parallel surveys using both molecular and traditional survey methods.

Development of new assays and/or implementation of existing assays for exotic pests is under way. This will facilitate expanded activities and broader surveillance coverage.

IMPLEMENTATION PLAN

Introduction

This project was designed to include a feasibility plan and business case, based on the need in the National System for the Prevention and Management of Marine Pests (the National System). The National System includes a monitoring strategy based on biennial traditional surveys at 18 National Monitoring Network (NMN) locations (Adelaide, Botany Bay, Brisbane, Bundaberg, Cairns, Hay Point (including Dalrymple Bay), Dampier, Darwin, Fremantle, Gladstone, Hobart, Melbourne, Newcastle, Portland, Port Kembla, Port Hedland, Sydney and Townsville) around Australia. During this project the National System was reviewed, including the monitoring strategy, and a number of issues were identified (Arthur *et al.* 2015). The main problem was that the expense of traditional surveys prevented implementation of the National System (Arthur *et al.* 2015). The monitoring strategy was designed with multiple aims, including to detect new incursions and range expansions of priority pest species (target species), to detect other introduced species that have invasive characteristics, and to inform policy decisions on marine pest management (National System for the Prevention and Management of Marine Pest Incursions 2010b). Arthur *et al.* (2015) noted that survey data were also intended to inform port status for the Australian domestic ballast water management system, which focused on presence/absence of seven species (Northern Pacific Seastar (*Asterias amurensis*), Asian Date Mussel (*Arcuatula senhousia*, formerly *Musculista senhousia*), European Green Crab (*Carcinus maenas*), Wakame (*Undaria pinnatifida*), European Fan Worm (*Sabella spallanzanii*), Pacific Oyster (*Crassostrea gigas*) and the Basket Shell Clam (*Corbula gibba*)) that have established populations in Australia. This list was key for prioritising target species for molecular assay development.

The review highlighted that these species that the National System surveys aimed to detect may not be suitable in the current context for setting controls on domestic ballast water. Although the domestic ballast water system is likely to be initially implemented using these species as indicators and the NMN locations as survey sites, the target species and locations are being reviewed. It is not possible, in the absence of knowing the survey sites and target species, to assess likely need for surveillance or a testing service, and it was therefore not practical to develop a feasibility plan and business case. This implementation plan was substituted and comprises an outline and instruction for designing molecular surveys for marine pests.

Conducting surveys following the approach and instructions detailed here will provide data that facilitates a robust assessment of pest presence/absence to inform domestic ballast water management, and contribute to determining the feasibility of future monitoring strategies to support the domestic ballast water management system.

Survey design

Background

The National System includes a Monitoring Manual and Guidelines, and a Monitoring Design excel Template (MDeT) was produced to facilitate survey design (current version MDeT 2.5) and to ensure surveys were conducted with known, adequate sensitivity. The MDeT developed for the National System surveys allows each port to be divided into representative sublocations, and calculates sample numbers per sublocation required to achieve a specified sensitivity for a range of applicable survey techniques, given data on the survey location, species of interest, and methods. Default species and method data are contained in the MDeT, but the latter can be modified with regard to area or volume collected (or surveyed) per sample. Appendix 1 includes MDeT species data for the seven target species for the National System ballast water system. Modifying the sublocations/areas of interest retains survey comparability between sites better than modifying method or species data. Relevant data on the survey location, including areas of hard and soft substrate, depth, and a range of hydrological data needs to be compiled and entered into the MDeT to calculate sample numbers.

A range of problems with the MDeT were identified by Arthur *et al.* (2015). The target population sizes are poorly defined, target population densities are not considered, and analytical sensitivity and specificity are assumed to be 100%. MDeT is also problematic because of its complexity, and the opaqueness of its outputs. The National System surveys aim to detect presence/absence of target pests with calculated sensitivity for a given population size, but the calculation of sensitivity relies on several assumptions that may not be valid, and density of pest larvae within ballast water uptake zones may be a more applicable measure of port status for the Australian Ballast Water Information System (ABWMIS) than presence/absence of a given population size within the broader port location (Arthur *et al.* 2015). Designing an alternative monitoring system targeting ballast water uptake zones would, however, require investigation of the best methods for surveying the seven species of interest, and identifying appropriate standards for assigning port risk status. MDeT aims to address the complicated needs of the monitoring strategy and provide a stable basis for designing comparable surveys at different sites. MDET also attempts to provide

many of the critical parameter values and calculations required by the current NMS (Arthur *et al.* 2015). We, therefore, recommend surveys using the molecular tools be designed using MDeT, with plankton tows used as the sampling method for molecular surveys. For assays that have been validated for other matrices, scrapes, cores, grabs and dredges may be suitable for molecular analysis.

