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Molecular surveys in 2020 of priority marine pests for the ports of Bunbury, Kwinana, Fremantle and Geraldton

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

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SARDI Aquatics Sciences
PO Box 120 Henley Beach SA 5022

December 2020

Report to the Department of Agriculture, Water and the Environment

SOUTH AUSTRALIA

Australian Government
Department of Agriculture, Water and the Environment

Government of South Australia
Department of Primary Industries and Regions

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Cover photograph: Plankton sampling in Fremantle, provided by Cardno (Perth) Pty Ltd

South Australian Research and Development Institute
SARDI Aquatic Sciences
2 Hamra Avenue
West Beach SA 5024
Telephone: (08) 8207 5400
Facsimile: (08) 8207 5415
http://www.pir.sa.gov.au/research

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Author(s):	Wiltshire K.H., Theil M.J., Giblot-Ducray D. and Deveney M.R.
Reviewer(s):	Grammer, G. and Kirkwood, R.
Approved by:	A/Prof Tim Ward Science Leader – Marine Ecosystems
Signed:	
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EXECUTIVE SUMMARY

Shipping is a major vector for marine pest introductions, and ports 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. Few ports around Australia have been surveyed for introduced marine species however, the high cost of implementing surveys using traditional methods (e.g. dives, trawls/dredges, trapping) is recognised as a major impediment. Molecular techniques for marine pest surveillance offer cost and time savings over traditional techniques because they require less than half the person-hours and field cost of traditional surveillance while providing higher survey sensitivity.

The South Australian Research and Development Institute (SARDI) has developed a plankton sampling method and molecular assays for the species of concern for domestic ballast water: Northern Pacific Seastar (*Asterias amurensis*), Asian Date Mussel (*Arcuatula senhousia*, formerly *Musculista senhousia*), European Green Crab (*Carcinus maenas*), Japanese kelp (*Undaria pinnatifida*), European Fan Worm (*Sabella spallanzanii*), Pacific Oyster (*Crassostrea gigas*, also known as *Magallana gigas*) and Basket Shell Clam (*Corbula gibba*), and for two pests that are exotic to Australia but are a risk for introduction: New Zealand green-lipped mussel (*Perna canaliculus*) and Black-striped mussel (*Mytilopsis sallei*). Surveillance using this method and suite of assays has been field validated with parallel molecular and traditional surveys. Assays for additional exotic pests of concern: Asian paddle crab (*Charybdis japonica*), Brown and Asian green mussel (*Perna perna, P. viridis*) and Harris mud crab (*Rhithropanopeus harrisii*) were developed more recently and are implemented in the SARDI system. These assays were also applied in this project to obtain data on field performance of the new assays, and to assess the status of these species at surveyed ports.

This report describes the design and results of molecular surveys for Bunbury, Kwinana, Fremantle and Geraldton, Western Australia (WA). Surveys were designed using the Monitoring Design Excel Tool version 2.5 using survey sensitivities of 0.6 and 0.8; and collection was based on the 0.8 sensitivity. Seasonality of molecular detections is not fully understood, but for assays that target the species for domestic ballast water management, field validation studies showed that a combination of sampling in late summer – early autumn and late autumn – early winter provided the greatest detection likelihood. Sample sets were therefore collected in summer and autumn in each port, with 23 samples per season collected in Bunbury, 60 in Kwinana, 33 in Fremantle and 15 in Geraldton. Fremantle and Geraldton ports consisted of a single sublocation

each. At Bunbury, the Inner and Outer Harbour areas were surveyed. At Kwinana, locations surveyed were the Alcoa Jetty, Bulk Terminal, Oil refinery and Bulk Jetty, and Grain Jetty. Summer samples were collected in February 2020 for Bunbury and Geraldton, and in March 2020 for Kwinana and Fremantle. Autumn samples were collected in May 2020 at each port.

