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Molecular surveys for priority marine pests in 2018–2019 for the ports of Gove, Weipa, Hay Point, Newcastle, Botany Bay, Port Kembla and Devonport

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

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Field sampling and data collection was performed by Jacobs Group (Australia) Pty Ltd in 2018 and by Cardno (NSW/ACT) Pty Ltd in 2019. Extractions, testing and reporting were undertaken at the Root Disease Testing Laboratory (RDTS) at SARDI Sustainable Systems under the guidance of Drs Alan McKay and Kathy Ophel-Keller. Sample preparation and processing for the molecular survey method was performed at SARDI Aquatic Sciences with assistance from Nhu Y Lieu, Ian Moody, and Leonardo Mantilla. A/Prof. Jason Tanner and Prof. Gavin Begg provided guidance and support. The report was reviewed by Drs Gretchen Grammer and Paul van Ruth (SARDI) and approved for publication by A/Prof Tim Ward.

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EXECUTIVE SUMMARY

Shipping is a major vector for marine pest introductions, and port areas are at high risk of new marine pest introductions. Knowledge of pest occurrence around ports is required for management, including to ensure compliance with ballast water regulations. Surveys for introduced marine species (IMS) at most ports around Australia, however, have rarely been conducted, with the high cost of implementing surveys using traditional methods (e.g. dives, trawls/dredges, trapping) recognized as a major impediment. Molecular techniques for marine pest surveillance offer cost and time savings over traditional techniques and so are receiving increasing attention. SARDI has developed qPCR assays to detect ten marine pests, including seven priority pests for ballast water management, and a method of plankton collection for testing with these assays. The system has been tested and refined using seasonal sampling from ports around Australia. Analyses of the results demonstrated that a combination of sampling in late summer–early autumn and late autumn–early winter provided the greatest detection likelihood across the target species.

This report describes the design and results of molecular surveys for Gove, Weipa, Hay Point, Newcastle, Botany Bay, Port Kembla, and Devonport. The surveys targeted six of the seven pests of concern for ballast water management:

- 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*, also known as *Magallana gigas*)

Sampling was conducted in autumn 2018 and summer 2019 in Gove, Weipa, Hay Point and Devonport, and in summer and autumn 2019 in Newcastle, Botany Bay and Port Kembla.

Arcuatula, Asterias, Carcinus and *Crassostrea* were detected in Devonport. *Asterias* has not been reported from Devonport although it is widely distributed on the south-east coasts of Tasmania, while *Arcuatula, Carcinus* and *Crassostrea* are previously recorded from Devonport. A follow-up traditional survey did not detect *Asterias* in Devonport, but it is possible that a population exists outside the surveyed port area, with DNA advected into the port by currents. *Sabella* was detected at low abundance during dive surveillance; this species is also recorded from Devonport but was

not detected by the current molecular surveys, probably due to low population size. *Crassostrea* and *Sabella* were detected in Botany Bay, with *Crassostrea* also detected in Newcastle and Port Kembla. These species are known to occur in New South Wales (NSW), with records of *Crassostrea* from both Botany Bay and Newcastle, and of *Sabella* from Botany Bay. Port Kembla has not been surveyed for marine pests recently and is within the region of NSW where *C. gigas* is broadly established. Low-level detections of *Undaria* occurred in 1-2 samples in summer in the NSW ports; these are likely to be of transient material, and no detections occurred in autumn. A single detection of *Sabella* occurred in Port Kembla in summer; this is also possibly a transient detection, especially as the detection was relatively low-level. *Arcuatula* was detected in the tropical ports of Gove, Weipa and Hay Point, where it has not been previously recorded, although surveillance targeting this species in these ports is lacking. It is likely that the current *Arcuatula* assay cross-reacts with native tropical relatives; investigation of the specificity of this assay and identification of better sequence targets for assay redesign is ongoing. Further investigation of the samples with *Arcuatula* detections will be conducted in parallel to the assay redesign to determine whether these detections are of *A. senhousia* or a native relative.

These surveys established pest status of the seven surveyed ports for Australian Sourced Ballast Applications in the Maritime Arrivals Reporting System. The findings reinforce the strengths of molecular surveillance in high sensitivity and low overall cost but include results that probably comprise transient detections or detections of non-viable material, and detections of pests where the population occurs outside the survey area. The results provide further data on the effects of PCR inhibition, supporting that sensitivity of some assays is reduced by higher levels of inhibition (scale factors > 5), but the scarcity of samples with high inhibition from ports with pests present means that effects of inhibition cannot be fully assessed with current data. The results also provide further evidence that the diagnostic specificity of the *Arcuatula* assay is inadequate, but all other assays performed as expected.

1. INTRODUCTION

1.1. Background

Marine pests affect fishing and aquaculture, amenity and infrastructure, undermining recreational, community and indigenous values of marine systems, and placing communities that depend on those systems at risk (Hayes and Sliwa 2003; Schaffelke and Hewitt 2007; Molnar *et al.* 2008; Hewitt *et al.* 2011). Surveillance is a key component of managing incursion risk. Early and reliable detection maximises the likelihood of responses to incursions being successful and supports minimisation of spread of established pests, supporting sustainable management of marine systems (Department of Agriculture and Water Resources 2018). New international and domestic regulations for ballast water management came into force in September 2017, and surveillance is required to support port status for the assessment of Australian Sourced Ballast Applications (ASBA) in the Maritime Arrivals Reporting System (MARS), which focuses on seven species that have established populations in Australia (Arthur *et al.* 2015; Department of Agriculture and Water Resources 2018). A surveillance strategy for Australian ports was established in the 2000s, but its implementation was limited. The strategy was based on traditional methods such as dredge sampling, trapping and visual surveys (National System for the Prevention and Management of Marine Pest Incursions 2010a, b). A review of that monitoring strategy identified that lack of surveillance was largely due to the expense of traditional surveillance methods (Arthur *et al.* 2015).

Molecular techniques for marine pest surveillance offer cost and time savings over traditional techniques, and technical advances have provided a platform for the development of practical, specific, sensitive and rapid molecular surveillance tools for marine pests (Bott *et al.* 2010b; Deveney *et al.* 2017; Department of Agriculture and Water Resources 2018). The South Australian Research and Development Institute (SARDI) has developed laboratory validated quantitative polymerase chain reaction (qPCR) assays for detection of 10 marine pest species (Ophel-Keller *et al.* 2007; Bott *et al.* 2010a; Bott and Giblot-Ducray 2011a, b, 2012), and developed and refined plankton sampling and preservation methods and quality controls for molecular marine pest surveillance (Wiltshire and Deveney 2011; Giblot-Ducray and Bott 2013; Deveney *et al.* 2017). Field performance of this molecular surveillance method was initially assessed by the Australian Testing Centre for Marine Pests (ATCMP) project (Deveney *et al.* 2017), which involved application of the assays to plankton samples collected from six ports around Australia in two seasons each. All established target pests were reliably detected in

Adelaide, Melbourne, Hobart and Sydney. The *Corbula gibba* assay, however, displayed problems with specificity when applied to samples from Cairns and Darwin due to cross-reaction, probably with a native tropical corbulid (Deveney *et al.* 2017), and is being re-designed. Further field validation of the molecular methods was carried out by conducting parallel molecular and traditional surveys targeting six of the priority pests (*Asterias amurensis*, *Arcuatula senhousia*, *Crassostrea gigas*, *Carcinus maenas*, *Undaria pinnatifida* and *Sabella spallanzanii*) in four ports (Gladstone, Brisbane, Melbourne and Hobart) over 2017–2018 (Wiltshire *et al.* 2019a). Results from the parallel surveys demonstrated that the molecular approach is fit-for-purpose for marine pest surveillance. Molecular methods provided higher survey sensitivity than traditional methods, while requiring less than half the person-hours and 22–45% of the field cost of traditional surveillance. Detection likelihood of most of the target pests varied between seasonal sampling sets, with the highest likelihood of detection corresponding to the known spawning season for the species with the most pronounced seasonal patterns (Wiltshire *et al.* 2019a). For detection of the six target species in temperate locations, a combination of sampling in later summer and late autumn would provide the overall highest likelihood of detection for a given number of samples, or equivalently, require the fewest samples to achieve a given survey sensitivity (Wiltshire *et al.* 2019a).

Molecular surveys were designed and conducted for seven ports using the methods applied in Wiltshire *et al.* (2019a). Two plankton collections were conducted in each port to cover summer and autumn sampling periods. Surveys were conducted in Gove, Weipa, Hay Point and Devonport in autumn 2018 and summer 2019; and in Newcastle, Botany Bay and Port Kembla in summer and autumn 2019. This report describes the design and outcome of these surveys.

1.2. Objectives

- Apply molecular surveillance for priority marine pests to Gove, Weipa, Hay Point, Devonport, Newcastle, Botany Bay and Port Kembla;
- Map detections and compare to previous records of detected species;
- Assess results using information on test diagnostic performance and considering potential effects of sampling volume, sample mass and PCR inhibition.

2. METHODS

Surveys were designed for each port using the Monitoring Design Excel Tool (MDeT) version 2.5, which was originally developed as part of the National System surveillance strategy. The MDeT was used despite recognition of several issues with the calculation of survey sensitivity, because alternative tools for survey design are lacking, and because it will give a standardised measure of design sensitivity across ports, even if this is not the true value. The MDeT is populated with a range of data that was compiled during its development, including data on the target species and methods. The parameters and calculations in the MDeT were developed through consultation with the high level working groups who developed the National System surveys (Arthur *et al.* 2015). Method data should be modified as appropriate to reflect the specific equipment and sampling techniques used. Plankton tow was the only sampling method used (see section 2.2) for these surveys as we were specifically interested in using plankton for the molecular detection of pest species in the water column. In addition to the species and method data, data on each sampling location is required. Surveying an entire port is typically infeasible, so representative sublocations within each port were selected for sampling (following National System for the Prevention and Management of Marine Pest Incursions 2010b), and the MDeT used to calculate sample numbers per sublocation.

2.1. Target species and MDeT species data

The surveys were designed considering the seven target species of relevance for ballast water management:

- 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*, also known as *Magallana gigas*)
- Basket Shell Clam (*Corbula gibba*)

Molecular (qPCR) assays have been developed by SARDI for all of these species (Ophel-Keller *et al.* 2007; Bott *et al.* 2010a; Bott and Giblot-Ducray 2011a, b, 2012). The National System surveys, for which the MDeT was built, targeted 55 species (National System for the Prevention and Management of Marine Pest Incursions 2010b), including these seven. The default MDeT is populated with data on all 55 species. Data for other species was deleted so that only the seven

species of current interest were used in the design calculations, but the MDeT species data for the current seven target species was not modified.

The 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 target population sizes. 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. The full MDeT species data used is provided in Wiltshire and Deveney (2017). Since the current survey specifically targets the planktonic stage (gametes/larvae) of species development, the applicable habitat to sample is the water column, the volume of which is calculated within MDeT using the area of subtidal habitat and average water depth. Calculated sample numbers are higher for larger locations 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. The water body residence time, provided in the location data, is also included in calculations for planktonic stages, with a shorter residence time resulting in higher sample numbers since this is considered to reduce likelihood of detection due to propagules not being retained within the area. Full detail of the calculations used in the MDeT is included in the MDeT instructions (provided as appendix in Wiltshire and Deveney 2017). Species data is lastly used to flag any species that may be intolerant of the temperature or salinity at each location. The MDeT lists these species and automatically excludes them from sample number calculations for locations where their tolerance limits are exceeded. For each location, the final sample number shown is the highest across the seven target species. *Corbula gibba* was retained in the MDeT for the purpose of design even though the current assay for this species was not used pending redesign, since collected samples may later be tested with the redesigned assay.

2.2. Ports data

Ports surveyed in 2018–2019 were Northern Territory: Gove, Queensland: Weipa, Hay Point, Tasmania: Devonport, and New South Wales: Newcastle, Botany Bay, and Port Kembla. At each port, wharves where ballast exchange is likely to occur were targeted for surveillance. A 250 m buffer around wharves was used to define the outer extent of each location.

For each location, the data required for MDeT were obtained. Hydrological data needed for the MDeT include temperature and salinity ranges for each location, to determine whether these are outside the tolerances of any of the target species, and data used in sample calculations for the plankton tow method: subtidal substrate area, water depth, and residence time (Table 1). Hydrological data were compiled from a literature review for each port, with the data sources used shown in Table 1. The planar surface area of submerged substrate was determined in ArcGIS 10.6 using rasterized versions of hydrographic charts (AusGeoTIFF) obtained from the Australian Hydrographic Office (hydro.gov.au). As detailed depth data was not available, the depth for each location was taken as the dredged wharf depth (or average depth for locations with multiple wharves). Location data used in the MDeT is shown in Table 2.

Table 1. Sources for hydrological data used to populate the MDeT for each port.

Port	Data	Source
All	Seasonal temperatures	http://seatemperature.org/australia-pacific/australia
	Tidal ranges	http://www.bom.gov.au/oceanography/projects/ntc/ntc.shtml
Gove	Residence time, Salinity	Wolanski *et al.* (2013)
Weipa	Residence time	Heggie and Skyring (1999)
	Salinity	Biofouling Solutions (2015)
Hay Point	Residence time, Salinity	GHD (2005)
Newcastle	Residence time, Salinity	Roper *et al.* (2011)
Botany Bay	Residence time, Salinity	Roper *et al.* (2011)
Port Kembla	Residence time, Salinity	Roper *et al.* (2011)
Devonport	Residence time, Salinity	Hydro Tasmania (2007)

2.3. Sample numbers and locations

For each location, sample numbers were calculated using the default MDeT predicted survey sensitivity of 0.8, and for a predicted sensitivity of 0.6, as applied in the field validation project (Wiltshire *et al.* 2019a). The generated sample numbers were divided by four and rounded up to the next whole number to give the required number of samples per sample set, based on four seasonal sampling events, as per Wiltshire *et al.* (2019a). For Hay Point, where the calculated sample number was very high due to the size of the location and short residence time (Table 2), the total number was divided by six rather than four, since the best approach to detect a target that may vary temporally in abundance is to sample more frequently, rather than to collect a larger number of samples at fewer time points. Only two sample sets were, however, collected at each

port, hence Hay Point was sampled at a lower sensitivity than the other ports. The sample sets were collected in summer and autumn, based on these seasons providing the highest detection likelihood across the target species, at least in temperate locations (Wiltshire *et al.* 2019a). The total sample numbers generated by MDeT for each location are shown in Table 3.

Table 2. Location data used in the MDeT for each port.

Port Sublocation	**Depth (m)**	**Tidal Range (m)**	**Residence Time (days)**	**Area subtidal soft substrate (m^2)**
Gove		2.2	50	
Bulk Wharf	14			379 980
Cargo Wharf	10			183 830
Perkins Wharf	5			121 531
Weipa		2.2	100	
Evans Landing	12			137 388
Humbug Point	9			144 264
Lorim Point	12			285 788
Hay Point	15	3.6	2	1 516 710
Newcastle		1.9	20	
Kooragang-Walsh Precincts	15			1 136 853
Mayfield Precinct	15			250 331
Dyke Wharves	15			306 244
Carrington Precinct	15			305 030
Botany Bay		1.3	40	
Port Botany	16			1 136 853
Kurnell Wharf	13			250 331
Port Kembla		1.3	21	
Inner Harbour	15			1 315 770
Outer Harbour	11			277 159
Devonport	7	2.5	3	460 710

In all cases the number of plankton samples processed and analysed from each location was that based on MDeT calculated sensitivity of 0.6, but additional samples were collected in some cases. In autumn 2018 sampling of Gove, Weipa, Hay Point and Devonport, and summer 2019 sampling of Gove, Weipa and Devonport, the total number of samples collected at each port was that based on MDeT sensitivity of 0.8. For all other sampling events, all collected samples were processed.

To assign proposed sample locations for plankton tows, we used the *samplePts* function from the *R* (R Core Team 2019) package *geospt* (Melo et al. 2012) to generate the required number of sample points using a hexagonal grid over the area of subtidal substrate for each location. The area within which points were generated was derived from a shapefile of the subtidal habitat as

used in MDeT calculations, but with a 50 m buffer around wharves and the shoreline (low tide mark) to reduce the likelihood of sample locations falling within areas that would be infeasible or inaccessible to sample. Where the number of samples to process was fewer than the number collected in a location, a subset of the samples were selected for processing. This subset was selected by choosing a subset of proposed sample points that were as evenly distributed throughout the sampling area as possible. Proposed sample locations are shown in Figures 1–4.

Table 3. MDeT generated sample numbers and proposed numbers per sample set for each port for MDeT calculated sensitivities of 0.8 and 0.6. Total number is the number predicted by the MDeT. Numbers per sample set were calculated assuming the total sample number would be collected over four approximately seasonal sample sets except for Hay Point, where bimonthly sampling (6 sets per year) was assumed.

	MDeT sensitivity = 0.8		**MDeT sensitivity = 0.6**	
Port Sublocation	**Total**	**Per sample set**	**Total**	**Per sample set**
Gove				
Bulk Wharf	57	15	32	9
Cargo Wharf	20	5	12	4
Perkins Wharf	7	2	5	2
Weipa				
Evans Landing	18	5	10	4
Humbug Point	14	4	8	3
Lorim Point	37	10	21	8
Hay Point	840	140	478	80
Newcastle				
Kooragang-Walsh Precincts	180	45	103	26
Mayfield Precinct	40	10	23	6
Dyke Wharves	49	13	28	7
Carrington Precinct	49	13	28	7
Botany Bay				
Port Botany	230	58	131	33
Kurnell Wharf	58	15	33	9
Port Kembla				
Inner Harbour	208	52	119	30
Outer Harbour	33	9	19	5
Devonport	80	20	46	15

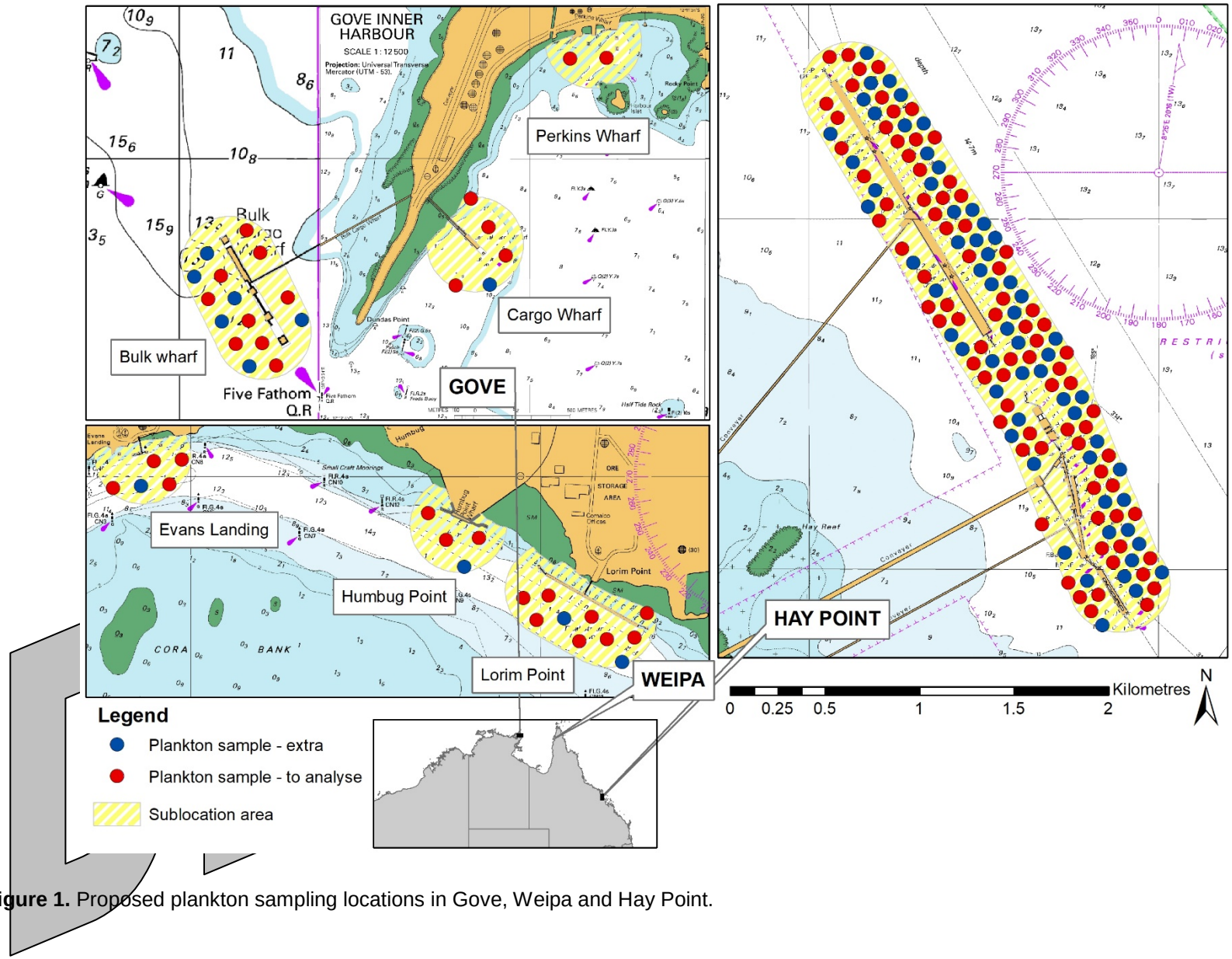

Figure 1. Proposed plankton sampling locations in Gove, Weipa and Hay Point.