MDeT species data includes a definition of suitable habitat (hard, soft epifaunal, soft infaunal or planktonic) for each life stage (larva or gamete and juvenile/adult), including whether the species is found in the intertidal; whether the species is conspicuous (suitable for visual surveys), sessile or motile, and probability of the species fleeing and thus escaping detection if the latter; duration of each life stage; salinity and temperature tolerances where known; and the minimum population size that can be detected with a specified confidence. The target population sizes are set to 2 000 juvenile or adult stages and 100 000 gametes or larvae for each of these target species in the MDeT. Sample number calculations are based on the probability of detecting a population of the target size, given the area/volume of suitable habitat type in each sublocation. Calculated sample numbers are higher for larger sublocations since the target is of a population size rather than density, and individuals are assumed to be evenly distributed throughout the area/volume of suitable habitat. Sample numbers for methods targeting adult stages, will also be higher for species that occur in both the subtidal and intertidal (here, *Carcinus* and *Arcuatula*) than those that are strictly subtidal, at least in sublocations with a substantial area of intertidal, due to the greater area of suitable habitat. The species data also determines the relative suitability of different sampling techniques, for example, visual surveys are best suited to conspicuous and particularly sessile targets, while traps are only effective for motile species. Life stage duration is also included in the calculations, with resulting sample numbers being higher for species with short duration. For planktonic stages (gametes/larvae), the water body residence time, provided in the location data, is also included in calculations. Calculations used in the MDeT are described in the monitoring manual (National System for the Prevention and Management of Marine Pest Incursions 2010b). Species data are lastly used to flag any species that may be intolerant of the temperature or salinity at each sublocation. The MDeT lists these species and automatically excludes them from sample number calculations for sublocations where their tolerance limits are exceeded. For each sublocation, the final sample number shown is the highest across all seven target species.

Sampling design: locations and frequency

Sublocations for each survey site should be chosen to include all commercial wharves where ballast exchange occurs plus surrounding areas of substrate suitable for the IMS of interest. There are limited data on the best seasons for collection of plankton tows for molecular detection of most target species. The optimal sampling season is likely to vary between species, for example, data from this study suggest that summer sampling is best for *Crassostrea* and winter for *Sabella,* although not all locations with these pests were sampled in all seasons, and sampling effort between seasons was not consistent since these experiments were designed to assess methodological aspects, not seasonality. Reproductive biology can, furthermore, differ between their native and different invasive ranges (Munguia and Shuster 2013). It is likely, however, that a single set of samples at one time will be inadequate to detect all pests, and at least two sampling times will be required if molecular sampling is implemented as a future monitoring strategy. A current project is identifying optimal times for sampling, to provide recommended plankton collection times at each port.

Sampling methods

SARDI identified plankton tows as a suitable approach for marine pest surveillance (Giblot-Ducray and Bott 2013). Plankton sampling methods for molecular analysis have been refined by the current project and the MDeT method data used to determine molecular sample numbers were based on the method developed. For the surveys as designed here, 100 m sub-surface tows should be collected with a conical plankton net of 0.5 m mouth diameter, 1.5 m length and 50 µm mesh size. The net should be fitted with a flow meter to allow effective water volume sampled to be determined. Field data should be recorded as outlined in the National System standard data sheets for traditional methods, with the addition of flow meter readings. The collection method and data recording requirements are described in further detail in the SARDI standard operating procedures (SOPs) for plankton sample collection and transport for molecular analysis (Appendix 2), but note that these SOPs cover a range of sampling scenarios. If samples from these surveys are to be processed and analysed with the SARDI-developed qPCR assays by SARDI, additional sample handling protocols should be followed. After collection, the sample should be rinsed down into the cod end, concentrated to a volume of not more than 40 ml and immediately added to 80 ml of a sulfate-based preservative. Samples should be kept cold after collection but not frozen. Note that the methods described in the SOPs may be further refined, and updated copies of SOPs should be obtained prior to the commencement of any sampling.

Survey design sensitivity

The default sensitivity used in the MDeT is 0.8, but taking adequate samples to achieve this sensitivity may be infeasible. We therefore routinely calculate sample numbers required to achieve a survey sensitivity of 0.6. Surveying at lower sensitivity than 0.6 is not recommended, and identifying smaller sublocations to achieve the desired sensitivity is preferable to surveying a larger area at a lower sensitivity. To ensure all surveys are comparable, the sensitivity chosen needs to be defined and equivalent across ports, ensuring sample numbers are feasible and resulting surveys cost-effective. Arthur *et al.* (2015) identified numerous problems with the MDeT approach to sensitivity of particular utility for molecular surveys, including lack of documentation for target population sizes, inadequate accounting for clustering of pest populations and poor relationship descriptors for numbers of adults and larvae. Grey *et al.* (unpublished data) found that around 15 samples are needed to exhaustively survey a single site using molecular methods, and it is likely that the MDeT approach over-samples when targeting plankton and therefore for molecular methods. Refining sample number requirements to achieve target sensitivities is a priority for further work.

Analysis

Accurate analysis of environmental samples requires efficient extraction of large samples. Variation should be minimised and total extract volume and DNA concentration need to be carefully documented. Subsequent testing artefacts, including inhibition as indicated by control results, should be carefully monitored to understand outputs from laboratory systems. Testing with a sufficient volume of sample DNA appears to be important for environmental testing to obtain high sensitivity. The current lack of consistency in process, testing and reporting between laboratories undertaking molecular testing for marine pests poses a problem for understanding the comparability of results. Developing criteria for assessing normality of amplification curves for reporting qPCR results and comparing between laboratories is important. National projects following on from this study will provide a more consistent approach to use of assays through validation, but concentrating on developing more uniform and comparable approaches to the total diagnostic process is a priority.

Reporting

Outputs of analytical processes need to be contextualised for reporting. Current best practice involves providing detection data including, for qPCR, Ct value, sample target DNA concentration and a measure of inhibition. The predictive value (PV) is the probability that a sample that has

been tested produces an accurate result and that the test outputs reflect its true diagnostic status. The probability of obtaining correct positive and negative results for the assays discussed here are not understood, but should be further defined through validation processes.