Detections of *Arcuatula senhousia* DNA occurred in Kwinana and Fremantle in both sample sets; in autumn *Sabella spallanzanii* DNA was detected in Bunbury and Kwinana, and *Crassostrea gigas* was detected in Geraldton; and in summer a single detection of *Rhithropanopeus harrisii* DNA was recorded in Fremantle. *Arcuatula senhousia* and *S. spallanzanii* are recorded from the ports where detections occurred for each. The assay for *A. senhousia* appears to cross-react with DNA from a native relative in tropical Australia, lending some doubt as to the species detected by this assay, but detections by this assay in temperate locations have occurred only in areas with known occurrence of *A. senhousia*, as is the case in the current survey. *Crassostrea gigas* is not known to occur in WA, and follow up surveillance is required to determine the status of this species in Geraldton. *Rhithropanopeus harrisii* is exotic to Australia, and the single low level detection of this species is likely to be of transient material, i.e. DNA present from a temporary source such as ballast water or hull-fouling, rather than from an established population. The assay for *R. harrisii* has not been fully validated, but if the detection is of a cross-reaction with non-target DNA, then the species causing the cross-reaction is rare in the area. Further validation and field testing using this assay is needed to confirm its specificity.

Keywords: Marine pests, qPCR, plankton, ports, surveillance, Western Australia

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): Asian Date Mussel (*Arcuatula senhousia*, formerly *Musculista senhousia*), Northern Pacific Seastar (*Asterias amurensis*), European Green Crab (*Carcinus maenas*), Japanese kelp (*Undaria pinnatifida*), European Fan Worm (*Sabella spallanzanii*), Pacific Oyster (*Crassostrea gigas*, also known as *Magallana gigas*) and Basket Shell Clam (*Corbula gibba*). Three of these species: *Asterias amurensis*, *Carcinus maenas*, and *Undaria pinnatifida*, are also on the Australian Priority Marine Pest List (APMPL, https://www.marinepests.gov.au/what-we-do/apmpl). A national surveillance strategy for Australian ports was established in the 2000s, 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), but its implementation was limited. 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 over 2015 – 2016 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 the other six priority pests in four ports (Gladstone, Brisbane, Melbourne and Hobart) over 2017 – 2018 (Wiltshire *et al.* 2019a). 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 incurring less than half the field costs and requiring less than half the person-hours for collection and post-collection processing. Detection likelihood of most of the target pests varied between seasonal sampling sets, with the highest likelihood of detection for the species with pronounced seasonal patterns of detectability corresponding to their known spawning season (Wiltshire *et al.* 2019a). For detection of the six target species in temperate locations, a combination of sampling in late summer and late autumn provides the overall highest likelihood of detection, or requires the fewest samples to achieve a given survey sensitivity (Wiltshire *et al.* 2019a).

In addition to the assays for priority pests for domestic ballast water management, SARDI has developed assays for two pests currently exotic to Australia but of concern for introduction: New Zealand (NZ) green-lipped mussel (*Perna canaliculus*) and black-striped mussel (*Mytilopsis sallei*) (see Bott and Giblot-Ducray 2011b; Bott *et al.* 2012). Assays for additional exotic pests: Asian paddle crab (*Charybdis japonica*), Harris mud crab (*Rhithropanopeus harrisi*), brown mussel (*Perna perna*) and Asian green mussel (*Perna viridis*) have been developed (Simpson *et al.* 2018) and are now implemented in the SARDI testing system. With the exception of *Charybdis japonica*, these species are also on the APMPL.

The Department of Agriculture, Water and the Environment has commissioned molecular surveillance for marine pests at a number of ports of interest around Australia (Wiltshire *et al.* 2019c). In 2020, Molecular surveys were designed and conducted for the ports of Bunbury, Kwinana, Fremantle and Geraldton, Western Australia (WA) using the methods applied in Wiltshire *et al.* (2019a, c). Two plankton collections were conducted in each port to cover summer

and autumn sampling times. Samples were tested for the priority pests for domestic ballast water management and for the exotic pests for which assays are available. This report describes the design and outcome of these surveys.