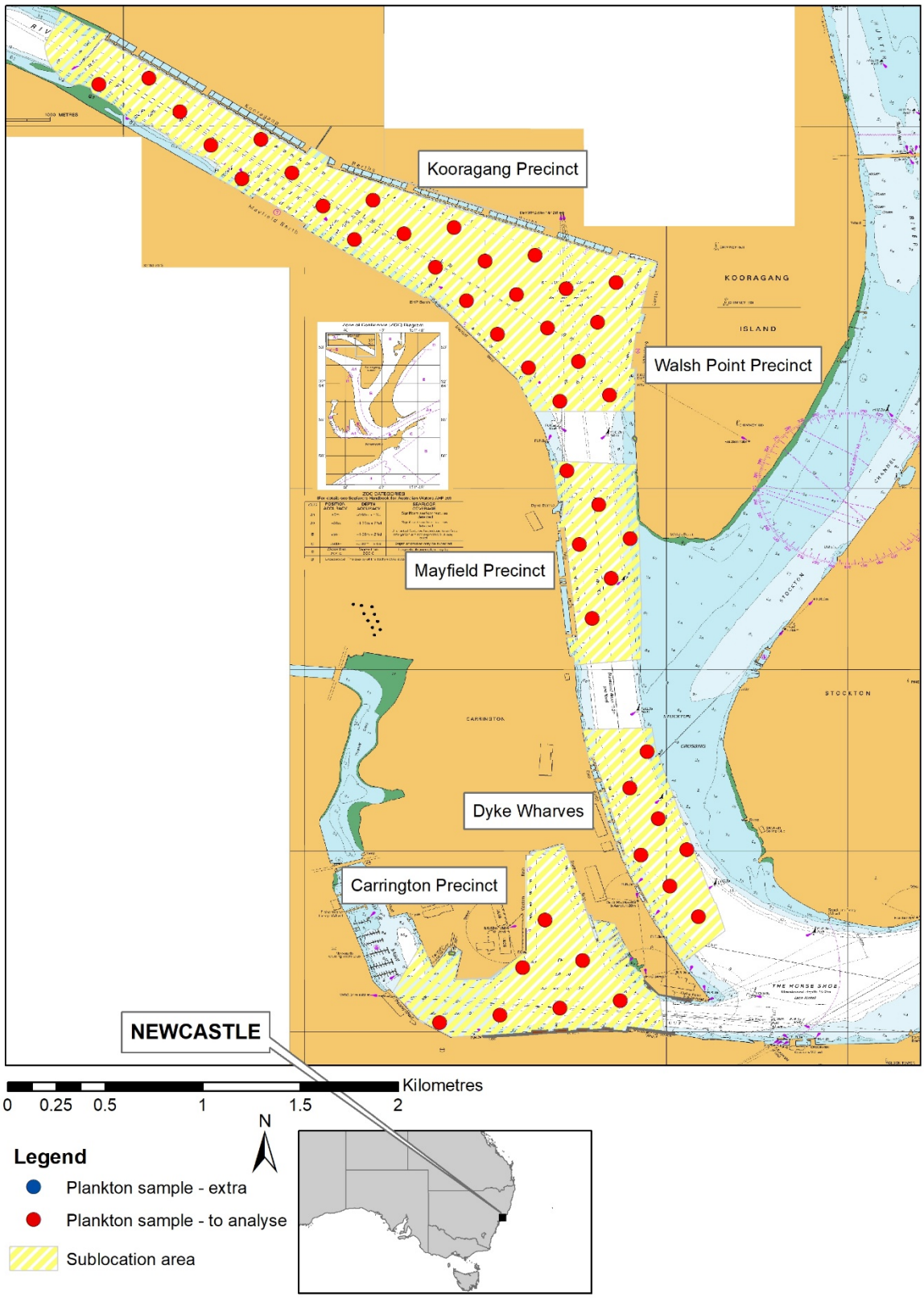

Figure 2. Proposed plankton sampling locations in Newcastle

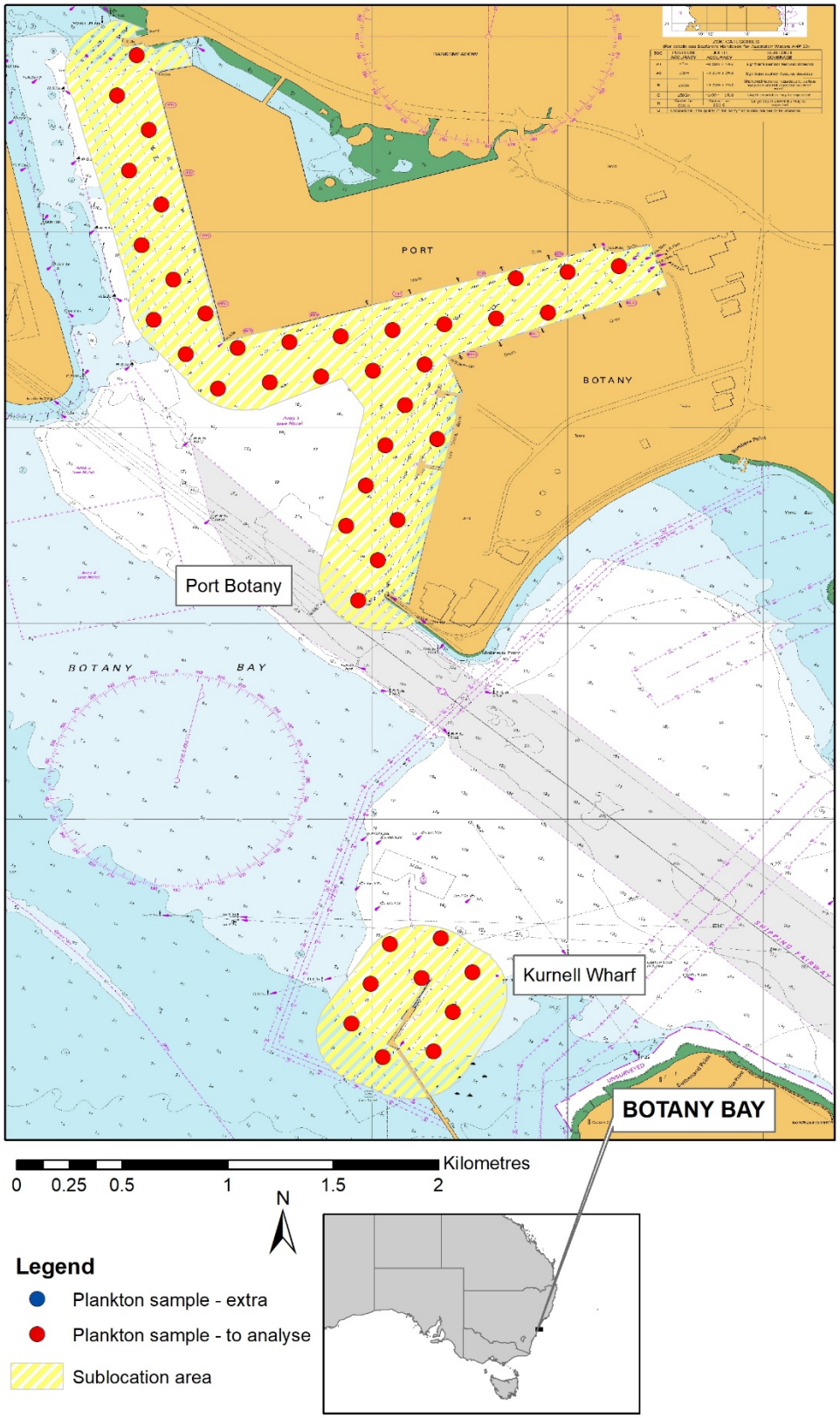

Figure 3. Proposed plankton sampling locations in Botany Bay

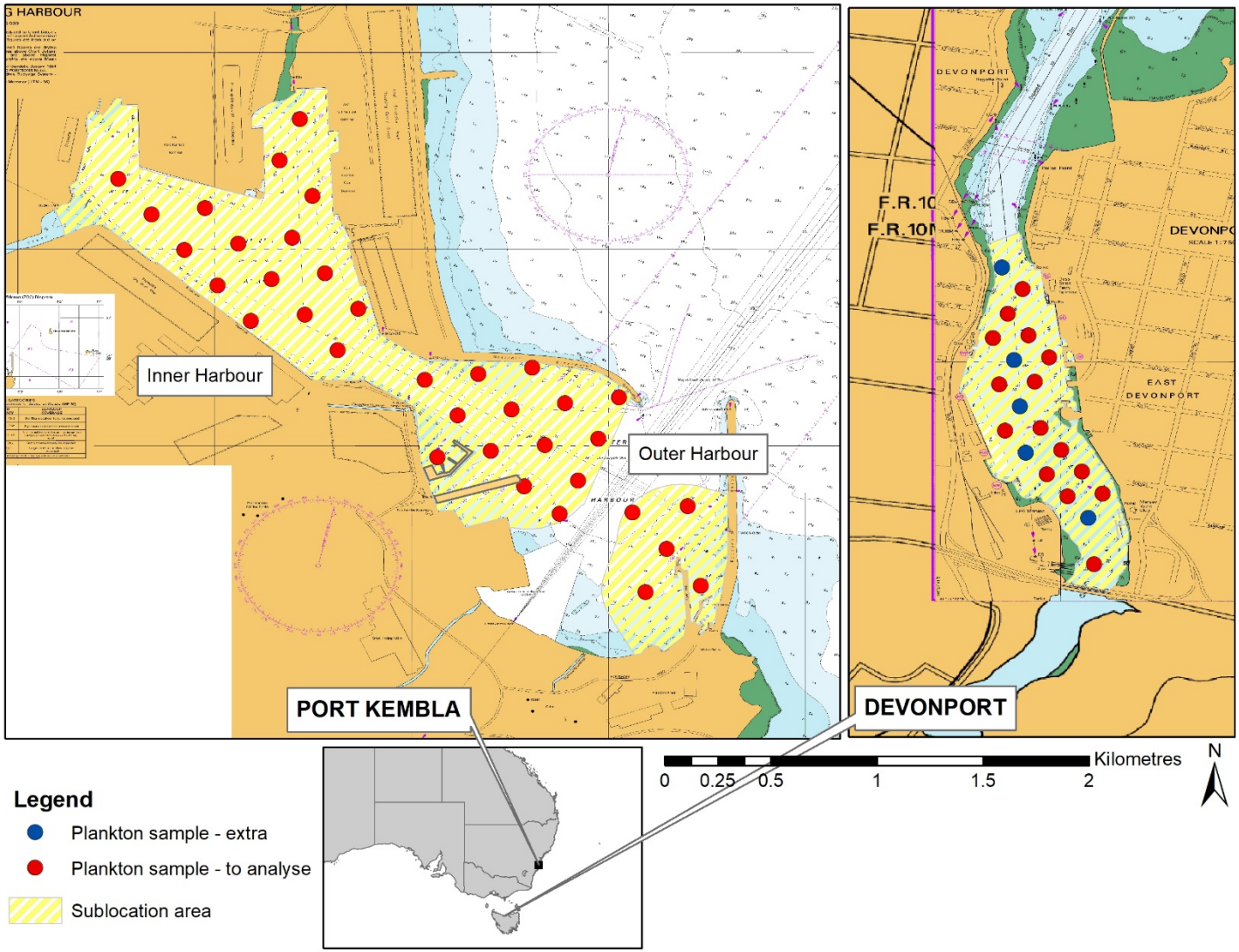

Figure 4. Proposed plankton sampling locations in Port Kembla and Devonport.

2.4. Plankton tow collection

Plankton samples for molecular analysis were collected based on the methods developed by Giblot-Ducray and Bott (2013) and refined by the ATCMP project (Deveney *et al.* 2017). A conical mesh plankton net with mouth diameter 0.5 m, length 1.5 m and 50 µm mesh (Aquatic Research Instruments AQ-150-50-50 or Sea-Gear 90-50x3-50) fitted with a flowmeter (Sea-Gear MF315) was towed behind a vessel at a speed of ~1–1.5 m s^{-1} and depth of 0.5–1 m for a target distance of 100 m. After collection, plankton samples were concentrated down to a volume of ~40 mL by filtering through the mesh windows of the plankton net cod end and transferred to 120 mL tubes containing 80 mL sulfate based preservation buffer (similar to Stanford University 2015). Samples were kept cool in an insulated container with gel ice packs or refrigerator after collection and for overnight delivery to the South Australian Aquatic Sciences Centre (SAASC) where they were stored in a cool room at ≤ 4 °C until processing (see section 2.5).

Proposed sampling locations (see section 2.3) could be altered if necessary due to field conditions, or access or logistical restraints. The final selection of sample locations was at the discretion of the third-party field sampling organisation, and actual sample locations, including plankton tow start and end points, were recorded. Field sampling and related data collection was conducted by Jacobs Group (Australia) Pty Ltd (Jacobs) for the autumn 2018 sample set and by Cardno (NSW/ACT) Pty Ltd (Cardno) for the 2019 summer and autumn sample sets. Field data comprised sampling waypoints, plankton tow flowmeter readings, and notes pertaining to field conditions and individual samples. Sample tube identifiers and flowmeter readings were recorded on hard copy data sheets, and waypoints were recorded using either a handheld or the vessel GPS. All field data were provided to SARDI for compilation.

2.5. Processing and analysis of molecular samples

The plankton samples selected for processing from each sampling event were filtered in the laboratory at SAASC using a manifold and sterile single-use filter cups with 0.45 µm filters (Thermo Scientific™ Nalgene™). Filter papers were transferred to 50 mL centrifuge tubes, frozen at −20 °C and freeze dried until completely dehydrated prior to DNA extraction. DNA extraction and qPCR analysis were carried out by the SARDI Root Disease Testing System laboratory (RDTS). DNA was extracted from samples using the method developed by SARDI Molecular Diagnostics, with 20 mL of DNA extraction buffer containing an internal control (exogenous organism added to each sample at a standardised amount) added to each sample before physical disruption (Ophel-Keller *et al.* 2008). The efficiency and consistency of SARDI's method to extract

DNA from environmental samples has been confirmed in comparison to commercial methods (Haling *et al.* 2011). Final elution volume of the DNA was 160 µL in elution buffer. Each DNA extract was then tested in singleplex quantitative polymerase chain reaction (qPCR) performed on QuantStudio7 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SARDI developed assays for the target marine pests (Table 4) plus two exogenous organisms that were added to samples as controls. Assays are referred to hereafter by the genus name of the target.

Table 4. Target pest species, assay gene target, and reference for assay.

Species	Gene target	Reference
Arcuatula senhousia	28S rDNA	Bott and Giblot-Ducray (2011)
Asterias amurensis	Cox1	Bott et al. (2010a)
Carcinus maenas	Cox1	Bott et al. (2010a)
Crassostrea gigas	Cox1	Bott and Giblot-Ducray (2012)
Sabella spallanzanii	28S rDNA	Ophel-Keller et al. (2007)
Undaria pinnatifida	Cox1	Bott et al. (2010a)

Testing included negative controls and the appropriate calibration standard for each target pest, except for *Sabella*, for which a positive control was used instead of calibration standards, since a standard curve had not been developed for this assay. One of the additional controls was a sampling quality assurance control to test for sample degradation post-collection. The sampling quality assurance control was a 50 µL aliquot of *Artemia salina* (Ocean Nutrition™ Instant Baby Brine Shrimp; hereafter *Artemia*) which was added to the preservation buffer of a subset of sample tubes prior to sampling. *Artemia* yield from these samples was determined using an *Artemia* qPCR assay (Mackie and Geller 2010) and compared to that of laboratory control samples, which consisted of a 50 µL aliquot of *Artemia* in preservation buffer and stored at ≤ 4 °C in the laboratory until processing. The other additional control was used to assess PCR inhibition. The inhibition control consisted of an exogenous organism added to each sample at a standardised amount prior to extraction. For each PCR analysis batch, reference samples that are known to not cause inhibition were also extracted and tested by qPCR after addition of the inhibition control organism. A scaling factor was calculated for each plankton sample by comparing the yield of inhibition control DNA detected in that sample to that in the reference samples. For those assays with a standard curve (all target pests, except *Sabella*), the scale factor for a sample is used as a multiplier to correct the apparent DNA concentration as calculated from the cycle threshold (C_T) value for the effects of inhibition (Ophel-Keller *et al.* 2008).

Four of the surplus samples collected from Devonport in autumn 2018 were subsequently processed and analysed as part of a project investigating diagnostic performance of the SARDI

qPCR assays (Wiltshire *et al.* 2019b). Analysis of these samples followed the method applied to other samples with the exception of inhibition assessment, which was not performed, i.e. the inhibition control was not added to samples, and no scale factor was calculated.

To avoid cross-contamination, samples from different locations were processed on different days, and all benchtops and apparatus, including freeze-drier shelving, were decontaminated using LookOut® DNA Erase between sample sets.

2.6. Mapping and statistical methods

Field data collected by Jacobs and Cardno during plankton sampling, and molecular analysis results from the RDTS, were compiled with qPCR results linked to recorded field sampling locations. During field sampling, the length of plankton tows were determined based on GPS coordinates; however, coordinates marking the end point of tows during the autumn 2018 sampling were not always recorded accurately. Actual tow length calculation was compromised for this sample set, and was not considered as a factor in analyses. For results presented in this report, locations of autumn 2018 plankton samples were mapped based on sample start point, while for summer and autumn 2019, tow midpoint, calculated using the average of start and end point latitude and longitude, was used to map tow locations.

2.6.1. Relative field DNA

The cycle threshold (C_T) results from qPCR give a relative measure of the concentration of target DNA present in the sample, with lower C_T values corresponding to exponentially higher sample DNA. Standard curves, which allow the yield of target DNA to be determined from the C_T value of a positive detection, are available for the controls and most of the pest assays but not *Sabella*. For the purpose of the current project, knowing the actual pest DNA yield in a sample is of limited importance, but we were interested in how relative DNA concentration varied between sample sets. A measure of relative DNA (relDNA) for each sample was calculated from the C_T value for each pest detection as: $relDNA = \exp[(45 - C_T) \times \ln(2)]$, and relDNA was considered to be zero for non-detections. This measure of relative DNA is equal to one for a C_T value of 45, this being the maximum number of PCR cycles run during the molecular analyses.

Rasters of interpolated relative DNA were generated for each pest with at least 5 detections in a sample set, with the exception of *Arcuatula* in tropical locations. It is not currently confirmed whether these detections are of the target *A. senhousia* or the result of a cross-reaction with a native related species (Wiltshire *et al.* 2019a), hence, further analysis of these data was not

performed. The calculated relative pest DNA in a sample will be less than the actual DNA yield where PCR inhibition is present. For the purpose of mapping, calculated relative DNA was multiplied by the scaling factor for each sample to give scaled relative DNA for each detection, but it should be noted that it is unclear whether the scaling response is linear for scaling factors above ~2. The four additional autumn 2018 Devonport samples which were analysed by Wiltshire *et al.* (2019b) did not have inhibition assessed and so were not included in the data used to generate maps of relative DNA. The volume of water filtered by the plankton net is needed to relate DNA present in a sample to field DNA concentration. Volume filtered is directly proportional to flow meter distance since the same specification net was used for all samples. Relative field DNA was calculated as scaled relative DNA divided by flow meter distance. Interpolation was based on inverse distance weighting accounting for barriers (coastline) using the *R* package *ipdw* (Stachelek 2018). Maps of interpolated DNA and summarizing detections per method were generated using ArcGIS 10.6 (Esri Inc).