Practical factors impact the fitness for purpose and utility of assays and testing systems for application. These include the diagnostic suitability of the assay, but also its acceptability to scientific and regulatory communities and clients, and feasibility of use in a practical setting (OIE 2016). We chose qPCR because of relative costs, high throughput, rapid turnaround and robustness, and regard it as more practical for validation and implementation than high throughput sequencing approaches. Further system validation should concentrate on making reporting more uniform.

5. CONCLUSIONS

This study has shown that molecular surveillance for marine pests using qPCR can form the basis of a functional surveillance system. The approach we describe here can rapidly and accurately identify invasive species from the environment, giving the best chance of eradication or minimising impacts and costs of invasions, and providing information to rapidly introduce effective control measures. Conventional surveillance techniques are expensive, slow and require specialist taxonomic expertise. These factors were initially offset by a lack of robust molecular methods for sampling and DNA extraction, but this study and the assays described in references herein largely address these issues.

The system, as defined, can be implemented as a surveillance tool. At a National Workshop in Adelaide in November 2016, it was agreed that *National Guidelines for validation of molecular methods for the detection of marine pests* would be developed, and these were finalised early in 2017. Our assays are currently undergoing additional work for them to be validated using this approach. Our *Corbula* assay will be redesigned in 2018, and our *Arcuatula* assay will be revised using further material from pest populations and native species. Assay validation provides further evidence of fitness-for-purpose and comparability of surveys across sites.

Parallel traditional and molecular surveys are under way, with seasonal sampling, to provide information on when to sample for target pests, how many samples are required to detect established pest populations and to refine the system as further required.

The greatest remaining need is for a revised National System with defined target species and surveillance sites. While SARDI can and will provide testing for external clients, without a framework it is impractical to reliably measure likely demand or number of assays likely to be used over time. Once these are defined, the cost-benefit and business suitability of deploying the system we outline here as a commercial service can be assessed. In the meantime, the molecular approach can be applied to monitoring as required.

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APPENDIX 1: MDET SPECIES DATA

Species_phylum	Species_name	Life_stage	Habitat	Sight_type	SubTida	InterTida	Behaviour	Min_duration	Pr_flee_pre	Pr_flee_nopre	Probe	VisID	Best_season
Echinodemata	Asterias_amurensis	Juv_adult	Hard_horizontal_vertical	Conspicuous	Yes	No	Sessile	730	0	0	Yes	Yes	1,2,3,4
Echinodemata	Asterias_amurensis	Gam_larv	Plankton_horizontal_vertical	na	Yes	No	Motile	92	0.05	0	Yes	Yes	3,4
Echinodemata	Asterias_amurensis	Juv_adult	Soft_epifauna	Conspicuous	Yes	No	Sessile	730	0	0	Yes	Yes	1,2,3,4
Crustacea/Brachyura	Carcinus_maenas	Juv_adult	Hard_horizontal_vertical	Inconspicuous	Yes	Yes	Motile	1095	0.8	0.05	Yes	Yes	1,2,3,4
Crustacea/Brachyura	Carcinus_maenas	Gam_larv	Plankton_horizontal_vertical	na	Yes	No	Motile	17	0.05	0	Yes	No	3,4
Crustacea/Brachyura	Carcinus_maenas	Juv_adult	Soft_epifauna	Inconspicuous	Yes	Yes	Motile	1095	0.8	0.05	Yes	Yes	1,2,3,4
Mollusca/Bivalvia	Crassostrea_gigas	Juv_adult	Hard_horizontal_vertical	Conspicuous	Yes	Yes	Sessile	730	0	0	Yes	Yes	1,2,3,4
Mollusca/Bivalvia	Crassostrea_gigas	Gam_larv	Plankton_horizontal_vertical	na	Yes	No	Motile	13	0.05	0	Yes	No	4,1,2
Mollusca/Bivalvia	Musculista_senhousia	Juv_adult	Hard_horizontal_vertical	Conspicuous	Yes	Yes	Sessile	365	0	0	Yes	Yes	1,2,3,4
Mollusca/Bivalvia	Musculista_senhousia	Gam_larv	Plankton_horizontal_vertical	na	Yes	No	Motile	14	0.05	0	Yes	No	4,1,2
Mollusca/Bivalvia	Musculista_senhousia	Juv_adult	Soft_epifauna	Conspicuous	Yes	Yes	Sessile	365	0	0	Yes	Yes	1,2,3,4
Annelida	Sabella_spallanzanii	Juv_adult	Hard_horizontal_vertical	Conspicuous	Yes	No	Sessile	1095	0	0	Yes	Yes	1,2,3,4
Annelida	Sabella_spallanzanii	Gam_larv	Plankton_horizontal_vertical	na	Yes	No	Motile	8	0.05	0	Yes	No	2, 3
Annelida	Sabella_spallanzanii	Juv_adult	Soft_epifauna	Conspicuous	Yes	No	Sessile	1095	0	0	Yes	Yes	1,2,3,4
Phaeophyta	Undaria_pinnatifida	Plant	Hard_horizontal_vertical	Conspicuous	Yes	No	Sessile	182	0	0	Yes	Yes	3,4,1
Phaeophyta	Undaria_pinnatifida	Gam_phyto	Plankton_horizontal_vertical	na	Yes	No	Motile	14	0	0	Yes	No	4,1
Mollusca/Bivalvia	Varicorbula_gibba	Gam_larv	Plankton_horizontal_vertical	na	Yes	No	Motile	7	0.05	0	Yes	No	4,1
Mollusca/Bivalvia	Varicorbula_gibba	Juv_adult	Soft_infauna	Inconspicuous	Yes	No	Motile	365	0.8	0.05	Yes	Yes	1,2,3,4