1.2. Objectives

- Apply molecular surveillance for priority marine pests (8 species on the APMPL, plus 4 additional species of ballast water concern) to Bunbury, Kwinana, Fremantle and Geraldton;
- Map marine pest 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
- Obtain further data on seasonality of pest detections

2. METHODS

Surveys were designed for each port using the Monitoring Design Excel Tool (MDeT) version 2.5. The MDeT was originally developed to design surveillance using the traditional methods applied in the previous national surveillance strategy. Wiltshire *et al.* (2019a) identified several issues in the MDeT calculation of survey sensitivity for plankton tows and the molecular method, but alternative tools for survey design are lacking. The MDeT is populated with data that were compiled during its development, including information on the target species and methods. The parameters and calculations in the MDeT were derived from a range of data and literature, and through consultation with experts and working groups who developed the national surveillance strategy (Arthur *et al.* 2015). Method data was modified in MDeT to reflect the specific equipment and sampling techniques used. Plankton tow was the only sampling method used (see section 2.2) for these surveys because we were 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 the Marine Pest Monitoring Manual (National System for the Prevention and Management of Marine Pest Incursions 2010b) describes how to choose representative sublocations within each port for sampling and the MDeT calculates sample numbers per sublocation.

2.1. Target species and MDeT species data

The surveys were designed considering the seven target species used for guiding ballast water management:

- Asian Date Mussel (*Arcuatula senhousia*, formerly *Musculista senhousia*)
- Northern Pacific Seastar (*Asterias amurensis*)
- European Green Crab (*Carcinus maenas*)
- Basket Shell Clam (*Corbula gibba*)
- Pacific Oyster (*Crassostrea gigas*, also known as *Magallana gigas*)
- European Fan Worm (*Sabella spallanzanii*)
- Japanese kelp (*Undaria pinnatifida*)

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 were deleted so that only the seven

species of current interest were used in the design calculations; data for these were 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); whether the species is intertidal; conspicuous (suitable for visual surveys), sessile or motile, and the probability of the species fleeing and escaping detection if it is motile; duration of each life stage; salinity and temperature tolerances; and target population sizes. The target population sizes are set within the MDeT v2.5 to 2 000 juvenile or adult stages and 100 000 microscopic stages (gametes or larvae). Lastly, species data are used to flag any species that may be intolerant of the temperature or salinity range at each location. The MDeT lists these species and automatically excludes them from sample number calculations for locations where their tolerance limits are exceeded. The full MDeT species data used for the designs is provided in Wiltshire and Deveney (2017).

The molecular method applied in this survey is designed to target the planktonic stage (gametes/larvae), so the applicable habitat to sample is the water column. Water column volume is calculated in MDeT using provided location data: area of subtidal habitat and average water depth. Sample numbers calculated by MDeT are higher for larger locations because the target is 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, also provided in the location data, is included in calculations for planktonic stages, with a shorter residence time resulting in higher sample numbers because water turnover reduces the likelihood of detection due to propagules being lost from the survey area. Full details of the calculations used in the MDeT is included in the MDeT instructions (provided as appendix in Wiltshire and Deveney 2017). 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 assay for this species was not used pending redesign, because samples from the survey may be tested later with the redesigned assay.

2.2. Ports data

The ports of Bunbury, Kwinana, Fremantle and Geraldton, WA, were surveyed in 2020. 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.

Location data needed for the MDeT were compiled from a literature review for each port (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 (http://hydro.gov.au/). Detailed depth data were not available, hence, 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
Bunbury	Residence time, Salinity	Parsons Brinckerhoff (2013)
Kwinana	Residence time, Salinity	D'Adamo (2002)
Fremantle	Residence time, Salinity	Huang *et al.* (2017)
Geraldton	Salinity	Bridgwood and McDonald (2014)
	Residence time	WA EPA (2002)

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)
Bunbury		0.5		
Inner Harbour	12		65	543 833
Outer Harbour	4		5	368 559
Kwinana		1	32	
Alcoa Jetty	12			284 505
Bulk Terminal	6.5			508 393
Oil refinery/Bulk jetty	11			1 147 778
Grain Jetty	14			288 499
Fremantle	14	1.2	14	872 269
Geraldton	12	1.1	7	382 689

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 by Wiltshire *et al.* (2019a, c). The sample numbers generated 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). A minimum sample size of 15 per sample set was applied for each port. Wiltshire *et al.* (2019a) demonstrated that sampling in summer and autumn only, using one quarter of total MDeT calculated sample numbers at each sample time, was sufficient for detection

of the target species in locations where they occurred. Only two sample sets were collected at each port, therefore, being in summer and autumn as per molecular port surveys in 2018-19 (Wiltshire *et al.* 2019c). The total sample numbers generated by MDeT for each WA port sampled are shown in Table 3.