2.6.2. Estimated true prevalence

Diagnostic performance of five of the qPCR assays applied in the current project (each species except *Arcuatula*) was recently assessed (Wiltshire *et al.* 2019b). Knowledge of test diagnostic performance aids the interpretation of survey results, allowing estimation of true prevalence (frequency of target DNA occurrence in samples) from the number of positive and negative test results in each case. The relationship between target DNA prevalence in plankton and adult pest population size is unknown, but estimation of true prevalence allows standardised comparison of results across species where test performance is not identical, and, where a target is not detected, estimation of the maximum prevalence that may be present. True prevalence for each species, except *Arcuatula*, at each port was determined in a Bayesian framework using the combined results across both surveys and the *truPrev* function of the *prevalence R* package (Devleesschauwer *et al.* 2014). Diagnostic sensitivity (DSe) and specificity (DSp) of each species assay used in the prevalence estimation were taken from Wiltshire *et al.* (2019b) using the lowest (most conservative) estimate in each case.

2.6.3. Temporal patterns of molecular detections and relative DNA

For *Crassostrea*, which was detected in both sample sets at several ports, patterns in likelihood of qPCR detection and relative DNA were analysed across sample sets and ports where detections occurred. For other species with detections across both sample sets at a port, the

effect of sample set on likelihood and detection and relative DNA was analysed using data from that port. Bayesian zero-altered lognormal (ZALN) models were run following Zuur and Ieno (2016) and including predictors for both the binary component, i.e. likelihood of detection, and continuous component, i.e. relDNA, which was calculated as in section 2.6.1.

Two models were run for each species, one to predict relative field DNA concentration, i.e. correcting for the effect of varying sample volume to determine DNA concentration per unit volume in the field, and one to predict the effective DNA yield in samples between sample sets. The apparent relative DNA in a sample as calculated from the C_T value may be less than the actual DNA present where PCR inhibition is present. The total relative DNA in a sample is given by: sampDNA = relDNA x Scale Factor. The Scale Factor, however, is calculated based on DNA yield of the internal control (see section 2.5), and it is possible that the response of pest DNA to inhibition varies so that Scale Factor may not be a perfect multiplier. The total sample DNA relates to the relative field DNA concentration as: sampDNA = fieldDNA x sample volume. Flow meter distance was used to represent volume as per section 2.6.1. For the continuous component of the ZALN, the logarithm of the response (sampDNA or fieldDNA) is taken to be normally distributed, with mean η dependent on predictors (see below), and variance σ. The models used were therefore:

(1) log(sampDNA) ~ $N(\eta, \sigma)$
hence: log(relDNA) ~ $N(\eta, \sigma) - \beta_{SF}$ x log(Scale Factor)

(2) log(fieldDNA) ~ $N(\eta, \sigma)$
hence: log(relDNA) ~ $N(\eta, \sigma) - \beta_{SF}$ x log(Scale Factor) + log(Flowmeter)

Where β_{SF} is a coefficient allowing for variation in the response of measured DNA to inhibition. Both options were run as zero-altered models, but the inclusion of the flowmeter offset does not affect predictions for the binary component; this was confirmed by examining model outputs. The binary component is therefore presented only once per species. Diffuse normal priors were used for coefficients for sample set and, in the *Crassostrea* model, for port and port x sample set for both model components. An informative normal prior with mean of one and standard deviation of 0.5 was used for β_{SF}. This prior reflects the expectation that pest DNA will respond to inhibition in the same way as the internal control, but allowing the multiplier to vary slightly from 1, specifically, this prior indicates 95% probability that the scale factor multiplier is between 0.02 and 1.98.

These analyses were run in *R* (R Core Team 2019) using package *rjags* (Plummer 2018) with 20,000 MCMC iterations thinned at a rate of 20, following 10,000 iterations for adaptation, and 70,000 for burn-in. Convergence was assessed by Gelman-Rubin convergence statistic, and confirmed by visual inspection of trace, density and autocorrelation plots generated using the *MCMCplots* package (McKay Curtis 2015). To visualise results, predictions for the models were made using mean flow meter distance for each port and season combination and scale factor = 1 (no inhibition).

2.7. Devonport traditional survey

Following molecular detections of *Asterias* in Devonport in autumn 2018 samples (see section 3.2), a follow up traditional survey was designed since this species is not previously recorded from this location. Trawl and diver visual surveys are appropriate for detection of *Asterias.* The number of samples proposed for these sample types were generated using the MDeT with the default survey sensitivity of 0.8, and also a survey sensitivty of 0.6, following the methods used by Wiltshire and Deveney (2017) and the port data originally collated to design molecular surveys (see section 2.2). Sample numbers were based on trawls being conducted with a 1 m wide trawl and 50 m long transect, while numbers for dives were calculated based on the default 100 m long transect and also for a 50 m transect, in both cases assuming visibility of 1 m. Two different dive survey lengths were applied since conducting a larger number of shorter transects is likely to increase the chance of detecting highly aggregated species, compared with fewer, longer transects. Generated sample numbers are shown in Table 5.

Table 5. Required trawl and dive sample numbers from MDeT for survey sensitivity (S_{SE}) of 0.6 and 0.8 for detection of *Asterias amurensis* in Devonport.

	Number of samples for MDeT sensitivity:	
Sample type	**0.6**	**0.8**
Trawl (50 m)	5	8
Dive (100 m)	3	5
Dive (50 m)	5	9

Proposed locations for trawl sampling were generated using a regular grid over the area of subtidal soft substrate in Devonport, using the same method as for plankton sample locations (section 2.3). In the 2017–2018 traditional surveys, visual surveys aimed primarily to detect target pests that occur on hard substrate (e.g. *Sabella, Crassostrea*), while trawl sampling was the

selected method targeting *Asterias*, which is associated with soft substrate (Wiltshire and Deveney 2017). Despite this, *Asterias* was detected in a high proportion of visual samples during the surveys of locations with established populations: Melbourne and Hobart (Wiltshire *et al.* 2019a), indicating that, while associated with soft sediment, this species is likely to aggregate around wharves and other structure. Aggregation of *Asterias* around wharves has also been observed in the Derwent estuary, Hobart (Whitehead 2008). Proposed locations for dive surveys in Devonport were chosen to be around wharves, but with the expectation that the focus of the surveys should be soft sediment areas around and beneath wharves, rather than wharf structures themselves. The proposed sampling locations are shown in Figure 5.

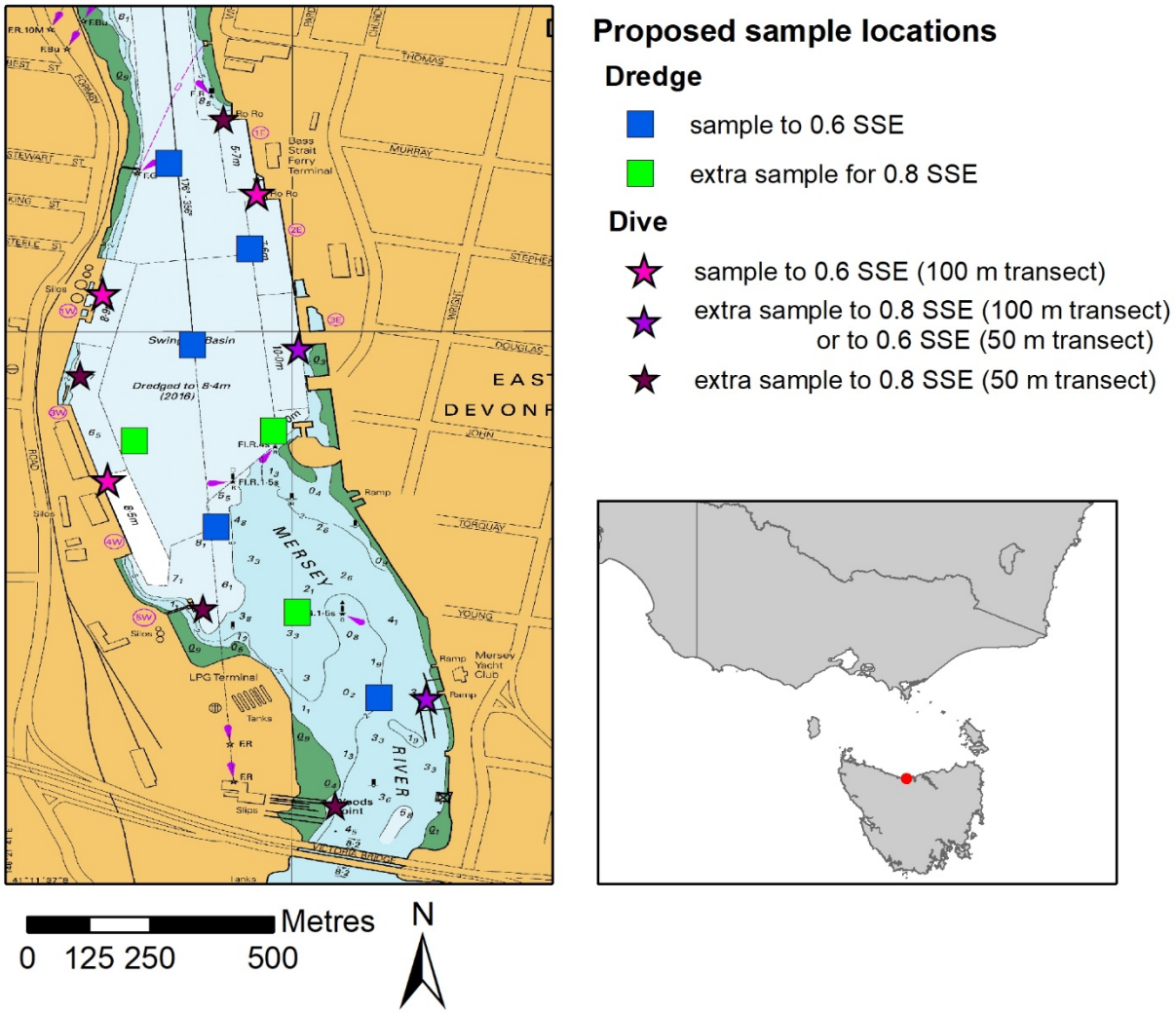

Figure 5. Proposed dredge and dive sampling locations for Devonport *Asterias* survey for MDeT calculated survey sensitivity (SSE) of 0.6 or 0.8.

Trawl and diver visual surveys in Devonport were conducted by Marine Solutions Tasmania Pty Ltd on 5th June 2019 (Marine Solutions 2019), using the sample numbers calculated using an MDeT sensitivity of 0.8 and proposed dive transect length of 50 m, i.e. 8 trawls and 9 dives. Trawls were collected with a modified KC rectangular dredge, with mouth width of 1 m (Marine Solutions 2019).

3. RESULTS

3.1. Samples collected and analysed

The number of samples collected and analysed in each sample set and dates of sample collection are shown in Table 6. The number of samples collected in autumn 2018 in Gove was 20 rather than the 22 proposed due to equipment failure which prevented the collection of the final two samples from the cargo wharf sublocation. Three samples were collected from this sublocation, but four were proposed to be analysed, therefore an additional sample from the Bulk wharf sublocation was analysed. Due to an error during export of sample location coordinates provided to the third-party organisation collecting the samples, the subset of 80 samples collected in Hay Point during summer 2019 were not evenly spaced, but were a subset of samples concentrated around the southern end of the location (Figure 7).

There was no evidence of sample degradation in any sample set based on the sampling quality control (*Artemia* yield from field samples).

Table 6. Number of plankton samples collected and analysed and sample dates for each sampling event. *15 samples from this set were originally analysed for the current project, with an additional 4 samples analysed by Wiltshire *et al.* (2019b) in January 2019.

Sample set	Port	Collected	Analysed	Start date	End date
Autumn 2018	Gove	20	15	25-May-18	25-May-18
	Weipa	19	15	21-May-18	22-May-18
	Hay Point	140	80	04-Jun-18	11-Jun-18
	Devonport	20	19*	06-Jun-18	06-Jun-18
Summer 2019	Gove	22	15	22- Feb-19	22-Feb-19
	Weipa	19	15	19-Feb-19	19-Feb-19
	Hay Point	80	80	12- Feb-19	14-Feb-19
	Newcastle	46	46	07-Mar-10	07-Mar-19
	Botany Bay	42	42	27-Feb-19	28-Feb-19
	Port Kembla	35	35	05-Mar-10	05-Mar-19
	Devonport	20	15	05-Mar-19	05-Mar-19
Autumn 2019	Newcastle	46	46	16-May-19	16-May-19
	Botany Bay	42	42	20-May-19	21-May-19
	Port Kembla	35	35	14-May-19	14-May-19

3.2. Species detections by qPCR and patterns in relative DNA

In Devonport autumn sampling conducted in 2018, *Carcinus, Crassostrea* and *Asterias* were detected, while in summer 2019 sampling, there were detections of *Carcinus, Crassostrea* and *Arcuatula* (Table 7; Figures 6–7). These species are recorded from this port (Thomson 1959; DPIPWE 2014, 2015a, c) with the exception of *Asterias*. *Sabella* has also been recorded in Devonport, although with low abundance (DPIPWE 2015b). The known range of *Asterias* in Tasmania is along the south east coast from Recherche Bay to Banks Strait, with largest populations occurring in the Derwent River (DPIPWE 2015c). The relatively high proportion of samples in which *Asterias* was detected (7/19 in autumn), with all sample C_T values being <40, suggests that these detections are not of transient material, and that a population of *Asterias* is present in or near Devonport. Estimated true prevalence (frequency of DNA occurrence in plankton samples) of *Asterias* in Devonport across both sample sets was 0.26 (95% credible interval 0.12–0.44) (Table 8). The positive samples from Devonport returned C_T values of 34.2–38.8. Samples collected from Hobart and Melbourne in May 2018 had C_T values of 27.8–39.4, and 21.1–30.7 respectively (Wiltshire *et al.* 2019a). A higher C_T value represents a lower yield of pest DNA, so these results show that relative DNA concentration of *Asterias* in Devonport was within, although at the lower end, of the range recorded in Hobart, and lower than the DNA concentration in Melbourne, although the detections were not low level (considered as $C_T > 40$). A lack of further detections of *Asterias* in summer 2019 sampling is unsurprising because *Asterias* spawns in autumn-winter (Dommisse and Hough 2004) and summer is likely to be a poor season for detection of this species; *Asterias* was rarely detected in summer samples from Hobart or Melbourne in previous molecular surveillance (Wiltshire *et al.* 2019a). Maps of relative DNA concentration show that the highest *Asterias* DNA concentrations were detected in the north of the area surveyed in Devonport, while *Crassostrea* DNA concentrations increased toward the south, and *Carcinus* DNA concentrations were generally lowest in the central area, increasing to the north and south of the survey area (Figure 14). *Crassostrea* showed higher DNA concentrations in summer than autumn in Devonport.

Crassostrea was detected in each of the NSW ports (Newcastle, Botany Bay and Port Kembla) in samples from both summer and autumn 2019 (Table 7; Figures 8–13). *Crassostrea* is recorded from the Hunter River, Newcastle, and from Botany Bay, with large populations present at each of these locations (NSW DPI 2012). Surveillance for this species (and introduced marine species generally) in Port Kembla has not been undertaken since 2000 (Pollard and Pethebridge 2002a). Overall prevalence of *Crassostrea* in plankton was estimated to be higher in Newcastle than

Botany Bay with Port Kembla having intermediate prevalence between these two ports, and similar prevalence to Devonport (Table 8). *Sabella* was also detected in Botany Bay samples from both sample sets with overall prevalence estimated to be 0.25 (95% credible interval 0.15–0.36) (Table 8). This species has been relatively recently recorded from Inscription Point in Botany Bay, approximately 1 km from Kurnell wharf and 2.5 km from Port Botany, although there is a lack of surveillance in the immediate port areas (Murray and Keable 2013; Ahyong *et al.* 2017). There was one qPCR detection of *Sabella* in Port Kembla in the autumn sampling. There is no record of this species in this port, but a lack of recent surveillance. The single detection suggests that this may be a detection of transient material particularly because the C_T was 38.9, i.e. close to being considered low-level. It is also possible that a population is present but at low abundance; estimated true prevalence based on this result is very low (0.03, 95% credible interval 0.00–0.09; Table 8). Low level detections of *Undaria* occurred in each NSW port in summer samples. This species is not recorded in NSW, but the low number and high C_T of the detections suggest these are transient material, and no further detections occurred in autumn sampling. Highest *Crassostrea* DNA concentrations in Newcastle occurred in the Carrington precinct in the south-west of the port area (Figure 15). In Botany Bay, there were areas of high *Crassostrea* DNA in Port Botany and around the Kurnell wharf (Figure 16). In Port Kembla, *Crassostrea* DNA concentrations were high throughout, but somewhat higher in the outer than inner harbor areas (Figure 17). In each NSW port, *Crassostrea* DNA concentrations were higher in summer than autumn (Figures 15–17). In Botany Bay, *Sabella* DNA concentrations were similar in summer and autumn, with areas of high concentration in Port Botany and near Kurnell (Figure 16).

Table 7. Total number of plankton samples, number with high (>5) scale factor (SF), and number of samples with a detection for each species. The number of samples with a low level detection (C_T > 40) is shown in brackets where > 0. *Note: only 15/19 tested samples from this sample set had SF assessed.

Sample Set Port	No of Samples (high SF)	Number of detections (number low level)					
		Arcuatula	*Asterias*	*Carcinus*	*Crassostrea*	*Sabella*	*Undaria*
Autumn 2018							
Gove	15 (0)	4 (3)	0	0	0	0	0
Weipa	15 (0)	12 (12)	0	0	0	0	0
Hay Point	80 (0)	0	0	0	0	0	0
Devonport	19 (0*)	0	7	5	6	0	0
Summer 2019							
Gove	15 (0)	4 (4)	0	0	0	0	0
Weipa	15 (6)	0	0	0	0	0	0
Hay Point	80 (35)	3 (2)	0	0	0	0	0
Newcastle	46 (2)	0	0	0	45	0	1 (1)
Botany Bay	42 (0)	0	0	0	42	10 (5)	1 (1)
Port Kembla	35 (0)	0	0	0	35	1	2 (2)
Devonport	15 (0)	2 (2)	0	9	15	0	0
Autumn 2019							
Newcastle	46 (2)	0	0	0	31 (1)	0	0
Botany Bay	42 (12)	0	0	0	2	7 (5)	0
Port Kembla	35 (7)	0	0	0	10	0	0

Detections of *Arcuatula* occurred in Gove and Weipa samples from autumn 2018, and in Gove and Hay Point samples from summer 2019 (Table 7; Figures 6–7). This species has not been recorded at any of these locations, but previous surveillance targeting this species is lacking. Marine pest surveillance using settlement plates has been conducted in Gove (Cribb *et al.* 2010) and Hay Point (NQBP 2018), but it is unclear whether this is a suitable method for detection of *Arcuatula*, which is primarily associated with soft sediments (Tait and Inglis 2016). Surveys for introduced marine species were conducted in Weipa in 2015, but did not consider *Arcuatula* as one of the target species (Biofouling Solutions 2015), although the methods used (dredging, pile scrapings) collected several bivalve species and hence may also have detected *Arcuatula* if present. It is possible that the *Arcuatula* assay cross-reacts with native relatives (Wiltshire *et al.* 2019a). Investigation of the specificity of this assay is ongoing and results of these investigations will assist in determining whether *Arcuatula* detections in tropical Australia are of the introduced *A. senhousia* or a native relative.

Table 8. Overall estimated prevalence cross sample sets for each species except *Arcuatula* at each port accounting for diagnostic test performance. Shaded cells indicate ports with species detections, excluding those where detections were likely transient material. Prevalence was not estimated for *Arcuatula* since diagnostic performance of this assay has not been assessed.