Summer	Autumn	Winter	Spring	MinTempTo	MaxTempTo	MinSalTol	MaxSalTol	Pop_size
1	1	1	1	0	25	19	41	2000
0	0	1	1	6	20 U	U		100000
1	1	1	1	0	25	19	41	2000
1	1	1	1	0	33	1	54	2000
0	0	1	1	11	26	26	39	100000
1	1	1	1	0	33	1	54	2000
1	1	1	1	2	35	3	56	2000
1	1	0	1	4	32 U	U		100000
1	1	1	1	5	30	7	39	2000
1	1	0	1	15	26 U	U		100000
1	1	1	1	5	30	7	39	2000
1	1	1	1	2	30	26	38	2000
0	1	1	0 U	U	U	U		100000
1	1	1	1	2	30	26	38	2000
1	0	1	1	0	25	20	34	2000
1	0	0	1	5	29 U	U		100000
1	0	0	1 U	U	U	U		100000
1	1	1	1	1	26 U	U		2000

APPENDIX 2: PLANKTON SAMPLING AND TRANSPORT SOPS

SARDI Aquatic Sciences
MISA Biosecurity Node

Field sampling SOP: Plankton sampling for molecular analysis

Date	December 2016
Review	December 2017 or as required
Author	K. Wiltshire
Approved	M. Deveney

Version control

Version	Date	Author	Amendments
1.0	January 2011	K. Wiltshire	Inception from old combined SOP.
1.1	July 2013	K. Wiltshire	Refinements, changed wording. Instructions made generic.
2.0	July 2015	K. Wiltshire	Changed wording and clarifications.
3.0	December 2016	K. Wiltshire	Refinements to cover more general sampling

Plankton sampling kit

Kit contents

Item	Qty included	Note
Sampling equipment		
Zooplankton net with flow meter + cod end[1]		
Rope with clip + weight[1]		
Aquarium net[1]		
2 L plastic beaker[1]		
Squirt bottle[1]		
Hand held GPS[1]		
AA Batteries (spare) [1]		
Data sheet		
Sampling design sheet (and optionally, maps)		
Optional spare waterproof paper blank		
120 ml sample jars (see design sheet)		
375 ml sample jars (see design sheet)[2]		
Syringes with Millipore filter[2]		

[1] Only included in the sampling kit where supplied by SARDI, otherwise, local equipment to be used

[2] May not be used with all sampling designs

Please refer to the enclosed packing list for details specific to your kit and to check that you have been sent all required items.

About the kit

The sample jars will be labelled on the side and on the lid. The label on the side will show the sample identification code, corresponding to the sampling design, while the number on the lid is the randomised field collection order where a random order is applied, or the same sample code as on the side of the jar otherwise. Jars that have not been assigned a specific code will be numbered sequentially on the lid and side.

The sampling design sheet will explain what type of sample is associated with each sample identification code.

The sampling data record sheet is printed on waterproof paper and shows the sample ID codes, which will be in randomised or systematic field collection order as required. For each sample, the

data sheet has fields to record flow meter readings (start and end, where used) and GPS waypoints plus any other notes pertaining to the sample. There is also an area on the data sheet for general sampling information to be recorded, and details of the net(s) used. If more than one net type is to be used for sampling (e.g. two different mesh sizes), the data sheet will also show which net to use for each sample. Where sampling takes place in multiple areas or subsites, there will be space for making notes specific to the area that may be relevant for sampling (e.g. highly turbid water, presence of jellies).

The jars should be kept refrigerated until the day of sampling. In the field, prior to sample collection, keep the sample jars cool; do not leave them in direct sunlight or exposed to heat sources (e.g. engines). If possible, keep the jars in an insulated bag, box or esky, ideally with ice or cool packs (e.g. gel ice) added.

Field collection protocol

Prior to sampling

- Check that you have the correct number of sample jars and that codes correspond to the sampling design and data sheets
- Ensure you have all required equipment available for collection and sample transport
- Place gel ice packs from the sampling kit into a freezer overnight or longer to ensure they will be frozen in preparation for sample transport
- Correspond with SARDI sample receiving staff to ensure sample transport is arranged for an appropriate time. Non-preserved samples should be transported on the day of, or the day after, collection. Ensure SARDI staff are aware of the date of sample arrival and available to take possession of samples as soon as they arrive
- Sort sample jars into field collection order
- Ensure you are familiar with the steps for sample collection and with the use of required equipment (e.g. GPS)
- Each plankton sample should be no more than ~40ml. Ensure you are familiar with the cod end of your plankton net so you can tell when the sample is of suitable volume. It can be useful to make a mark at a suitable height on the cod end as a guide.

Required equipment

To complete sampling, you will need all items listed under “sampling equipment” on the first page of this SOP.

Additionally, you will need (as a minimum):

- Pencil(s) for writing on water proof paper
- Esky with ice for field use
- Suitable PPE for your local conditions, including protection from marine stingers where appropriate. You may wish to consider:
    - Gloves to protect from chafing while handling tow rope and from strands of wire rope from towing bridle
    - Wet weather gear and boots to protect clothing from splashing seawater

Recommended items:

- Bucket for collecting seawater and washing down net
- Clip board for holding data sheet
- Pencil sharpener and eraser

Safety precautions:

Ensure you have all other equipment required to safely operate your vessel and undertake field work following your organisation's protocols and national and local maritime regulations. Follow the SOPs in this document. If sampling with SARDI staff or in the absence of local equivalent protocols, follow relevant SARDI SOPs for other aspects of field work.