To assign proposed sample locations for plankton tows, we used the *samplePts* function from the *R* (R Core Team 2020) 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 placed was derived from a shape-file 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.

Table 3. MDeT generated sample numbers and proposed numbers per sample set for each port (including by sublocation where port survey area included more than one location) for MDeT calculated sensitivities of 0.8 and 0.6. Per sample numbers were calculated assuming four approximately seasonal sample sets.

	MDeT sensitivity = 0.8		**MDeT sensitivity = 0.6**	
Port Sublocation	**Total**	**Per sample set**	**Total**	**Per sample set**
Bunbury	**90**	**23**	**53**	**14**
Inner Harbour	68	17	40	10
Outer Harbour	22	6	13	4
Kwinana	**248**	**60**	**142**	**37**
Alcoa Jetty	36	9	21	6
Bulk Terminal	35	9	20	5
Oil refinery/Bulk jetty	128	32	76	19
Grain Jetty	40	10	25	7
Fremantle	**129**	**33**	**74**	**19**
Geraldton	**49**	**15**	**28**	**7**

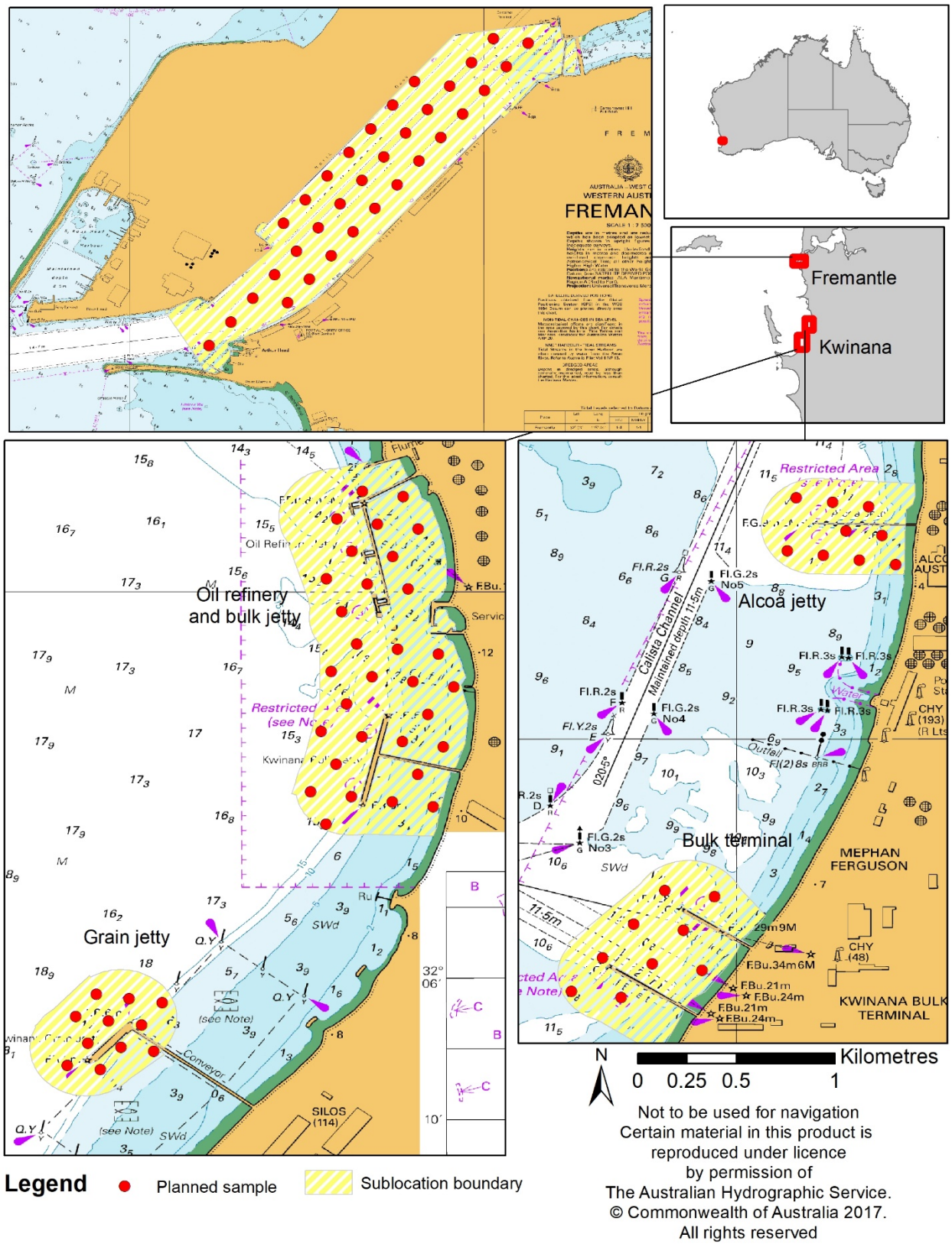

Figure 1. Proposed plankton sampling locations in Fremantle and Kwinana.