	Estimated true prevalence (95% credible interval)				
Port	*Asterias*	*Carcinus*	*Crassostrea*	*Sabella*	*Undaria*
Gove	0.04 (0.00–0.11)	0.04 (0.00–0.12)	0.03 (0.00–0.10)	0.04 (0.00–0.11)	0.04 (0.00–0.13)
Weipa	0.04 (0.00–0.11)	0.04 (0.00–0.12)	0.03 (0.00–0.10)	0.04 (0.00–0.11)	0.04 (0.00–0.13)
Hay Point	0.01 (0.00–0.02)	0.01 (0.00–0.02)	0.01 (0.00–0.02)	0.01 (0.00–0.02)	0.01 (0.00–0.03)
Newcastle	0.01 (0.00–0.04)	0.01 (0.00–0.04)	0.90 (0.81–0.98)	0.01 (0.00–0.04)	0.03 (0.00–0.08)
Botany Bay	0.01 (0.00–0.04)	0.01 (0.00–0.04)	0.58 (0.46–0.69)	0.25 (0.15–0.36)	0.03 (0.00–0.09)
Port Kembla	0.02 (0.00–0.05)	0.02 (0.00–0.05)	0.70 (0.58–0.82)	0.03 (0.00–0.09)	0.06 (0.01–0.13)
Devonport	0.26 (0.12–0.44)	0.52 (0.33–0.73)	0.68 (0.50–0.84)	0.03 (0.00–0.09)	0.04 (0.00–0.11)

3.3. Maps of sample locations, species detections and relative DNA

Maps of sample locations and detections are shown in Figures 6–13. Figures are grouped by location and sampling event. All maps show the port locations at the same scale (1:25 000). In each map, sample results are shown with a single point per sample coloured by species detected. Multiple species detections are shown as a pie-chart with a different coloured segment per species and size of chart proportional to the number of species detected, while samples with no detection appear in black.

Maps of relative DNA are shown by location in Figures 14–17. Port locations in these figures are all shown at the scale 1:50 000. Note that maps of relative DNA were only generated where at least five detections of a species occurred in a sampling event.

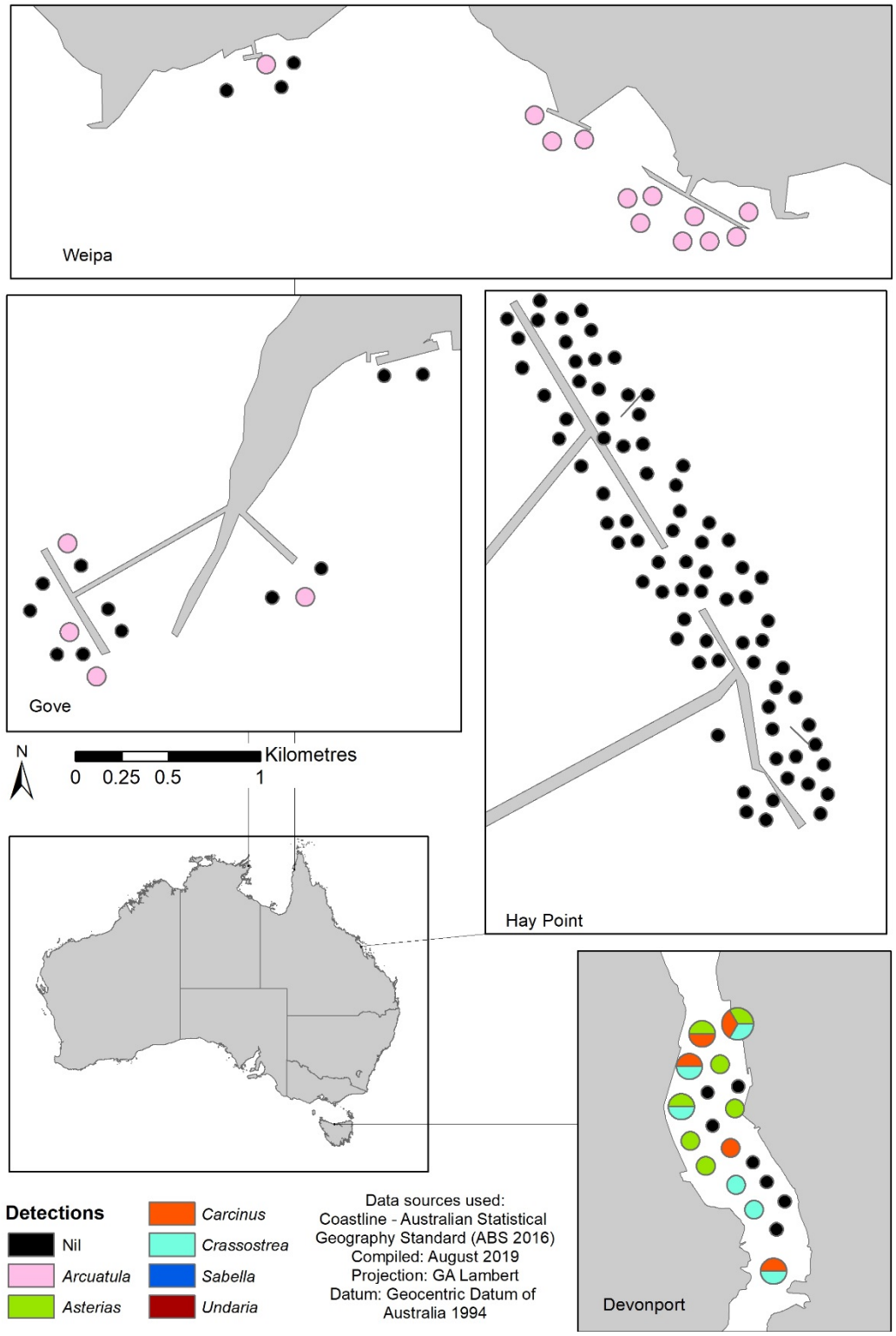

Figure 6. Map of plankton tow locations and molecular detections for Weipa, Gove, Hay Point and Devonport in autumn 2018.

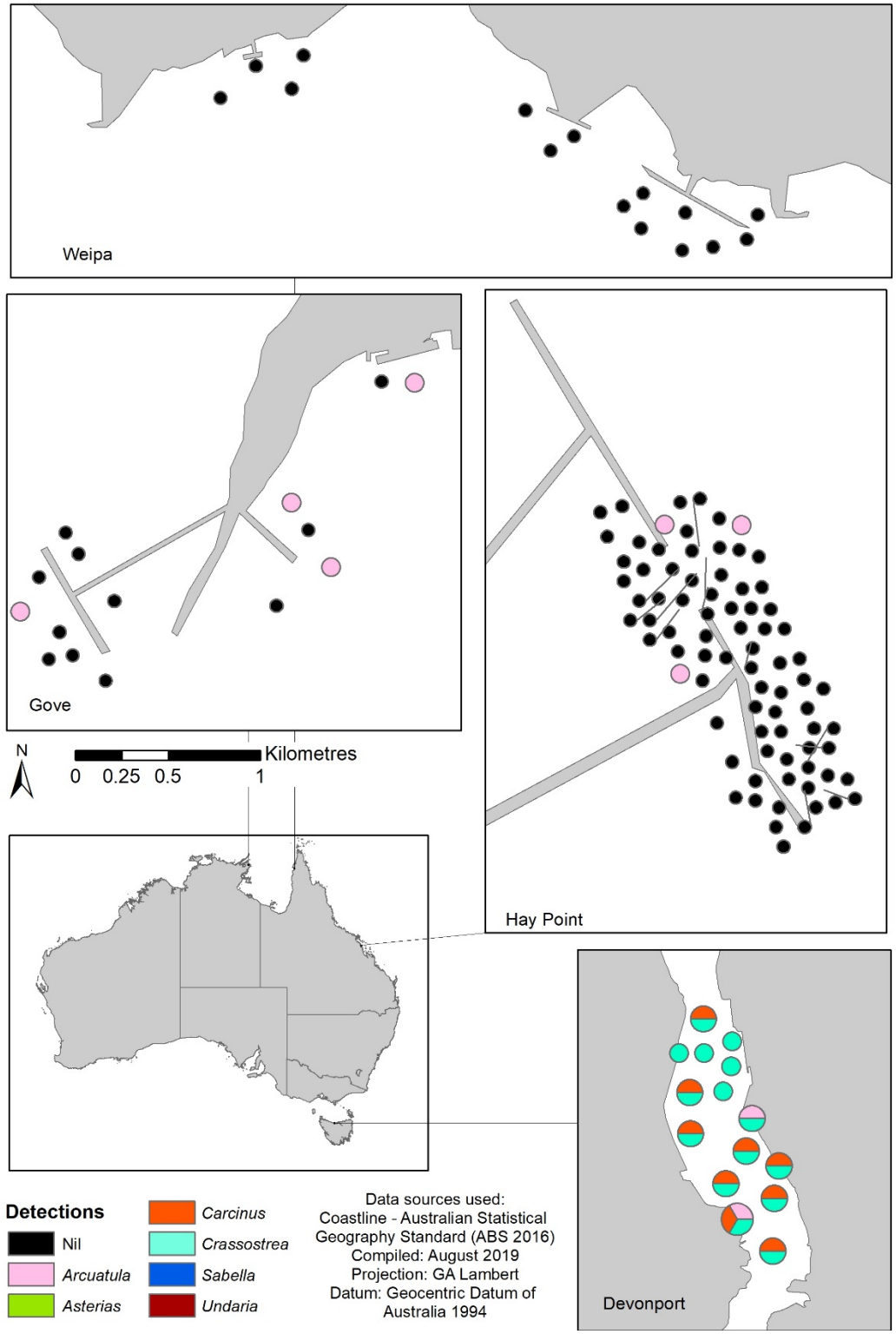

Figure 7. Map of plankton tow locations and molecular detections for Weipa, Gove, Hay Point and Devonport in summer 2018.

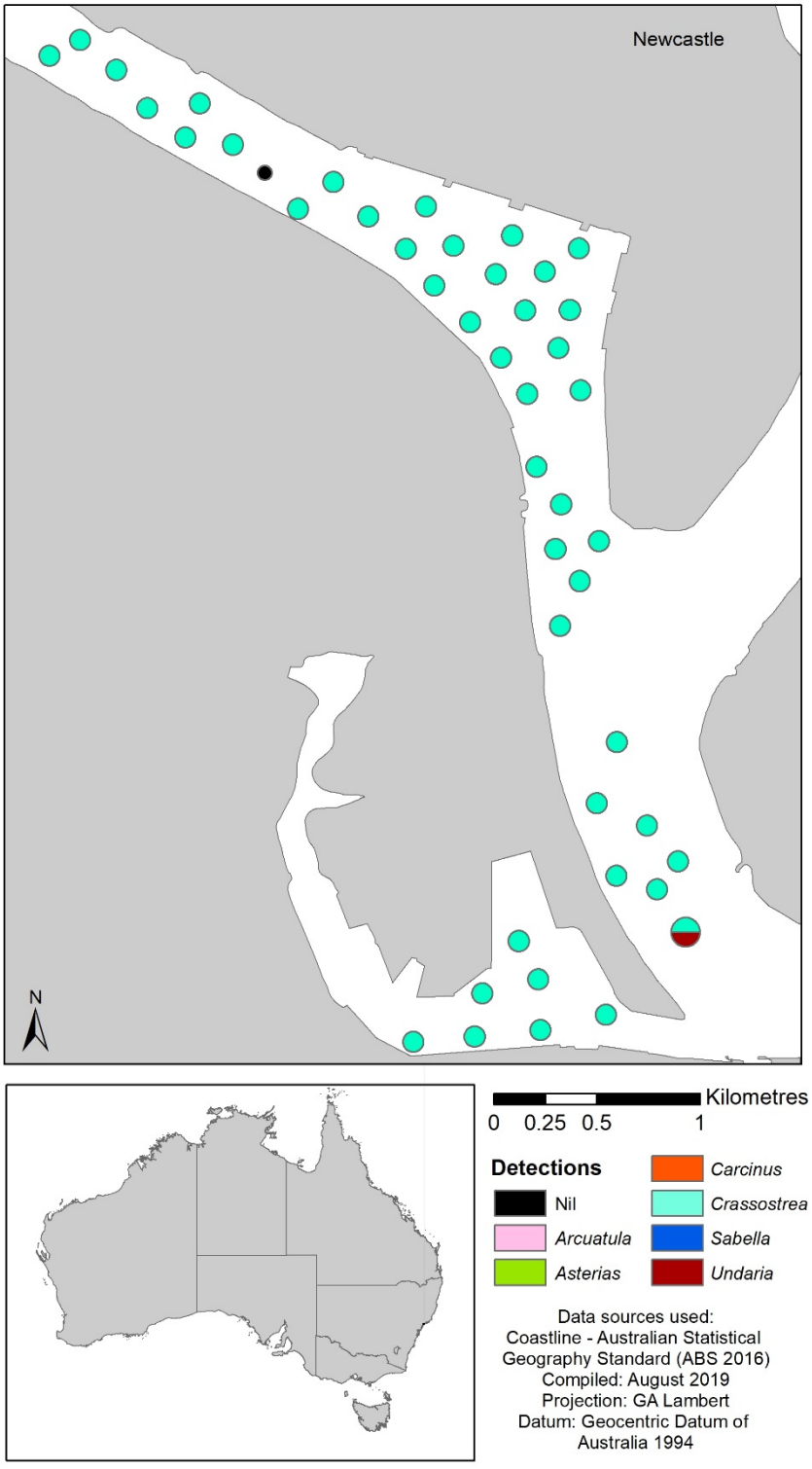

Figure 8. Map of plankton tow locations and molecular detections for Newcastle in summer 2019.

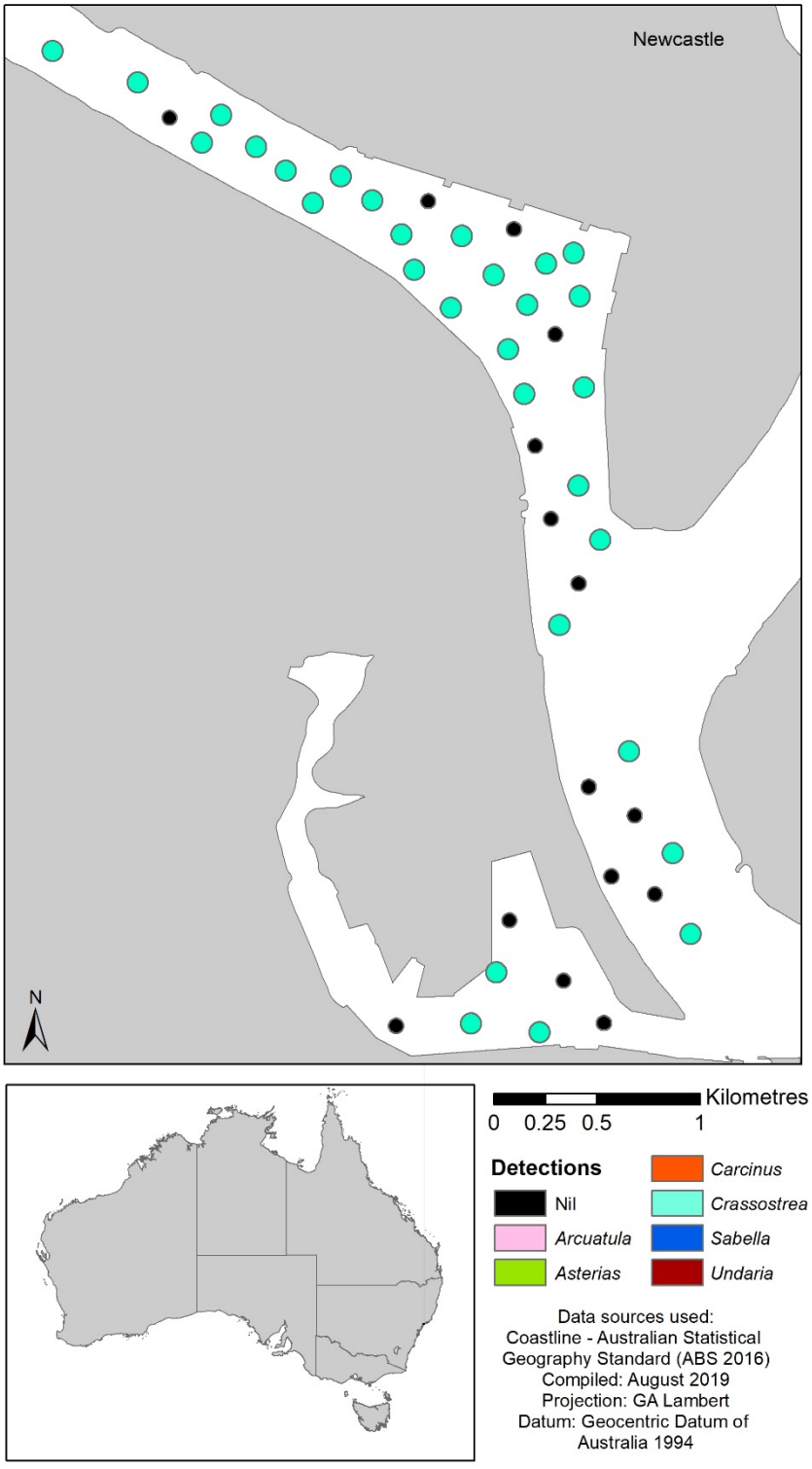

Figure 9. Map of plankton tow locations and molecular detections for Newcastle in autumn 2019

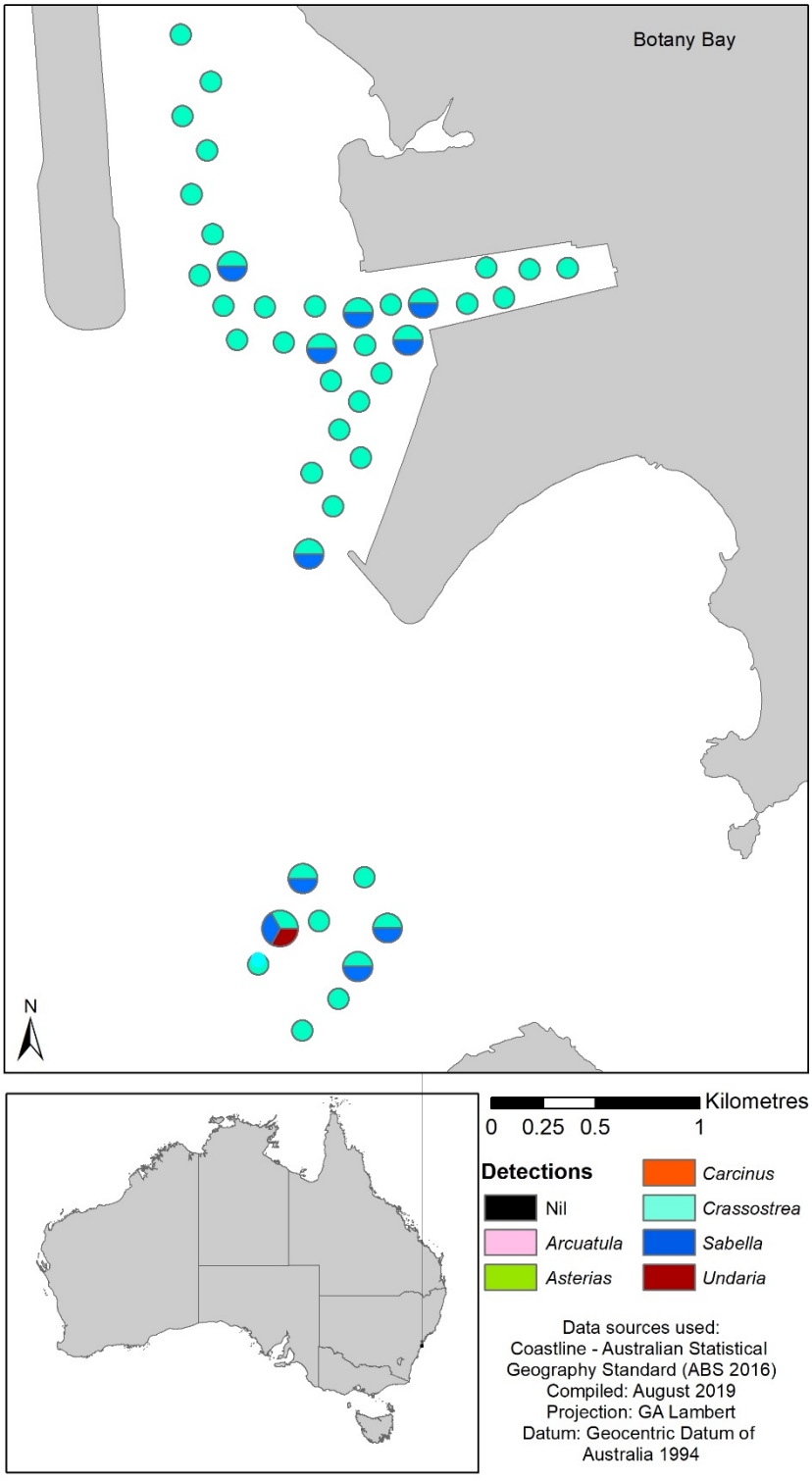

Figure 10. Map of plankton tow locations and molecular detections for Botany Bay in summer 2019.