Sampling can be conducted with a minimum of two persons – the vessel skipper and someone to conduct tows. The skipper can operate the GPS while the person conducting tows records other data. You may wish to have a third person to assist, in which case this person can operate the GPS, record data, and assist with sampling as needed.

Containment precautions:

Follow good scientific practice:

- Dispose of any waste and disposable equipment in sealed plastic bags. Double bag at the completion of field work and dispose of bags in biohazard (yellow) bins for incineration
- Clean and decontaminate all reusable equipment (see Aquavetplan Operational Manual: Decontamination for details)
- Use only field equipment
- Never take field equipment or soiled clothing into laboratories, aquaculture areas or other areas where live animals are held

Treat all samples as potentially health and environmentally biohazardous.

Filtered seawater controls

Filtered seawater controls may not be used with all sampling designs. Check the design sheets and sampling sheet.

Where filtered seawater controls are used, the sampling data sheet(s) has a line for each control jar to indicate at what point during the sampling it should be filled with filtered seawater. At each of the prescribed times, 40 ml filtered seawater should be added to the relevant jar following the below procedure. Note the time of filling of each control sample on the sampling data sheet.

The procedure for collecting filtered seawater is as follows:

1. Take the GPS waypoint of the site where seawater for controls is taken and record this on the datasheet along with the time
2. Take up seawater into the syringe to rinse syringe. Discard this water, then repeat to give 3 rinses.
3. Fill the syringe with seawater and place the Millipore membrane filter over the syringe outlet.
4. Add~40 ml seawater from the syringe through the membrane filter to the relevant sample jar. Remove the filter to refill the syringe.
5. Cap the control jar. Invert the sample with preservative several times to mix. Place on ice.

Seawater from your sampling location should be used, but it is not necessary to do a control sample for each sub-location if you are sampling at multiple sites.

Sample collection

Prior to starting plankton tows, record the sampling location, date, a contact name for field personnel (for reference if questions arise), and general sampling notes in the areas provided on the data sheet(s). Notes should include details of the net used where this has not been supplied by SARDI, weather conditions (air temperature, cloud cover, wind) and any other points of interest (e.g. notable water turbidity, run-off from recent rain, tidal cycle and tide times).

The sampling order has been randomised to ensure no systematic bias between sample types, e.g. all samples of one type taken on incoming tide and all others on outgoing tide. Therefore, please collect samples in the randomised order indicated. Samples will be listed on the data sheet

in this order, but as a safeguard, please ensure that GPS waypoints and flow meter readings are each recorded with the correct sample ID

1. Attach the plankton net tow bridle(s) to a suitable length rope for use with your vessel. It is recommended that a minimum 1 kg weight should be attached to the net mouth or towing bridle to assist in submerging the net. If multiple nets are to be used, a single rope with clip can be used to facilitate changing between nets. Ensure the correct net is used as appropriate for each sample.
2. Collect seawater from the location you will be sampling and fill the squirt bottle.
3. Lay the net out to ensure it is not twisted and check that the cod end is attached.
4. Record the starting value of the flow meter.
5. With the vessel moving slowly, lower the net over the vessel side with the mouth facing the direction of travel, and pay out rope as needed so that the net is clear of the vessel motors and is submerged just below the water surface. Ensure that the net has not twisted and that the flow meter is clear of the net sides. Secure the net on a suitable length of rope by tying or looping around a cleat or vessel structure or by holding it firmly. Make sure that the net will be clear of the vessel motors once underway Depending on your net and your vessel configuration, you may opt to tow the net alongside, rather than behind, the vessel. In this case, the vessel may be stationary as the net is lowered.
6. As the net enters the water, mark the starting point with a hand held or the vessel GPS and note the waypoint name/number on the data sheet. Use the GPS find or go to function to locate the point just marked and select a screen/option that shows distance to the point.

    To mark a waypoint with the Garmin GPSmap 78sc unit, press and hold “Enter” until the waypoint screen appears. Note the waypoint number.

7. Motor slowly (1-1.5 knots) so that the net remains submerged but fully extended.
8. Use the GPS to determine when a distance of 100m has been reached, moving the vessel in as straight a line as possible. Stop the vessel and mark the finish way point with the GPS. Record the end waypoint on the datasheet

    The SARDI GPS is a Garmin GPSmap 78sc.To find a waypoint with this GPS unit, press “find” and then use the arrow keys to select “Waypoints and press “Enter”. The most recent/nearest waypoint will usually be at the top of the list but, if not, scroll with the arrow keys until the correct waypoint is selected. Press “Enter” to select the waypoint and press “Enter” again to start navigation. Press page until “Compass” page is selected. “Distance to next is the distance from your selected waypoint. If using another type of GPS, ensure you are familiar with the process for determining distance from a marked waypoint.

9. Pull the net up with the rope until the metal ring at the top can be grasped. Lift the net by the ring primarily, taking care not to handle the mesh too roughly. Do not hold by the steel tow wires as these may have sharp ends or frayed wires. Be sure to use a suitable lifting technique to avoid back strain.