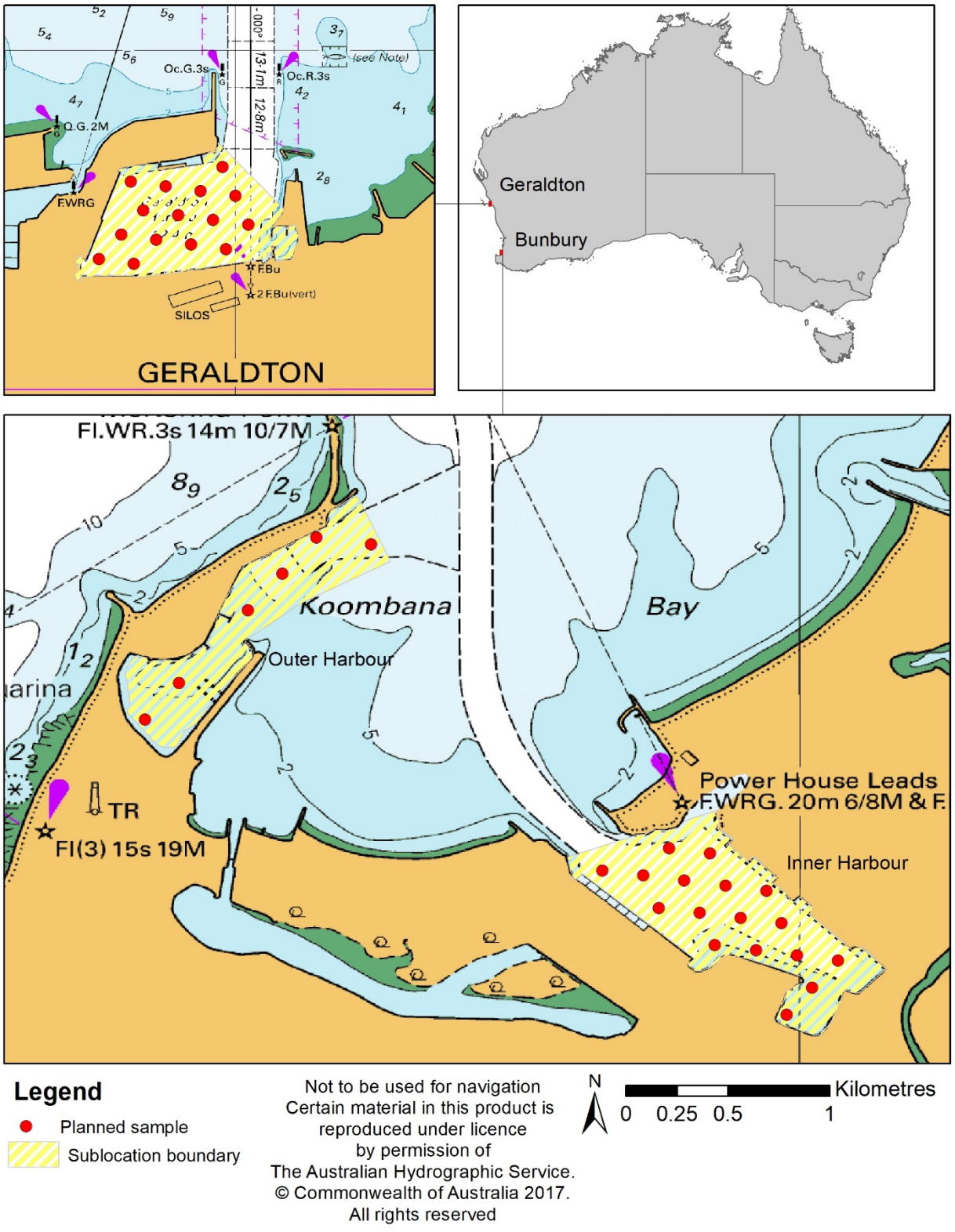

Figure 2. Proposed plankton sampling locations in Geraldton and Bunbury.

2.4. Plankton tow collection

The number of plankton samples collected at each port was that based on MDeT sensitivity of 0.8 (Table 3). All samples were processed.

Plankton samples for molecular analysis were collected based on the methods developed by Giblot-Ducray and Bott (2013) and refined by Deveney *et al.* (2017). A conical mesh plankton net with mouth diameter 0.5 m, length 1.5 m and 50 µm mesh (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). After collection, samples were kept cool in an insulated container with gel ice-packs or refrigerator 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).

Field collection, immediate storage and dispatch of plankton samples was undertaken by Cardno (WA) Pty Ltd (Cardno). Proposed sampling locations (see section 2.3) could be altered if necessary due to field conditions, access or logistical constraints. The final selection of sample locations was, therefore, at the discretion of the third-party field sampling organization. Cardno provided data on plankton-tow start and end points, which were recorded by a Garmin hand-held GPS, flowmeter readings, from which tow distance was calculated, and notes pertaining to field conditions and individual samples. All field data were provided to SARDI for compilation.

A sample quality assurance control consisting of a 50 µL aliquot of *Artemia salina* (Ocean Nutrition™ Instant Baby Brine Shrimp; hereafter *Artemia*), was added to the preservation buffer of one in every five sample tubes prior to sampling.