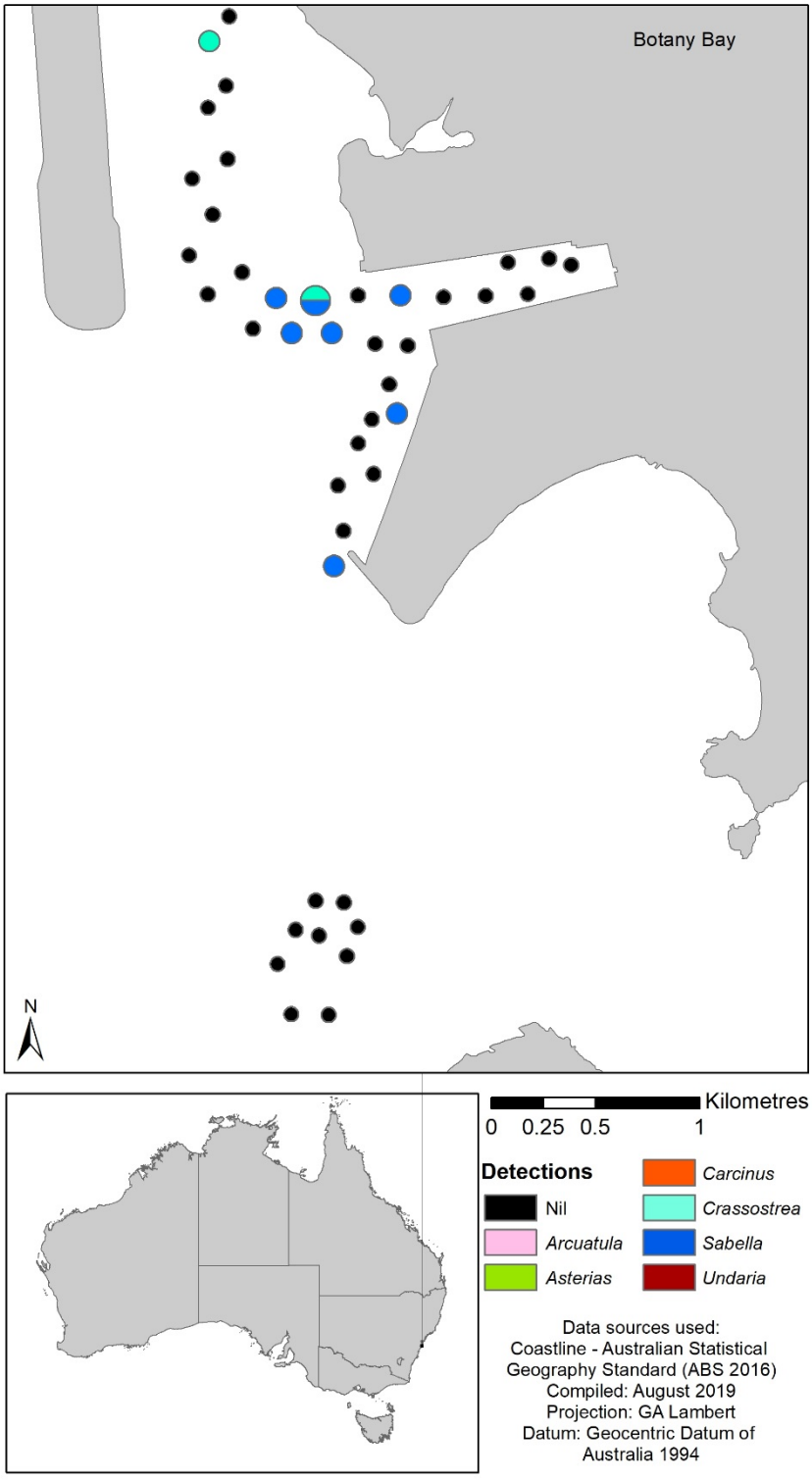

Figure 11. Map of plankton tow locations and molecular detections for Botany Bay in autumn 2019.

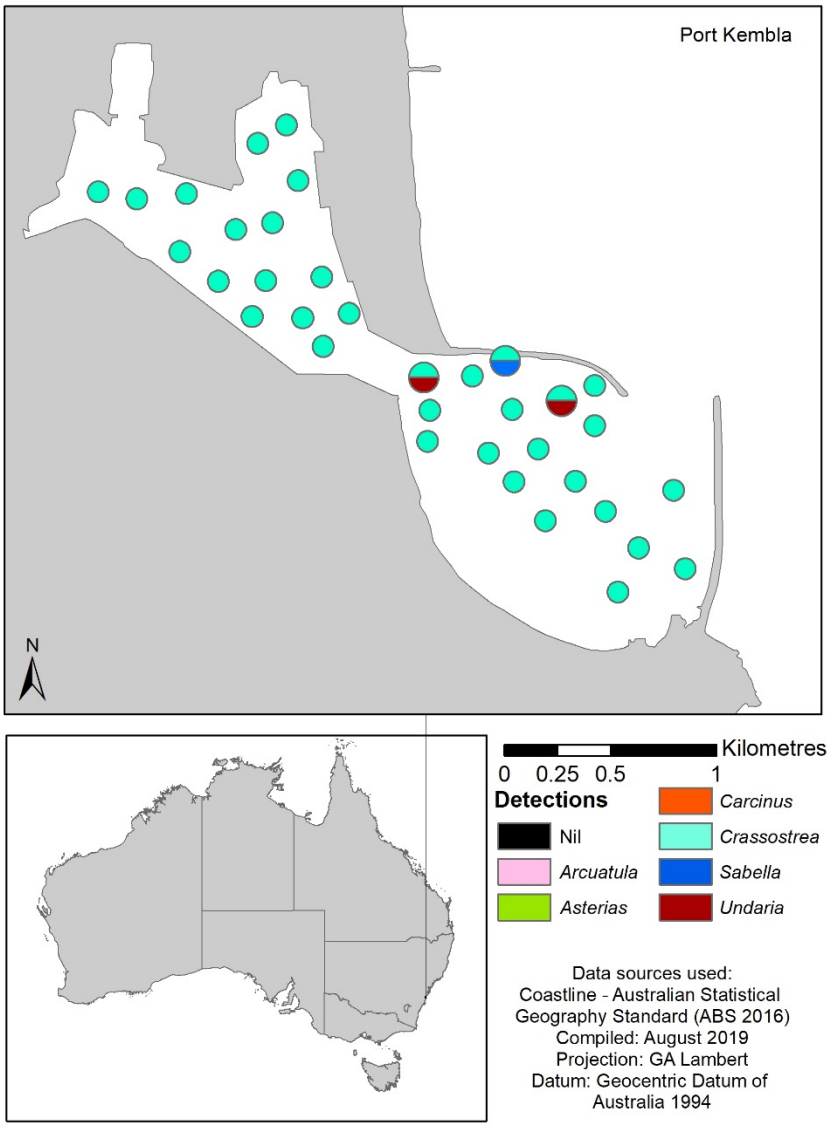

Figure 12. Map of plankton tow locations and molecular detections for Port Kembla in summer 2019.

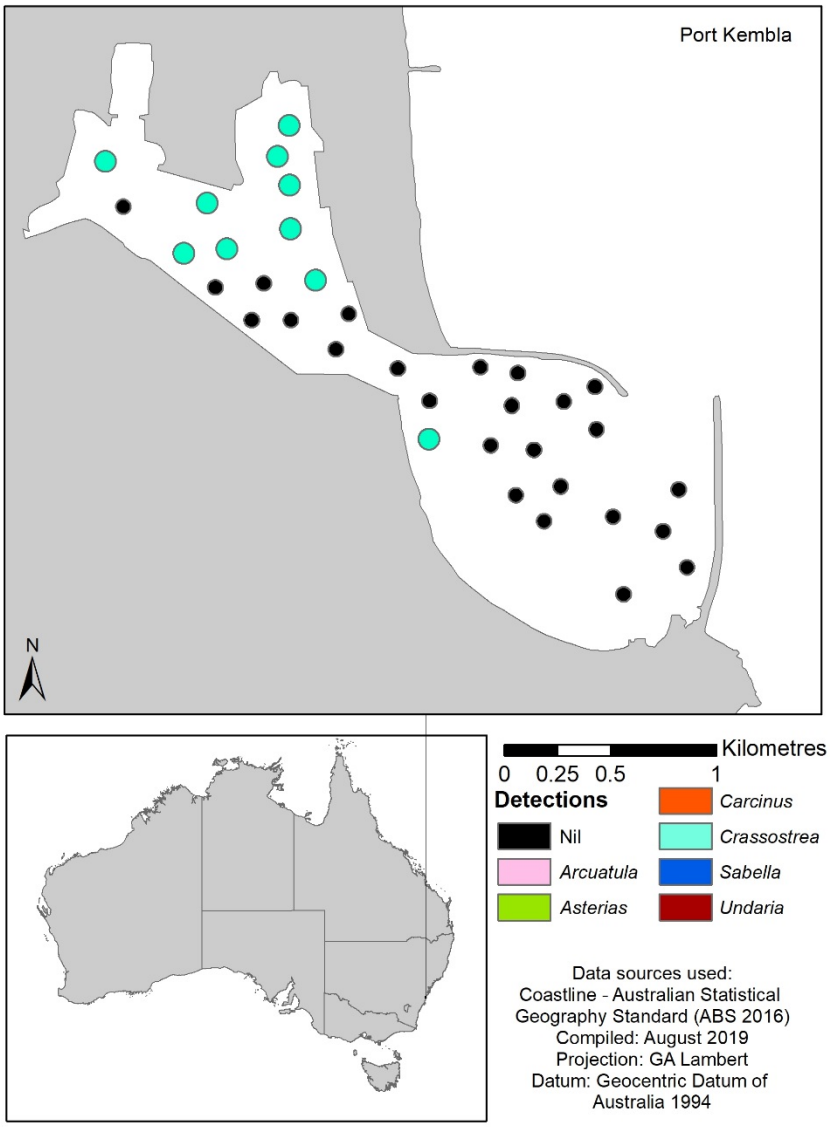

Figure 13. Map of plankton tow locations and molecular detections for Port Kembla in autumn 2019.

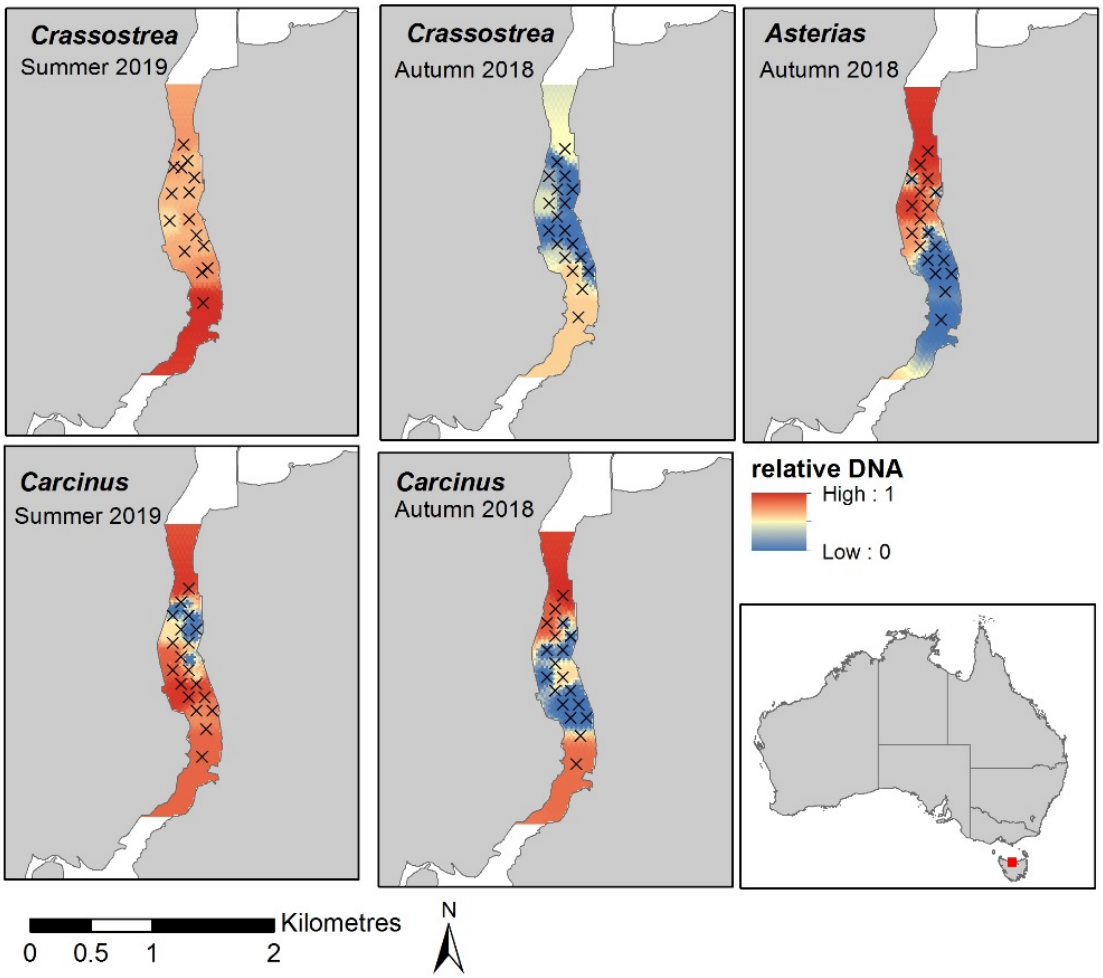

Figure 14. Map of relative DNA concentration for *Crassostrea, Asterias* and *Carcinus* in Devonport by sample set.

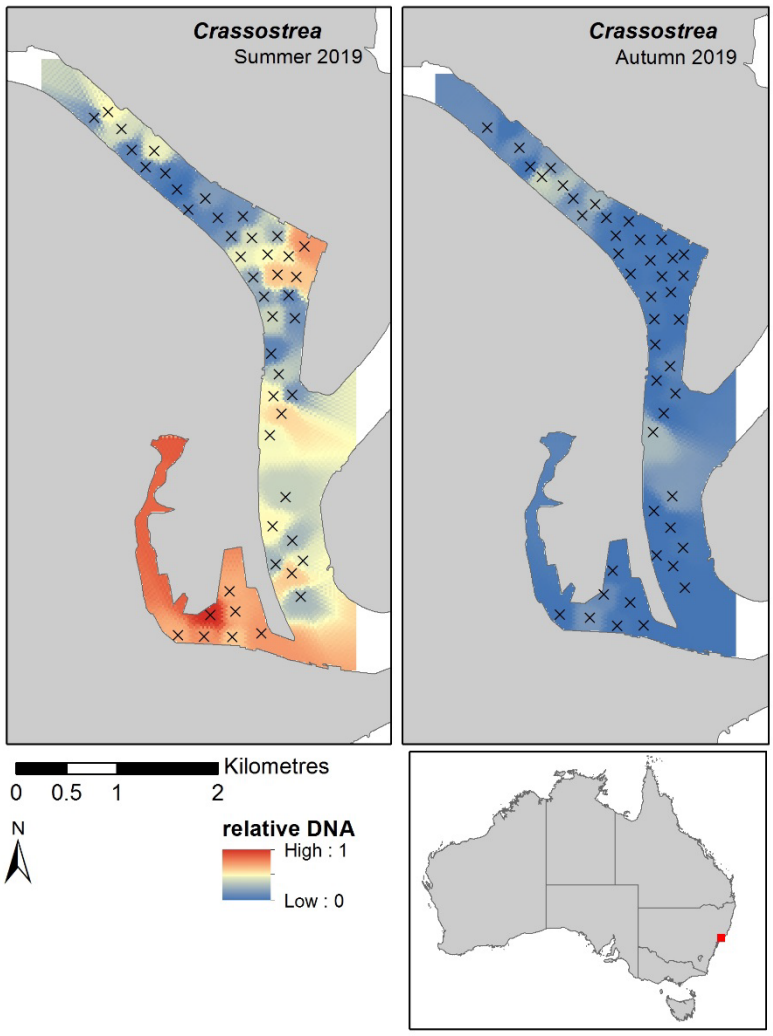

Figure 15. Map of relative DNA concentration for *Crassostrea* in Newcastle by sample set.

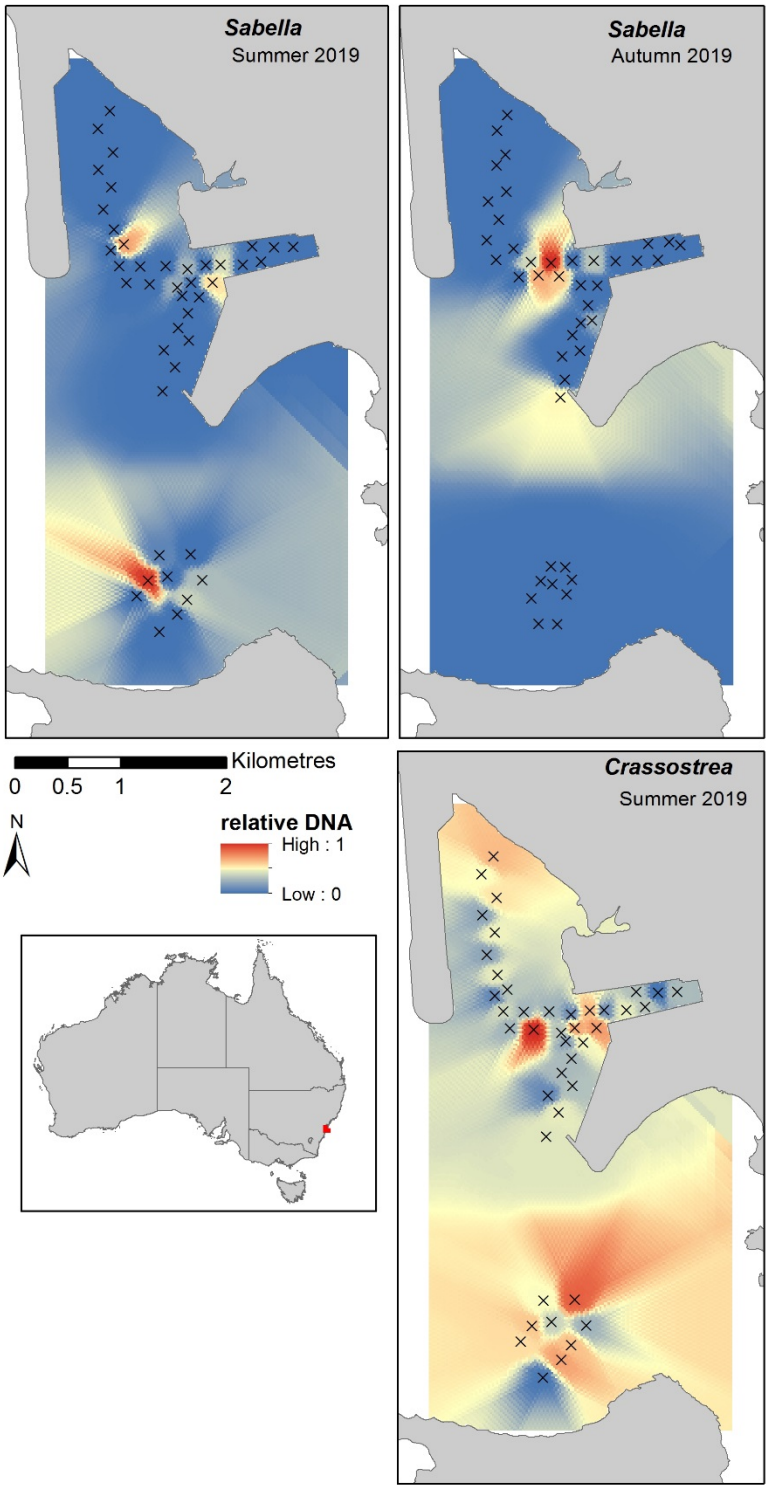

Figure 16. Map of relative DNA concentration for *Sabella* and *Crassostrea* in Botany Bay by sample set.