10. As the net is lifted, dip it up and down several times at the water surface to wash plankton down into the cod end.

11. Record the final value on the flow meter.

12. Once the net is on board, wash down the net sides with the seawater squirt bottle or bucket to wash plankton into the cod end. Let excess water drain from the cod end. Tilt or gently shake or swirl the cod end to help remove excess water

13. Remove the cod end, taking care not to tip out the contents.

14. Inspect the cod end contents to check for large detritus or organisms (e.g. seagrass leaves, jellyfish). If large detritus or organisms are present that will prevent the sample being concentrated down to the target volume (40-50 ml), follow steps 14a-c, otherwise, proceed to step 15.

    a) Pour the cod end contents into the clean beaker through the sieve or aquarium net, rinsing the inside of the cod end well with the squirt bottle of sea water so that all material is washed into the sieve or net.

    b) Use the squirt bottle to wash all plankton through the sieve or net while leaving detritus in the sieve or net.

    c) Pour the sample from the beaker back into the cod end and discard the large detritus from the net or sieve.

15. Carefully swirl the sample in the cod end to remove excess water and concentrate the sample into the bottom of the cod end.

16. Wash down the cod end windows with seawater from the squirt bottle or bucket. The total sample volume must be no more than 40ml.

17. Pour the sample into the appropriate labelled sample jar. Ensure that the sample jar label corresponds to that shown on the sampling data sheet. If any sample identification label (on the side of the jar) is damaged or smudged prior to or during sampling, please write the sample ID onto the jar lid with a permanent marker. Invert the sample several times to mix.

18. Place sample on ice in an esky and keep cold.

19. Record the GPS waypoint identifiers (start and finish), flow meter readings (start and finish) and any other relevant notes on the datasheet. Please make a note if any of the following occur: presence of detritus so that the sample needed pre-filtering, possible impedance of flow meter, change to sampling order, move to a new sub-location, or any problem with sample collection. Record the actual jar label used if different to that on the sample sheet, e.g. if a specified spare jar is used. Other useful notes include observations of tidal flow or wind conditions that may impact vessel speed, other location notes (proximity to a land mark, water inflow point), observations of water clarity, etc.

Space for notes is provided on the data sheet(s) and additional notes can be recorded on the spare waterproof paper provided.

20. If material remains in the plankton net, or if moving to a new sampling sub-location, wash the net down with a bucket or tow it a short way without the cod end in place to rinse. If changing location, use water from the new location to rinse the net, or perform this tow in the new location. Refill the squirt bottle with water from the new location. For some sampling designs, additional decontamination may be required when moving between locations. Where this is specified, clean the net following step 22 before commencing sampling at the new location. If local biosecurity protocols require additional measures are used for cleaning, ensure those protocols are adhered to.
21. Replace the cod end and repeat from step 3 to take further samples.
22. Once sampling is completed wash the net(s) and cod end(s) with hot freshwater and 20mL/L Decon 90. Dispose of washing solution on land or to sewer. Clean all equipment (squirt bottle, weights, bucket etc.) in 20mL/L Decon 90 and decontaminate in hypochlorite at a minimum of 200 ppm available chlorine.

After collection

Samples without preservative must be kept cold (on ice or with cold packs) until they are processed or packed for transport, but it is recommended to also keep preserved samples cold as an additional safeguard wherever possible. Unpreserved samples must be transferred to a refrigerator for overnight storage if to be processed and/or shipped the next day and kept at 1-4ºC at all times. Preserved samples should be kept in an insulated container in a cool area as a minimum, but ideally kept with cold packs or ice or refrigerated. See the Plankton sample processing for molecular testing SOP for samples requiring on site processing prior to transport. Package samples following the sample transport and handling SOP.

Download waypoints from your GPS and save in a format that shows the latitude and longitude associated with each waypoint identifier. This can be the native Garmin GPS format or as a .txt (or .csv) file. Email this file to your contact at SARDI.

Photocopy or scan the sampling data sheet to make a back-up copy. The original data sheet should be returned to SARDI with the samples.

Plankton sample packaging for transport

Date	December 2016
Review	December 2017 or as required
Author	K. Wiltshire
Approved	M. Deveney

Version control

Version	Date	Author	Amendments
1.0	December 2016	K. Wiltshire	Separated from combined transport and handling SOP. Updated transport methods for additional sample types

Equipment

Packaging for transport		
Insulated container or sturdy box[1]		
Gel ice packs[2]		
Parafilm strips		
Press-seal bags		
Plastic liner bag		
Rubber band		
Foam tray for tubes[3]		
Packaging tape		

[1] For cold or frozen samples, an insulated container must be used. If not supplied by SARDI, a suitable container should be requested from Critical Transport Solutions.
[2] Required for cold sample transport, optional for ambient transport. Dry ice (not supplied) must be used for frozen transport.
[3] For on-site processed samples only

Plankton sample handling and transport

Process for managing samples after field sampling and/or sample processing are complete.

SOP to pack samples to IATA Packing Instruction 650 standard, but without biohazard/UN3373 labelling (environmental samples are not classed as toxic or infectious to humans).

Sample transport

Sample transport should be booked at least 24 hours before it is required, additional notice may be needed for frozen sample transport from some areas. Call Critical Transport Solutions on 1300 487 944 to arrange pick up. Please request next-day delivery, and advise the pick-up location. For frozen samples, transport on dry ice is required. A 3kg quantity of dry ice is sufficient for transport of up to 20 samples. If not sourcing dry ice separately, this can be supplied by Critical Transport Solutions, but note that additional notice may be needed. If a suitable insulated container has not been supplied, request a suitable insulated container from Critical Transport Solutions. Ensure SARDI staff are aware of the shipment arrival date and will be available to receive samples as soon as they arrive.