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. DNA extraction and qPCR analysis were carried out by the SARDI Molecular Diagnostics laboratory. DNA was extracted from samples using the method developed by SARDI Molecular Diagnostics, with 20 mL of DNA extraction buffer containing an inhibition control (standardised quantity of an exogenous organism) 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 by comparison with commercial methods (Haling *et al.* 2011). The 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 a 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.

Table 4. Target pest species, assay gene target, and reference for assay. * Assay for which diagnostic performance has been determined (Wiltshire *et al.* 2019b). †Species on APMPL. Standard shows type used for standard curve: genomic (gDNA) or synthetic. C_T low-level shows the C_T value above which a detection is considered low level (see section 2.5.1).

Species	Gene target	Standard	C_T low-level	Reference
Domestic ballast water species of concern				
Arcuatula senhousia	28S rDNA	gDNA	44.7	Bott and Giblot-Ducray (2011)
*Asterias amurensis**†	Cox1	gDNA	38.2	Bott et al. (2010a)
*Carcinus maenas**†	Cox1	gDNA	37.4	Bott et al. (2010a)
*Crassostrea gigas**	Cox1	gDNA	37.0	Bott and Giblot-Ducray (2012)
*Sabella spallanzanii**	28S rDNA	synthetic	37.2	Ophel-Keller et al. (2007)
*Undaria pinnatifida**†	Cox1	gDNA	37.3	Bott et al. (2010a)
Exotic pests of concern				
Charybdis japonica	Cox1	synthetic	36.6	Simpson *et al.* (2018)
Mytilopsis sallei†	Cox1	gDNA	36.8	Bott *et al.* (2012)
Perna canaliculus†	IGS	gDNA	37.3	Bott and Giblot-Ducray (2011b)
Perna perna†	IGS	synthetic	37.8	Dias *et al.* (2013)
Perna viridis†	IGS	synthetic	37.2	Dias *et al.* (2013)
Rhithropanopeus harrisii†	Cox1	synthetic	36.1	Simpson *et al.* (2018)