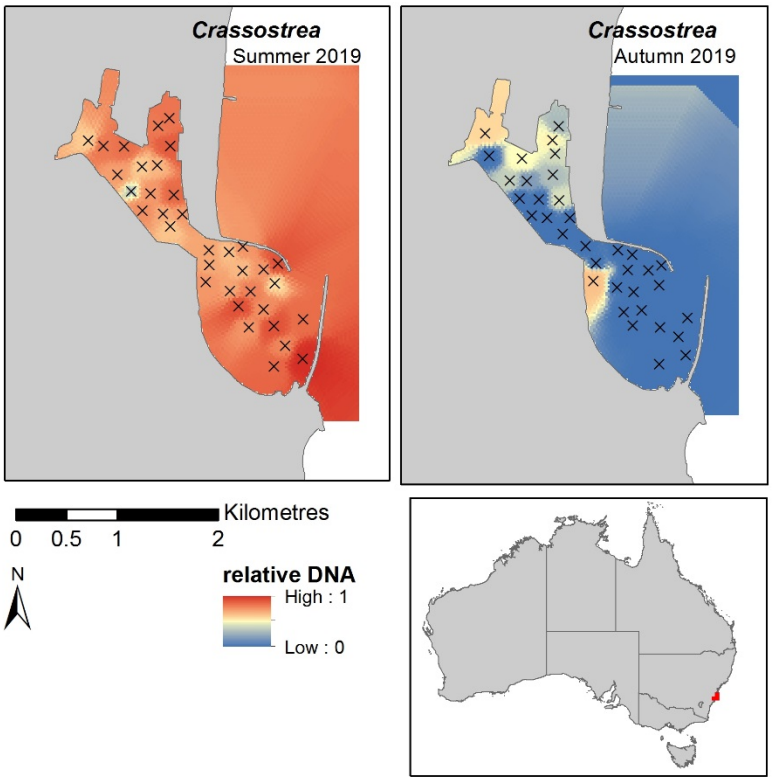

Figure 17. Map of relative DNA concentration for *Crassostrea* in Port Kembla by sample set.

3.4. Patterns in PCR inhibition, plankton tow sampling volume and sample dry weight

PCR inhibition, as measured by scale factor (see section 2.5), occurred in some samples from most sampling events, but without consistent patterns. Inhibition was more common in summer 2019 samples from Weipa and Hay Point than autumn 2018 samples from these ports, but in Botany Bay and Port Kembla, inhibition was observed more frequently in autumn than summer 2019 (Figure 18). Scale factors > 5 may reduce likelihood of detection by some of the pest assays (Wiltshire *et al.* 2019b). The sample sets most affected by inhibition were: the autumn set from Botany Bay, where 12 of 42 samples recorded scale factors >5, with a maximum of 598 and minimum of 1.5; Weipa in summer where 6 of 15 samples had scale factor >5, with a maximum of 411 and minimum of 1.3; and Hay Point in summer, where 35/80 samples had scale factor >5, with a maximum of 1408 and minimum of 1.3.

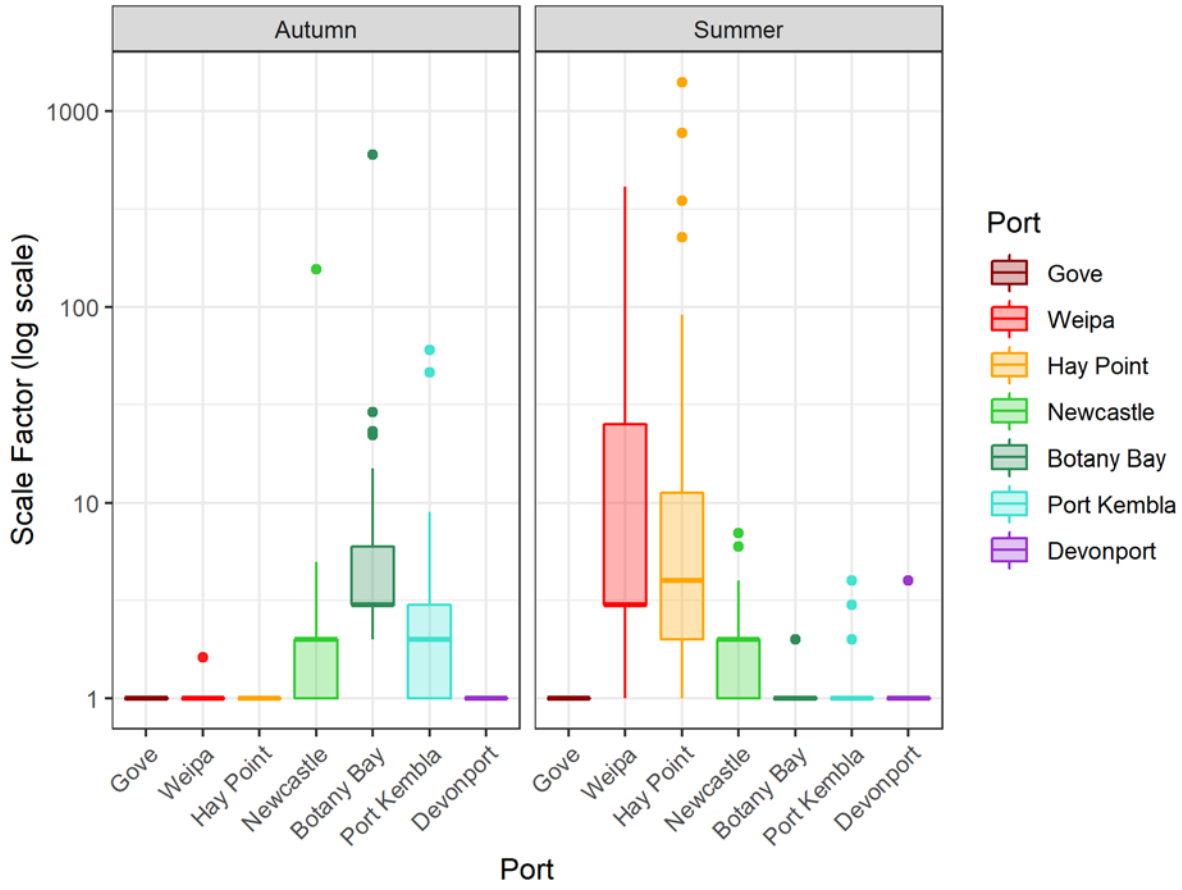

Figure 18. Boxplot of scale factor by port and collecting event. Autumn sampling was conducted in May-June 2018 for Gove, Weipa, Hay Point and Devonport, and in May 2019 for Newcastle, Botany Bay and Port Kembla. Summer samples were collected in February 2019 for all ports (see Table 4 for sampling dates).

Flow meter distance, hence sample volume, was generally similar across ports and sample sets (Figure 19). A sample length of 100 m, as measured by GPS, was targeted for the sampling, and flow meter distances were typically less than this, indicating some net clogging occurred. A few samples recorded flow meter distances > 100 m, which can occur if sampling is conducted into tidal or other currents, resulting in greater water flow through the net than expected based on distance travelled.

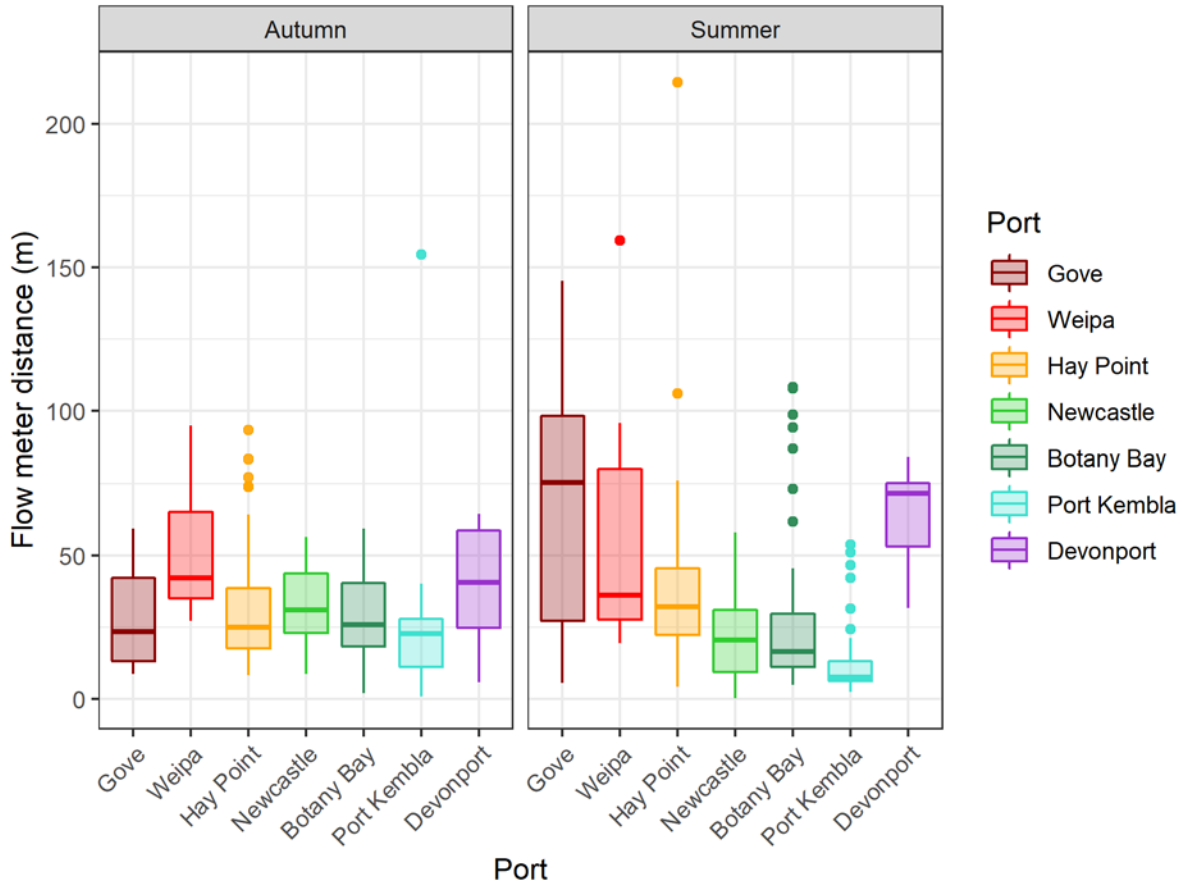

Figure 19. Boxplot of flow meter distance, as a proxy for sample water volume, by port and collecting event. Autumn sampling was conducted in May-June 2018 for Gove, Weipa, Hay Point and Devonport, and in May 2019 for Newcastle, Botany Bay and Port Kembla. Summer samples were collected in February 2019 for all ports (see Table 4 for sampling dates).

Sample dry weight was typically < 1 g, similar to that recorded in most ports and sample sets during the field validation project (Wiltshire *et al.* 2019a); summer samples from Gove and Weipa recorded greater dry weights (several > 2 g), while most autumn samples from Weipa and Devonport had sample dry weight > 1 g but < 2 g (Figure 20).

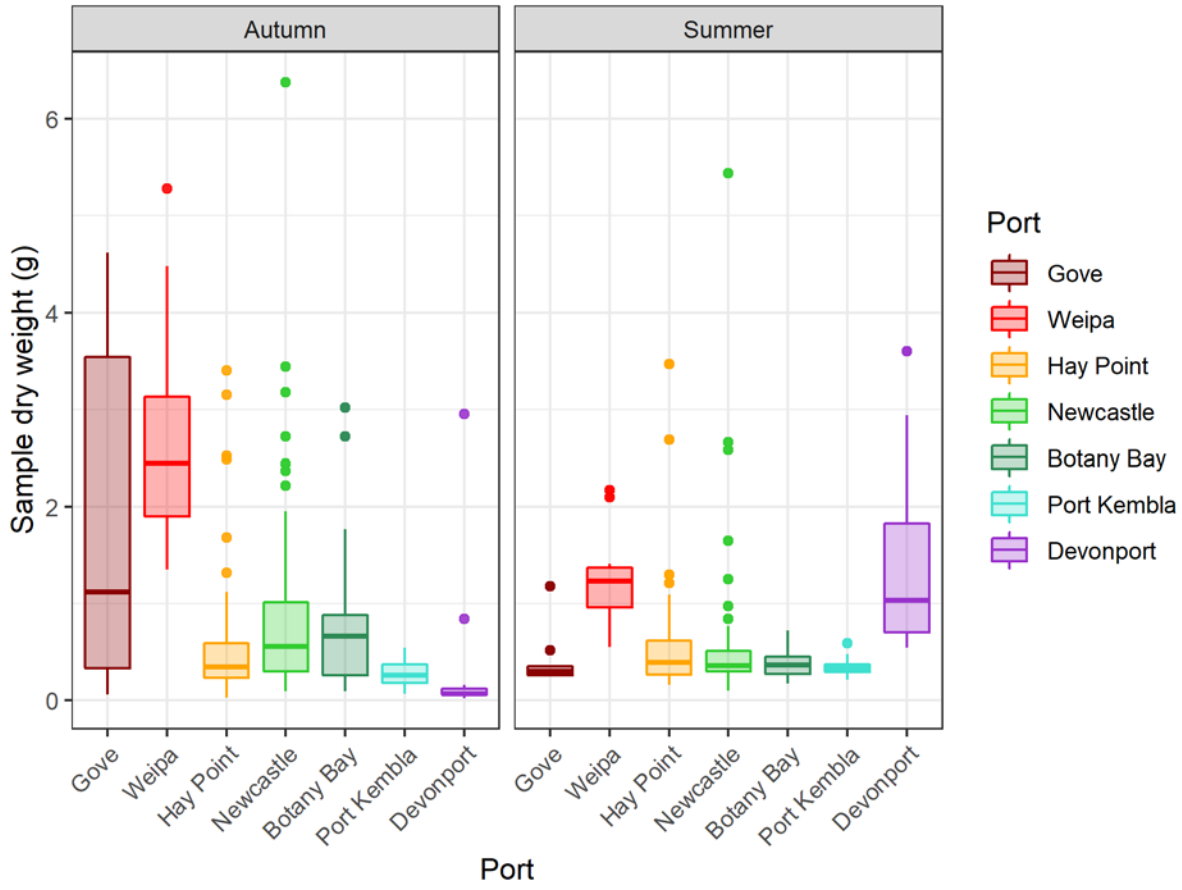

Figure 20. Boxplot of filtered sample dry weight by port and collecting event. Autumn sampling was conducted in May-June 2018 for Gove, Weipa, Hay Point and Devonport, and in May 2019 for Newcastle, Botany Bay and Port Kembla. Summer samples were collected in February 2019 for all ports (see Table 4 for sampling dates).

3.5. Temporal variation in molecular detections and relative DNA

3.5.1. *Crassostrea* in New South Wales ports and Devonport

The zero added log normal (ZALN) model used to analyse results for *Crassostrea* confirmed the clear differences in likelihood of detection and relative DNA of this species between sample sets, though with some variation in the pattern between ports. In each port where *Crassostrea* was detected, the likelihood of detection was higher in summer than autumn, being close to 1 in summer and <0.5 in autumn, except for Newcastle, where it was 0.73 (95% credible interval: 0.59–0.86) (Figure 21). Botany Bay recorded the lowest number of *Crassostrea* detections in autumn, and predicted detection likelihood was 0.11 (95% credible interval 0.02–0.31). Relative

DNA yield in samples was similar between ports within each season although slightly higher in Botany Bay than Newcastle in summer (Figure 22). When sample volume was accounted for, relative DNA concentration in Devonport in summer was lower than for the NSW ports, but, for all ports, field DNA concentrations were higher in summer than autumn (Figure 23).

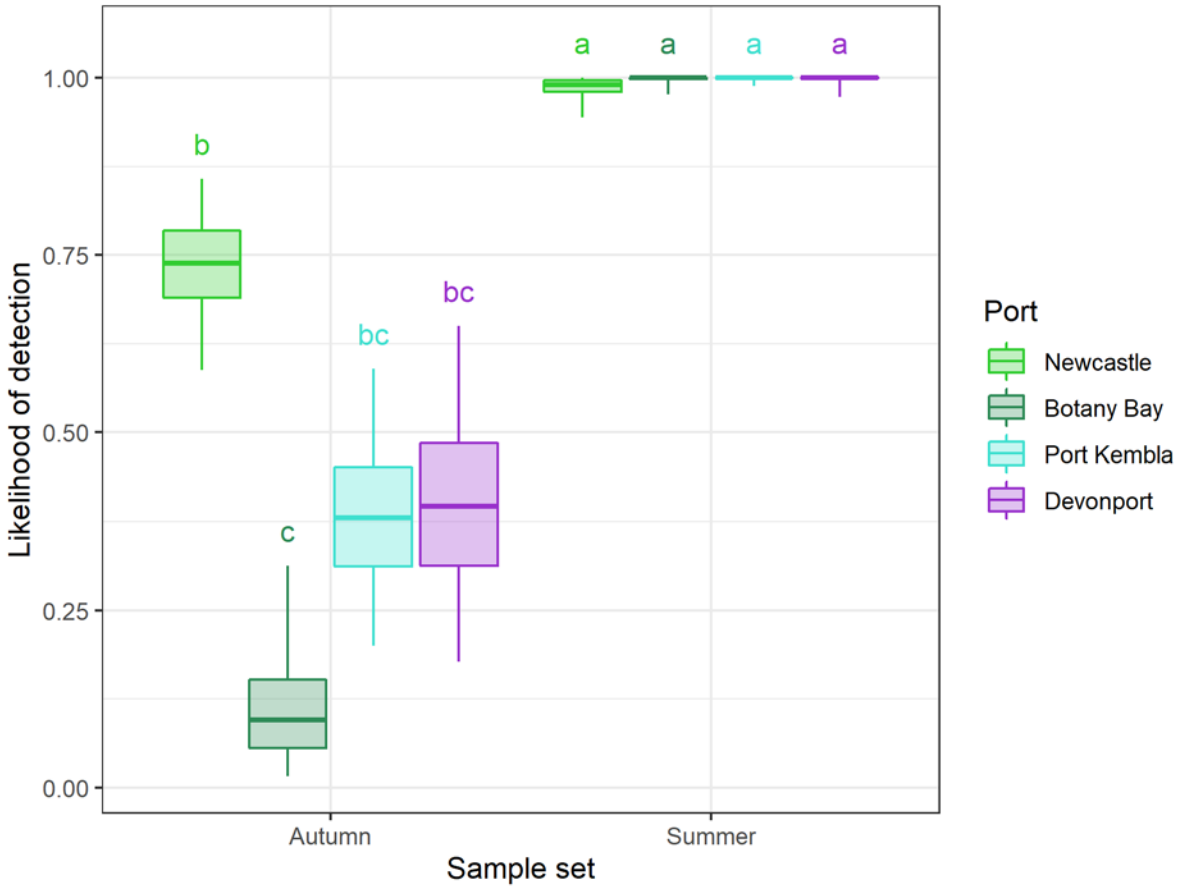

Figure 21. Posterior predictions of mean detection frequency for *Crassostrea* in Newcastle, Botany Bay, Port Kembla and Devonport samples by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

PCR inhibition, as measured by scale factor, was predicted by the ZALN to have a negative effect on detection likelihood for *Crassostrea* (posterior mean coefficient −1.04, 95% credible interval −0.02–−2.26), but to not affect relative DNA concentration, with the scale factor multiplier predicted to be 0.00 (95% credible interval −0.33–0.32). Inhibition occurred more frequently in autumn than summer samples from the NSW ports, particularly in Botany Bay. The sample with

highest scale factor and *Crassostrea* detection had scale factor 9.2. Only three samples from ports with *Crassostrea* present displayed higher scale factors than this, all of which were very high (>40).