Unfiltered unfrozen samples

Unfiltered unfrozen samples without preservative must be kept cold during transport. These should be transported in the supplied insulated container with gel ice packs to maintain temperature. Samples with preservative can be transported at ambient temperature, but use of an insulated container is still recommended to avoid temperature extremes. Gel ice packs should also be used to keep samples cool wherever possible as an extra safeguard against degradation. Where an insulated container is not available, a sturdy container, preferably hard plastic, should be used.

Prepare unfiltered samples for transport by wrapping each lid with a strip of parafilm and placing jar into press-seal bags (4-5 sample jars per bag). Use long parafilm strips for the lids of 375 ml jars (where present) and the standard length for 120 ml jars. Place a liner bag into the insulated container and arrange bags of samples upright inside the liner bag. Place frozen gel ice packs (where used) primarily on top of the samples (as cool air will sink, keeping samples cold). If practical, ice packs can also be placed between sample bags to cushion and secure these. Newspaper or bubble wrap may be used as additional packaging material if needed. Gooseneck

the top of the liner bag and secure with rubber bands. Place the lid on the esky and secure with packaging tape.

Filtered frozen samples

Keep frozen samples in the freezer until pick up. Where a suitable insulated container has been supplied and dry ice sourced, transfer samples to the supplied insulated box with dry ice. Packaging will be completed by the courier service to IATA standards.

APPENDIX 3: EXPERIMENTAL DESIGNS

Pre-processing method	Spike	RDTS processing method
		10g sand
		10g sand
		10g sand
		Direct extraction
		Direct extraction
		Direct extraction
		10g sand
		10g sand
		10g sand
		Direct extraction
		Direct extraction
		Direct extraction
		10g sand
		10g sand
		10g sand
		Direct extraction
		Direct extraction
		Direct extraction
		10g sand
		10g sand
		10g sand
		Direct extraction
		Direct extraction
		Direct extraction
		10g sand
		10g sand
		10g sand
		Direct extraction
		Direct extraction
		Direct extraction
		10g sand
		10g sand
		10g sand
		Direct extraction
		Direct extraction
		Direct extraction

i. Experimental design for Geographic sampling 1 Port Adelaide

Treatment	Pre-processing method	Spike	RDTS processing method
			10g sand
			10g sand
			10g sand
			Direct extraction
			Direct extraction
			Direct extraction
			10g sand
			10g sand
			10g sand
			Direct extraction
			Direct extraction
			Direct extraction
			10g sand
			10g sand
			10g sand
			Direct extraction
			Direct extraction
			Direct extraction
			10g sand
			10g sand
			10g sand
			Direct extraction
			Direct extraction
			Direct extraction
			10g sand
			10g sand
			10g sand
			Direct extraction
			Direct extraction
			Direct extraction

ii. Experimental design for Geographic sampling 1 for sites other than Port Adelaide

Treatment	Processing method	Spike
TOW (T)	Centrifuge (C)	100 ul Brine shrimp spike (SP)
		No spike (NS)
	Filter (F)	100 ul Brine shrimp spike (SP)
		No spike (NS)
	Raw, no treatment (R)	100 ul Brine shrimp spike (SP)
		No spike (NS)
PUMP (P)	Centrifuge (C)	100 ul Brine shrimp spike (SP)
		No spike (NS)
	Filter (F)	100 ul Brine shrimp spike (SP)
		No spike (NS)
	Raw, no treatment (R)	100 ul Brine shrimp spike (SP)
		No spike (NS)

iii. Experimental design for Geographic sampling 2 for the Port Adelaide method assessment sampling.

Treatment	mesh size	Tow length
Plankton tow (18 samples) ~40 ml plankton tow samples collected and spiked with a brine shrimp control and placed on ice.	150 micron	20m
		100m
		300m
	50 micron	20m
		100m
		300m
Plankton Pump (27 samples) ~40 ml plankton pump samples collected and spiked with a brine shrimp control and placed on ice.	150 micron	20m
		100m
		300m
	50 micron	20m
		100m
		300m
	5 micron	20m
		100m
		300m

iv. Experimental design for Geographic sampling 2 for the Port Adelaide sampling methods trial.

Treatment	Pre-processing method	Spike

v. Experimental design for Geographic sampling 2 for Cairns.

Treatment	Treatment/Pre-processing	Spike	Net Size
			50
			50
			50
			150
			150
			150
			50
			50
			50
			150
			150
			150
			50
			50
			50
			150
			150
			150
			50
			50
			150
			150
			150

vi. Experimental design for Geographic sampling 2 for Darwin, Hobart and Sydney.

Treatment	Pre-processing method	Spike 1	Spike 2	RDTS processing method
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**