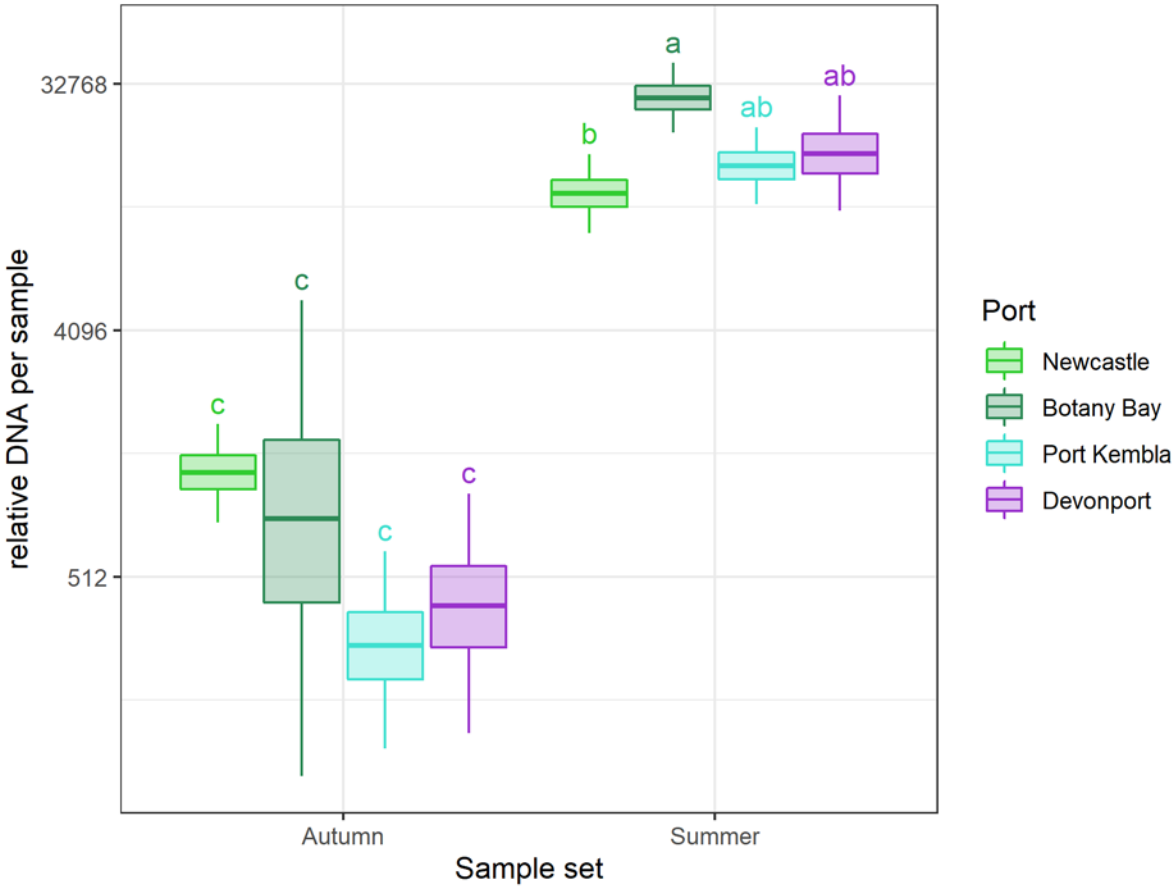

Figure 22. Posterior predictions of mean relative DNA yield for *Crassostrea* in Newcastle, Botany Bay, Port Kembla and Devonport samples by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap

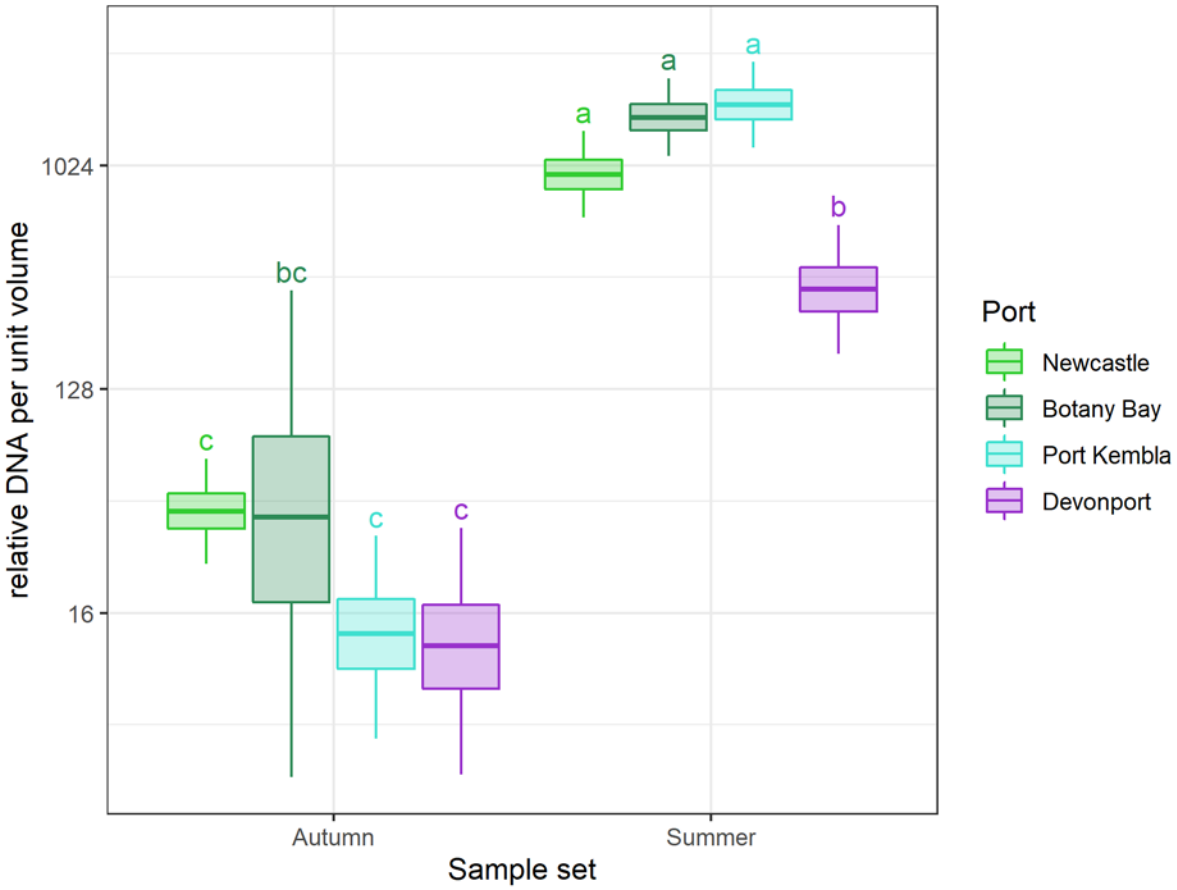

Figure 23. Posterior predictions of mean relative DNA field concentration for *Crassostrea* in Newcastle, Botany Bay, Port Kembla and Devonport by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap

3.5.2. *Sabella* in Botany Bay

For *Sabella* in Botany Bay, despite slightly more detections occurring in summer than autumn, the ZALN model predicted a higher likelihood of detection in autumn than summer (Figure 24), but note that this prediction is for samples with no inhibition. PCR inhibition was predicted to have a large negative impact on detection likelihood for *Sabella* (posterior mean coefficient −9.16, 95% credible interval −2.92–−16.9). There were 12 samples with scale factor >5 from autumn sampling in Botany Bay but in summer the maximum scale factor was 2.0. The highest scale factor recorded for a sample with *Sabella* detection, which was from Botany Bay in autumn, was 2.8.

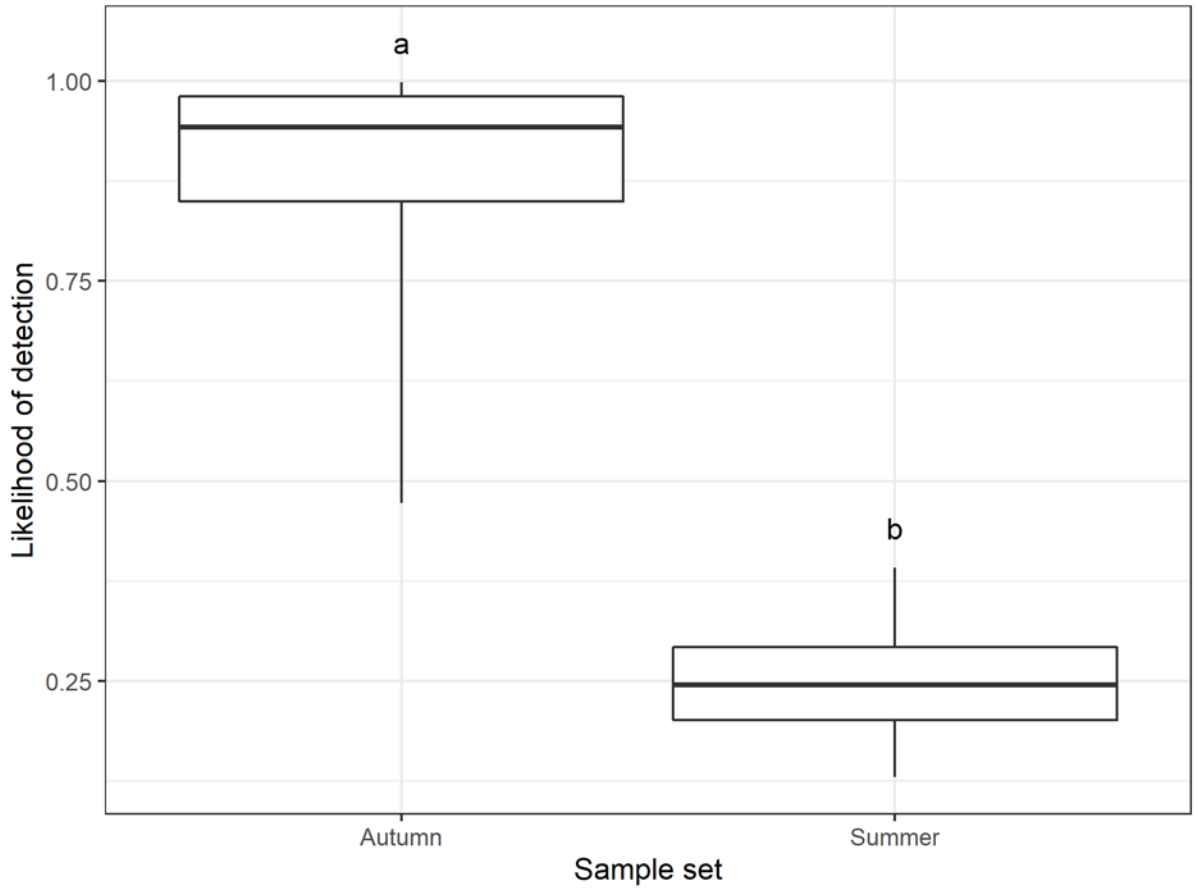

Figure 24. Posterior predictions of mean detection frequency for *Sabella* in Botany Bay samples by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap

The ZALN also predicted higher relative DNA in Botany Bay samples from autumn than summer (Figure 25); accounting for sample volume reduced this difference so that 95% credible intervals of the predictions overlapped slightly (Figure 26). The scale factor multiplier was predicted to be 0.85 (95% credible interval −0.08–1.79) suggesting that relative DNA for *Sabella* was similarly affected by inhibition as that of the control organism.

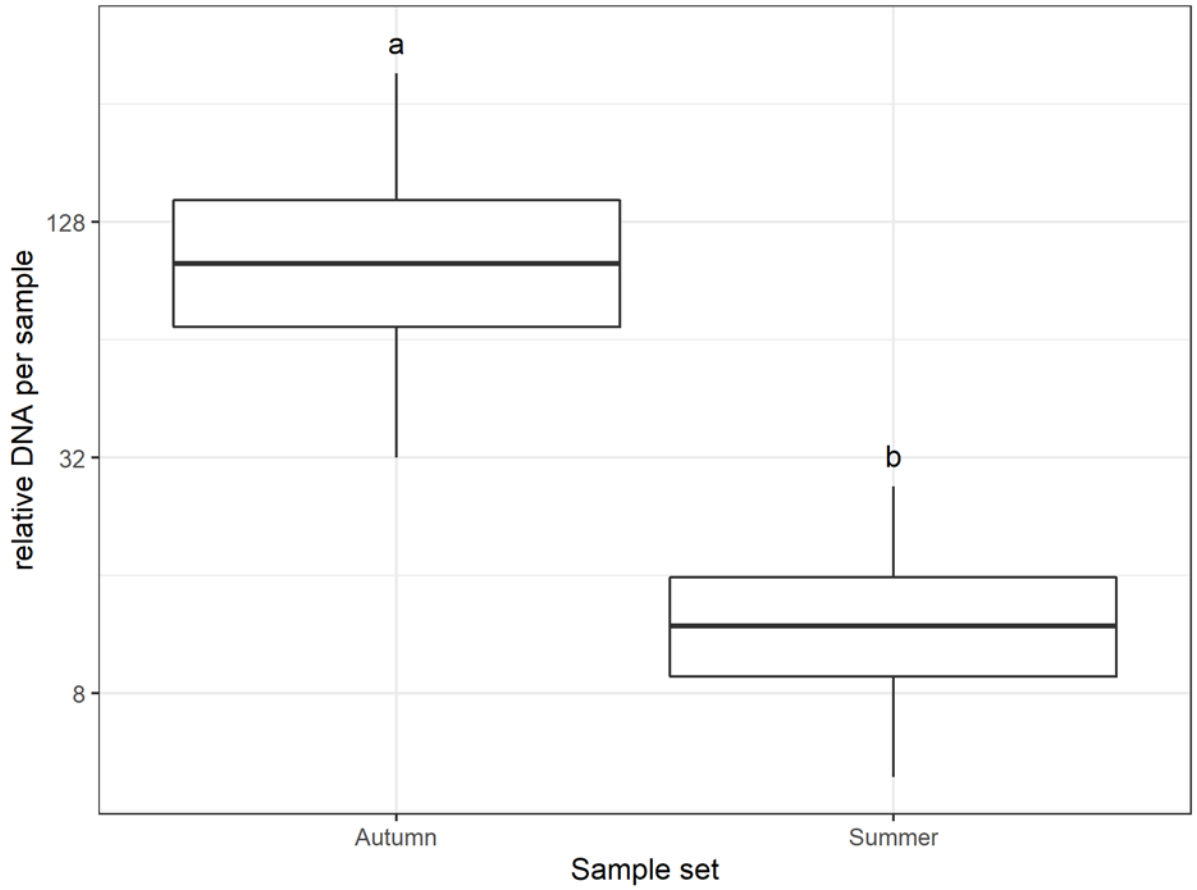

Figure 25. Posterior predictions of mean relative DNA yield for *Sabella* in Botany Bay samples by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap

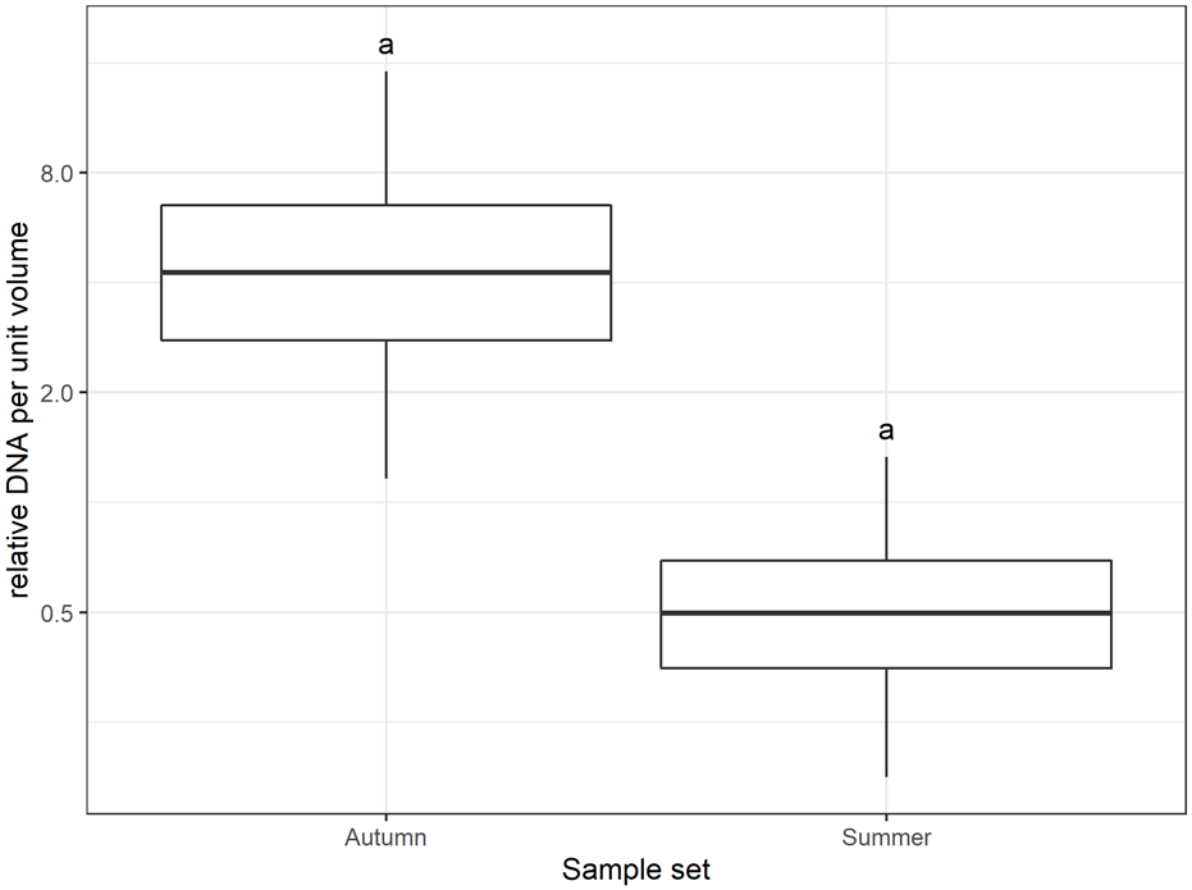

Figure 26. Posterior predictions of mean relative DNA field concentration for *Sabella* in Botany Bay by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap

3.5.3. *Carcinus* in Devonport

The ZALN for *Carcinus* in Devonport showed that detection likelihood and relative DNA yield in samples tended to be higher in summer than autumn, but with considerable overlap of 95% credible intervals. Accounting for sample volume, relative field DNA concentration was predicted to be similar across sample sets. Only one sample from Devonport had scale factor >1, hence effects of inhibition on detection and relative DNA could not be assessed. This sample, from the summer sample set, recorded a scale factor of 3.7, and had detections of *Carcinus* and *Crassostrea.*

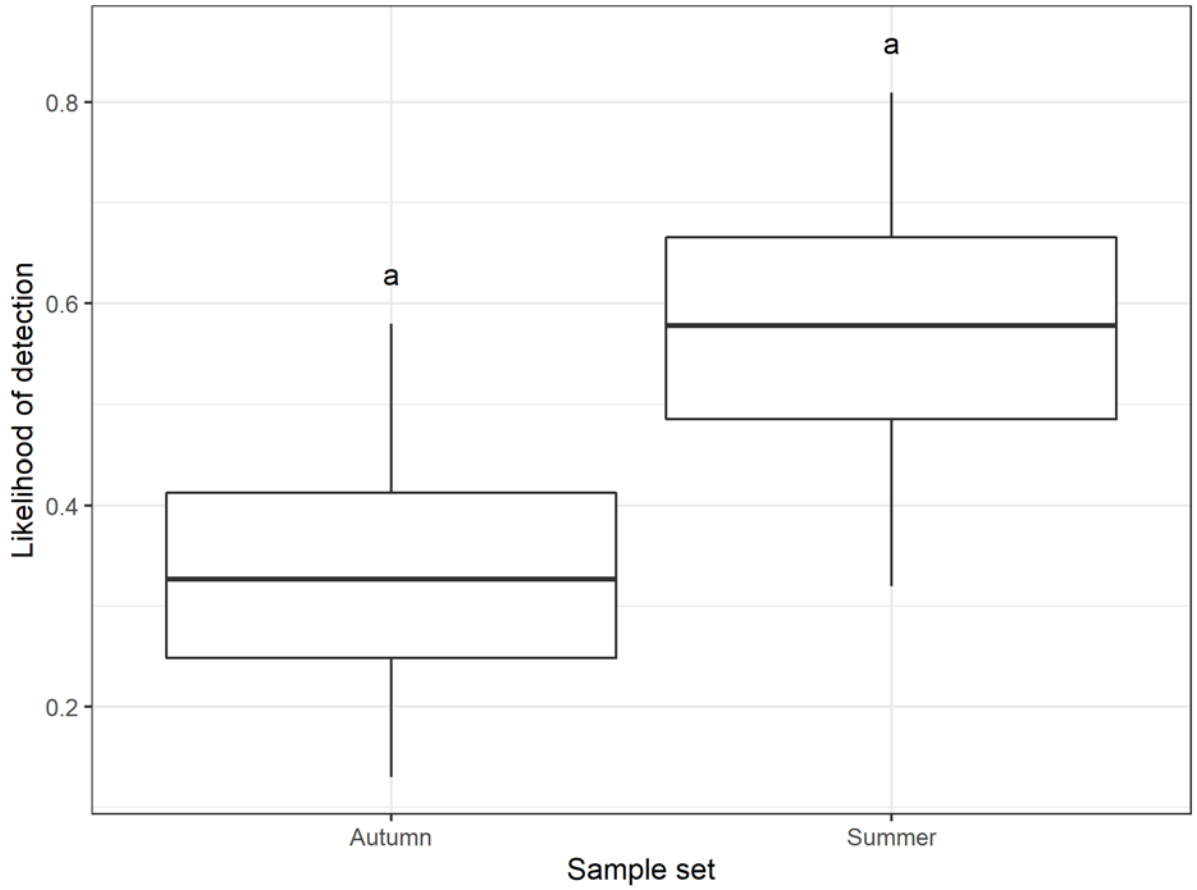

Figure 27. Posterior predictions of mean detection frequency for *Carcinus* in Devonport samples by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap

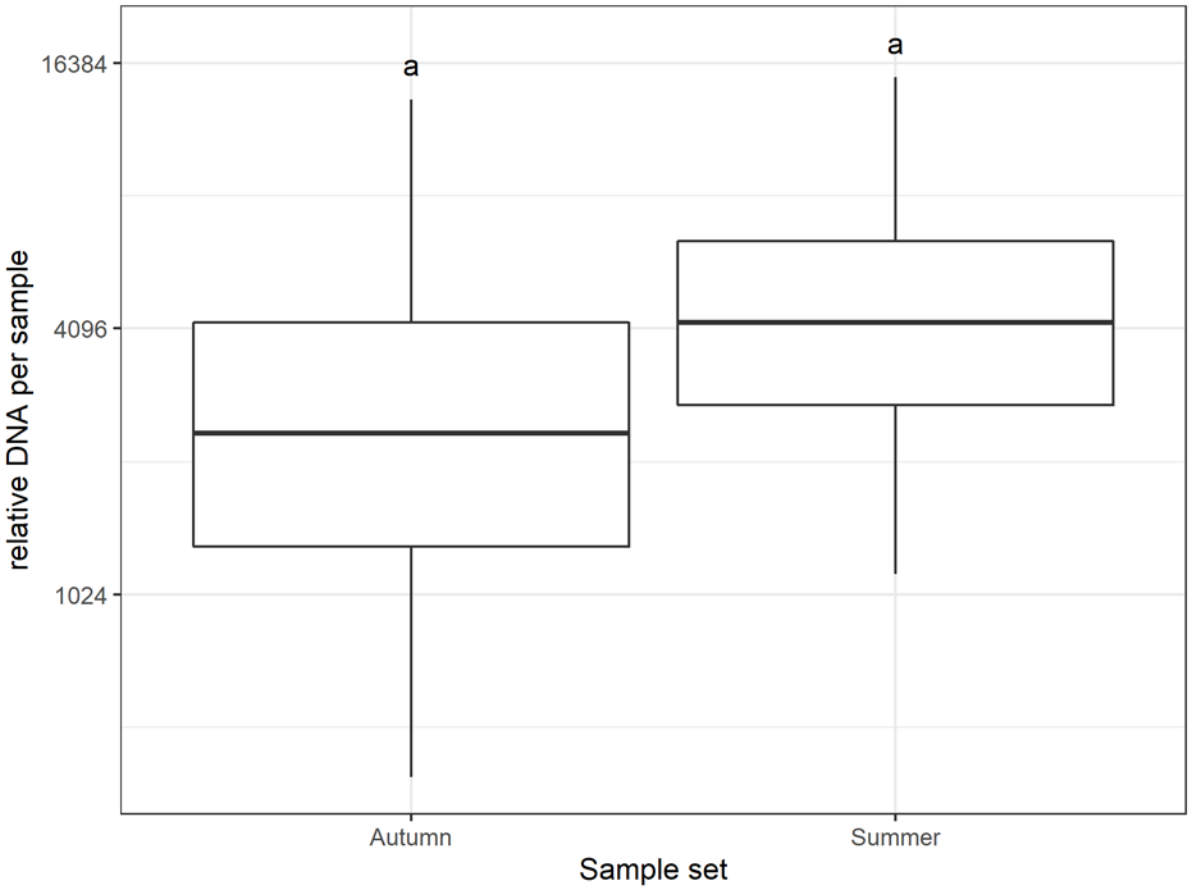

Figure 28. Posterior predictions of mean relative DNA yield for *Carcinus* in Devonport samples by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap

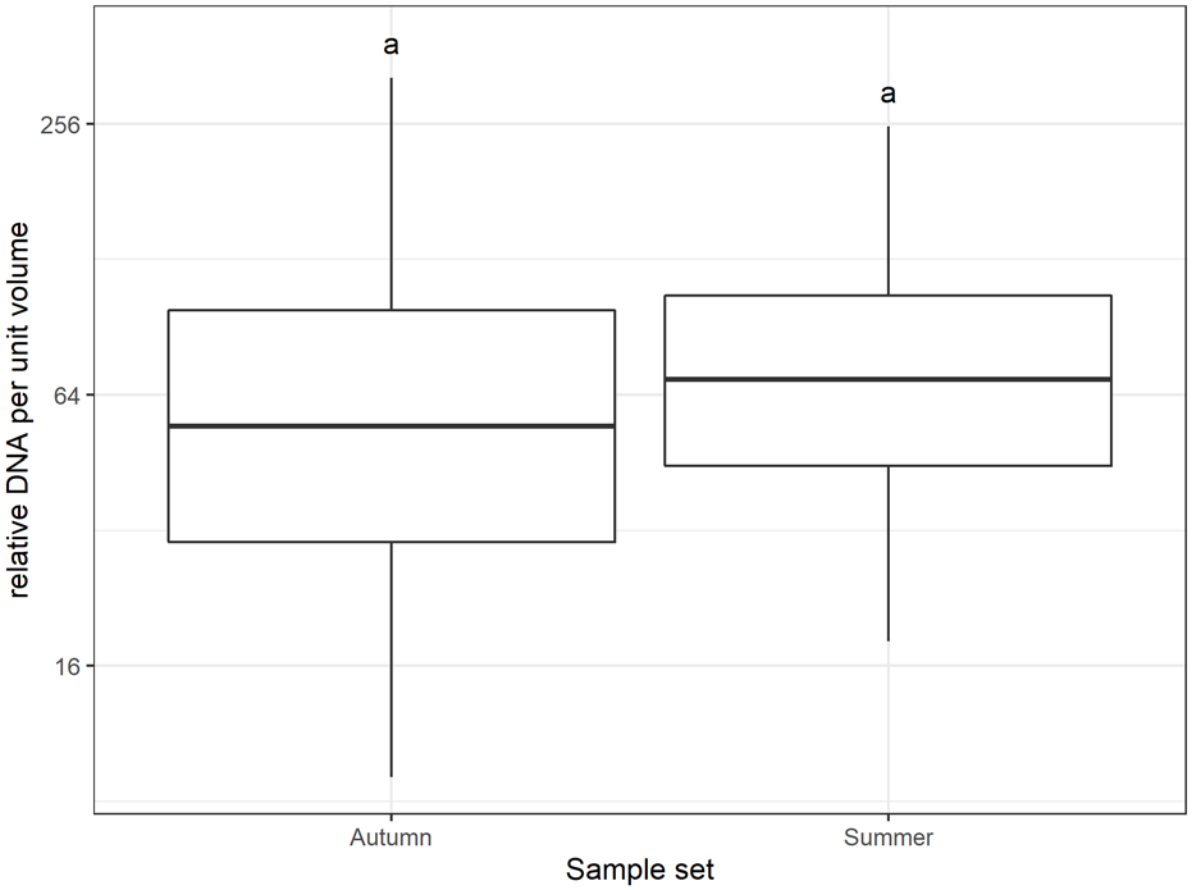

Figure 29. Posterior predictions of mean relative DNA field concentration for *Carcinus* in Devonport by sample set. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

3.6. Traditional sampling in Devonport

Dredge sampling and visual surveys in Devonport did not detect any *Asterias* (Marine Solutions 2019). While the survey was designed to target *Asterias*, other pests were noted where found. *Crassostrea* was observed during dive surveys, and *Sabella* was observed on one dive transect in the north-east of the survey area (Marine Solutions 2019). *Crassostrea* was noted to occur on the three most northern dive transects and on one transect to the south-west. In addition, *Crassostrea* shells were collected in one of the southern dredge samples (Marine solutions, pers. comm.). The locations of *Crassostrea* occurrence generally correspond with the location of qPCR detections of this species, although patterns of DNA concentration suggest that *Crassostrea* may be more abundant to the south, possibly also occurring further upstream from the survey area.

4. DISCUSSION

These molecular surveys provided updated information on the status of six pests of concern for ballast water management in the ports of Gove, Weipa, Hay Point, Newcastle, Botany Bay, Port Kembla and Devonport. Molecular surveillance detected the majority of pests known to occur at each location: *Arcuatula, Carcinus* and *Crassostrea* in Devonport; *Crassostrea* and *Sabella* in Botany Bay; *Crassostrea* in Newcastle. Port Kembla has not been surveyed for marine pests since 2000 (Pollard and Pethebridge 2002a), and was not included in recent surveillance for *Crassostrea* in NSW (NSW DPI 2012), but is within the known range of this species, which is common in adjacent NSW estuaries. The molecular survey detected *Crassostrea* in a large proportion of samples in Port Kembla and provided strong evidence that a population is established in this location. The Port Kembla samples had similar C_T values (hence, relative DNA) to Botany Bay and Newcastle, and overall prevalence intermediate between these two ports and similar to Devonport. *Asterias*, which is not previously recorded in Devonport, was detected by qPCR in 7/19 samples in autumn 2018. A follow-up dive and dredge survey did not detect this species within the port area. The number of detections and range of C_T values for *Asterias* in Devonport are unlikely to have resulted from transient material, therefore, despite the lack of detection in traditional sampling, it is likely that a population of *Asterias* is present in or near Devonport. Estimated prevalence of *Asterias* DNA in plankton was similar to that of *Sabella* in Botany Bay, and 95% credible intervals of the prevalence estimate did not include zero, indicating that complete absence is improbable. Non-detection in the traditional survey may have resulted from the population having low abundance and/or high aggregation, both of which reduce likelihood of detection by the traditional survey, or by the population occurring outside the immediate port area that was surveyed. Molecular detections may occur of DNA advected from populations outside the survey area. Data on local hydrodynamics would assist in determining the source locations for DNA advected into the Devonport survey area, but given *Asterias* detections by qPCR occurred in the north-east of the surveyed area in Devonport, with highest relative DNA concentrations in the north, the population may occur further downstream the Mersey River from the survey area, outside the downstream extent of the traditional survey.

Additional detections of pests not previously recorded at survey locations were: low-level *Undaria* detections in NSW ports in summer; a single near low level detection of *Sabella* in Port Kembla in summer; and detections of *Arcuatula* in Gove, Weipa and Hay Point. The lack of recent surveillance of Port Kembla means it is difficult to rule out the occurrence of *Sabella* in this port, especially given that this species also occurs in Botany Bay, and in the Port of Eden (Murray and

Keable 2013; Ahyong *et al.* 2017). If *Sabella* is present in Port Kembla, it is likely to occur at low abundance, but the single, high C_T detection means that it also may have been a detection of transient material. The *Undaria* detections are likely to have been of transient material, especially given that no further detections of this species occurred in autumn. Detections of this species occurred in a much high proportion of autumn than summer samples where it is established in Melbourne (Wiltshire *et al.* 2019a). Surveillance for marine pests, other than *Crassostrea*, has not been conducted in Newcastle since 1997 (Hewitt *et al.* 1999) or Botany Bay since 1998 (Pollard and Pethebridge 2002b), but based on results from Melbourne, qPCR detections would be expected in autumn if *Undaria* were established in any of the NSW ports. Overall prevalence estimated for *Undaria* was ≤ 0.06 in each case, and similar between ports with and without detections (Table 8). In Port Kembla the 95% credible interval for estimated prevalence was 0.01–0.13, but for other sites the 95% credible intervals included zero, and had upper limits ≤ 0.13 (Table 8). These data indicate that if present, abundance is likely to be very low. Sea surface temperatures in the NSW ports are suitable for *Undaria* survival, but are likely to exceed the upper limit for zoospore germination during summer (O'Loughlin *et al.* 2006; Summerson *et al.* 2007; Glasby and Lobb 2008) when *Undaria* typically reproduces (Schaffelke *et al.* 2005; Primo *et al.* 2010). Establishment of *Undaria* at these locations is therefore unlikely.

Specificity of the *Arcuatula* assay has not been assessed as thoroughly as the other assays applied in this project due to a lack of biological specimens and sequences of related taxa, including native relatives from tropical Australia. Investigation into the specificity of this assay is ongoing, but it is possible that *Arcuatula* qPCR detections in tropical locations are the result of cross-reaction with DNA of a native relative. Estimates of true prevalence were not possible for *Arcuatula* because diagnostic performance of this assay has not been assessed; the assay will be redesigned if it is not adequately specific (i.e. cross-reacts with native species) (Wiltshire *et al.* 2019b). The status of *Arcuatula* in tropical ports with qPCR detections remains unclear, but will be informed by these specificity investigations and sequencing of PCR product from samples with molecular detections. A lack of surveillance suitable for targeting *Arcuatula* in the ports of Gove and Hay Point means presence of this species cannot be confidently ruled out, but the more comprehensive pest survey conducted in Weipa (Biofouling Solutions 2015) could have been reasonably expected to detect this species if it was present, although it was not one of the specified target species of that surveillance. The *Arcuatula* detections in Devonport are, however, likely to be of *A. senhousia*, which is recorded from that location. Molecular detections of *Arcuatula* in Melbourne during previous surveillance were supported by samples of *A. senhousia* collected

by parallel dredge sampling (Wiltshire *et al.* 2019a), and no potentially false positive detections have occurred in other temperate ports (Deveney *et al.* 2017).

Sabella occurs in Devonport but was not detected by qPCR. *Sabella* was recorded on a single dive transect during the follow-up traditional survey, in a location where it has been observed in earlier surveys. Counts of this species were not made, but photographs from the dive transect show a sparse population (Marine Solutions 2019). It is likely that the current population size of this species in Devonport is below the detection limit of the current molecular survey. The overall likelihood of detecting a species that is present in an area is dependent on the likelihood of a target occurring in a sample volume or area (occurrence likelihood or encounter rate), the likelihood of successful capture of the target where present (capture efficiency, which, combined with the encounter rate gives the true prevalence, i.e. rate of target occurrence in samples), and on the likelihood of detection (or positive identification) of the target when it is present in a sample (Hayes *et al.* 2005). In the context of molecular surveillance, this final likelihood is the diagnostic sensitivity (DSe). Investigation of the diagnostic performance of the qPCR assays used for detection of marine pests has shown that DSe is typically high (> 0.8) for the assays applied in this project, and at least 0.84 for the *Sabella* assay (Wiltshire *et al.* 2019b). The validation project outcomes also suggest high capture efficiency for the molecular method across species (Wiltshire *et al.* 2019a). This study and Wiltshire *et al.* (2019b) suggest that DSe of the *Sabella* assay may be adversely affected by PCR inhibition, especially at scale factors ≥ 5. Inhibition is unlikely to have prevented detection in Devonport, where only one sample displayed scale factor >1. The other assays applied have demonstrated detections in samples with relatively high PCR inhibition (scale factors >10), although with some decline in performance likely especially at very high scale factors (current study; Wiltshire *et al.* 2019a). Where severe inhibition is not an issue, absence of target DNA from samples (i.e. low prevalence) is the most likely reason for non-detection at localities where a species is present.

The sample numbers predicted to provide a given survey sensitivity are calculated based on a target population density or prevalence. To detect a lower population density or prevalence, exponentially more samples are required to achieve equivalent survey sensitivity, becoming infinite as the target population size/prevalence becomes very small (Hayes *et al.* 2005; Furlan *et al.* 2016). Where a target is truly absent, it cannot be detected, regardless of the number of samples collected. It is therefore not possible to completely prove absence of a pest species, rather, absence of detection provides a level of confidence that a population, if present, is below a given population size/prevalence. The level of confidence is determined by the number of

samples collected and performance of the method used, with methods having high capture efficiency and DSe providing greater confidence for the same number of samples than a poorer performing method. In the current molecular surveys, the upper limits of the 95% credible interval for prevalence of species with no detections (and also for cases of likely transient detections) were ≤ 0.13, while lower limits for species with confirmed occurrence were ≥ 0.15, and the lower limit of the 95% credible interval was 0.12 for *Asterias* in Devonport. This suggests that the molecular surveys as performed are suitable for detecting a DNA prevalence in plankton of ~0.15. For detection of a lower prevalence with equivalent confidence, additional samples would need to be collected, as illustrated by Wiltshire *et al.* (2019b). It should be noted, however, that the concept of prevalence in plankton is complicated, because the likelihood of a sample containing DNA depends on the sampling method, the DNA concentration and whether DNA is randomly distributed or spatially clumped (Furlan *et al.* 2016). DNA concentration and prevalence is also likely to vary seasonally (Wiltshire *et al.* 2019a). The relationships between adult pest abundance and DNA prevalence in plankton has also not been established. It is likely that larger pest populations provide higher DNA concentrations and thus greater prevalence, and that therefore the likelihood of qPCR detection increases with increasing pest density, but the exact relationship is likely to be complex, differ between species and vary seasonally.

The results from these surveys also provide more data on the effects of PCR inhibition. Consistent with results of Wiltshire *et al.* (2019a), the effect of inhibition varied across assays. Sensitivity of the *Sabella* assay appears to be more affected that other assays, and detection likelihood for this species is reduced at scale factors > 5, as also demonstrated by Wiltshire *et al.* (2019b). Effects of inhibition on other assays are less clear, with detections occurring in samples with scale factors > 10. The relative scarcity of samples displaying inhibition from ports with pests present means that effects of inhibition cannot be fully assessed with current data. Inhibition should continue to be assessed in future to obtain more data on patterns in inhibition and effects on assay performance, and to identify any sample sets where inhibition is severe enough to impact overall survey sensitivity.

5. CONCLUSIONS

These surveys established pest status of the seven surveyed ports for Australian Sourced Ballast Applications in the Maritime Arrivals Reporting System. The surveys detected *Arcuatula* in Gove, Weipa and Devonport, *Asterias* and *Carcinus* in Devonport, *Crassostrea* in Devonport, Newcastle, Botany Bay and Port Kembla, *Sabella* in Botany Bay and *Undaria* in Newcastle, Botany Bay and Port Kembla. Molecular surveillance provides a specific, sensitive approach for detection of marine pests, and most detections are consistent with known pest distributions. The detections of *Undaria* in New South Wales appear to be associated with transient material, being few (1–2 samples), low-level, and occurring in only summer sampling. Summer temperatures of these ports are also likely to prevent successful reproduction and hence establishment of *Undaria.* Detections of *Asterias* in Devonport were not confirmed by a traditional survey, but the survey was not extended outside the port area targeted by the molecular survey, and it is likely the material detected by qPCR was advected into the survey area by currents. Occurrence of *Asterias* in Devonport is plausible given that this species occurs in south-eastern Tasmania and also in Port Philip Bay, Victoria, with regular vessel movements between these locations. Recommended approaches to interpreting detections regarded as potentially transient were provided by Wiltshire *et al.* (2019a). The *Arcuatula* assay appears to lack specificity and may detect a native *Arcuatula* or other closely-related species in northern Australia; this assay is under investigation and subject to redesign. Temperate detections by the assay, such as in Devonport, are, however, likely to be of *A. senhousia.* PCR inhibition was apparent in some sample sets, but did not prevent detection of the pests occurring in those locations. Survey results need to be interpreted in context and with all available information before translation into a management system.

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