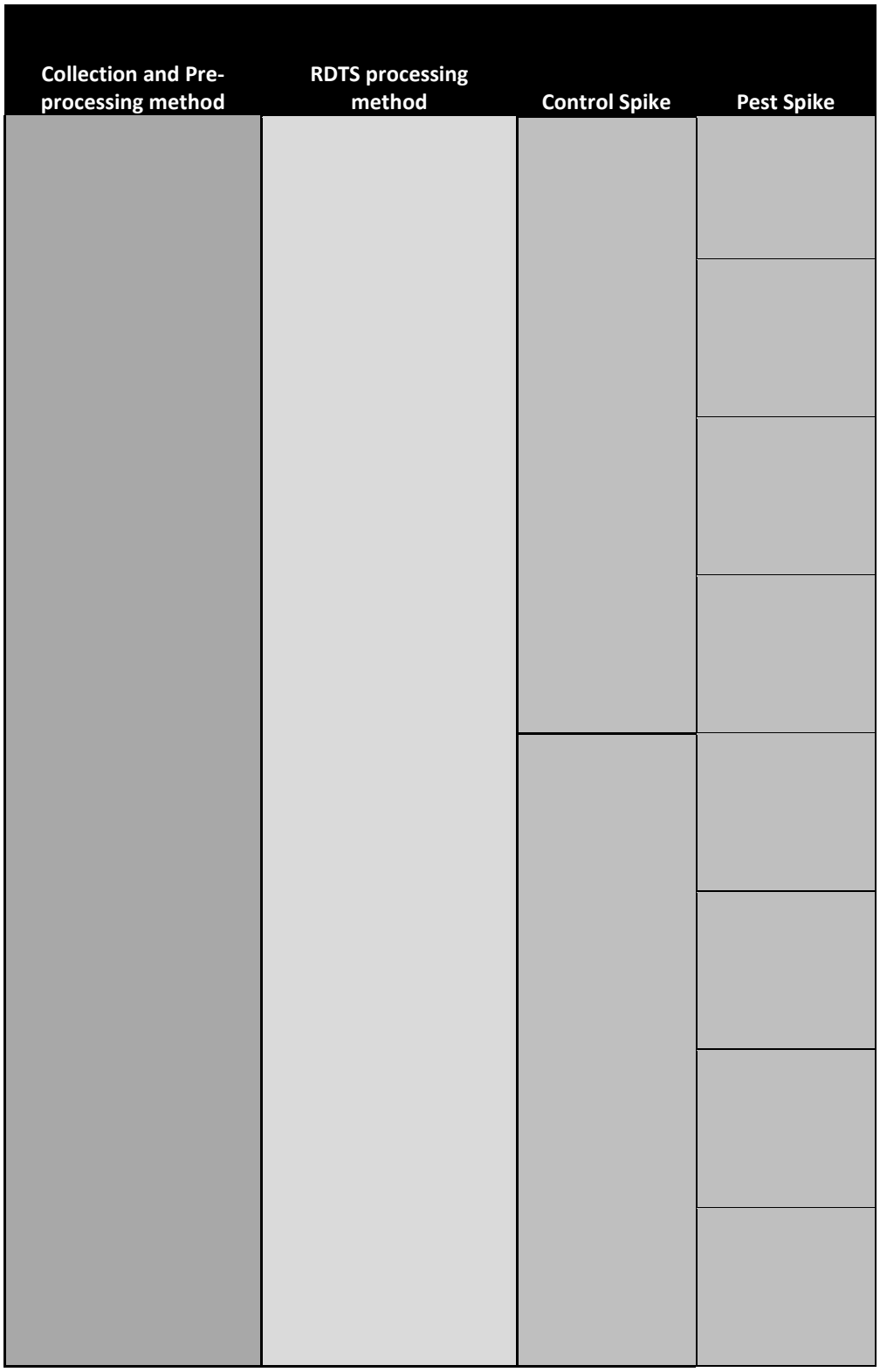
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**
			No Oyster larvae	**10g sand**
			Oyster larvae - 1	**10g sand**
			Oyster larvae - dil. 1 (100)	**10g sand**
			Oyster larvae - dil. 2 (1000)	**10g sand**

			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**
			No Oyster larvae	**Direct extraction**
			Oyster larvae - 1	**Direct extraction**
			Oyster larvae - dil. 1 (100)	**Direct extraction**
			Oyster larvae - dil. 2 (1000)	**Direct extraction**

vii. Experimental design for *Crassostrea* limit of detection 1 experiment.

	RDTS processing method	Sample Mass	Control Spike	Pest Spike
Plankton tow (24 samples) ~40 ml plankton tow samples collected and spiked with or without a brine shrimp control and pest larvae and placed on ice. **Filter & Freeze-dry**, filtered and the filter placed in tube with beads, frozen @ -20°C	Direct extraction	Low mass - 5ml plankton slurry	No brine shrimp (12 samples)	None
				1:1000 dilution
				1:100 dilution
				1:10 dilution
			50 ul brine shrimp commercial slurry	None
				1:1000 dilution
				1:100 dilution
				1:10 dilution
Plankton tow (24 samples) ~40 ml plankton tow samples collected and spiked with or without a brine shrimp control and pest larvae and placed on ice. **Filter & Freeze-dry**, filtered and the filter placed in tube with beads, frozen @ -20°C	Direct extraction	medium mass- 25 ml plankton slurry	No brine shrimp (12 samples)	None
				1:1000 dilution
				1:100 dilution
				1:10 dilution
			50 ul brine shrimp commercial slurry (12 samples)	None
				1:1000 dilution
				1:100 dilution
				1:10 dilution
Plankton tow (24 samples) ~40 ml plankton tow samples collected and spiked with or without a brine shrimp control and pest larvae and placed on ice. **Filter & Freeze-dry**, filtered and the filter placed in tube with beads, frozen @ -20°C	Direct extraction	high mass - 125 ml plankton slurry	No brine shrimp (12 samples)	None
				1:1000 dilution
				1:100 dilution
				1:10 dilution
			50 ul brine shrimp commercial slurry	None
				1:1000 dilution
				1:100 dilution
				1:10 dilution

viii. Experimental design for *Crassostrea* limit of detection sample mass experiment.



ix. Experimental design for *Asterias* limit of detection experiment.