Testing included negative controls, the appropriate calibration standard for each target pest, the sample quality assurance control, which was tested using an *Artemia* qPCR assay (Mackie and Geller 2010), and the inhibition control. For each PCR analysis batch, reference samples that are known to not cause inhibition were also extracted after addition of the inhibition control organism and tested by qPCR. 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. The scale factor for a sample is used as a multiplier to correct the apparent DNA concentration as calculated

from the C_T value for the effects of inhibition (Ophel-Keller *et al.* 2008). *Artemia* yield from the samples containing the sampling quality assurance control was 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.

To avoid cross-contamination between samples from different locations, 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.

2.5.1. Classification of low level detections

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. We (Wiltshire *et al.* 2019a; Wiltshire *et al.* 2019c) have regarded detections with a C_T value > 40 as low-level. PCR efficiency, however, varies across the assays from 74.9 – 99.8%, which means that the DNA yield providing a C_T of 40 is not the same for each species. Standard curves, which allow the yield of target DNA to be determined from the C_T value of a positive detection, have been developed for each assay, but the curves use different standard types, with genomic DNA standards used for assays developed in 2010 – 2012, and synthetic standards used for the subsequently developed assays and *S. spallanzanii* for which a standard curve was developed in late 2019 (Table 4). The use of different standards means that calculated DNA yield is comparable within, but not between, species. We therefore determined the C_T value for each assay that would result from an equivalent number of DNA molecules as a C_T value of 36 using a perfectly efficient (100%) assay, assuming that the perfect assay would return a C_T of 45 for a single molecule. These C_T low-level criterion values are shown in Table 4.

2.6. Mapping and statistical methods

Field data collected by Cardno during plankton sampling and molecular analysis results were compiled with qPCR results linked to recorded field sampling locations. For results presented in this report, tow midpoint, calculated using the average of start and end point latitude and longitude, was used to map each tow location.

2.6.1. Relative field DNA

Rasters of interpolated relative field DNA were generated for each pest with at least 5 detections in a sample set. The relative DNA used to generate rasters was the sample DNA yield calculated

from the standard curve for each species, multiplied by the scaling factor for each sample to account for inhibition where present. To relate DNA in a sample to field DNA concentration, the volume of water filtered by the plankton net needs to be considered. The same specification net was used for all samples, therefore, the water volume filtered is directly proportional to the flow-meter distance. Relative field DNA was therefore calculated as sample 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 detections were generated using ArcGIS 10.6 (Esri Inc).

2.6.2. Estimated true prevalence

In environmental surveillance, prevalence is the likelihood that a sample will contain a target, i.e. the frequency of occurrence of target DNA in plankton samples. Apparent prevalence is the proportion of samples returning a positive detection; where assay diagnostic performance has been assessed, an estimate of true prevalence, which adjusts apparent prevalence for possible false positives and false negatives, can be made (Speybroeck *et al.* 2013). Diagnostic performance has been assessed for five of the qPCR assays applied in this project (Table 4) (Wiltshire *et al.* 2019b), permitting estimation of true prevalence for these species from surveillance results. Bayesian analysis provide estimates of true prevalence with credible bounds (based on 95% of the posterior probability), given the sampling effort and assay performance (Speybroeck *et al.* 2013). The upper credible limit shows the maximum plausible prevalence, which provides the basis for Bayesian proof of freedom approaches for species that are not detected (Low-Choy 2013; Stanaway 2015). The relationship between DNA prevalence in plankton and adult pest population size is unknown, but an estimation of true prevalence allows a standardised comparison of results across species for which test performance is not identical. Prevalence for each species at each port was determined in a Bayesian framework using code modified from the *truPrev* function of the *prevalence R* package (Devleesschauwer *et al.* 2014) to simultaneously estimate prevalence for multiple species. Beta priors were used for diagnostic sensitivity (DSe) and specificity (DSp) of each species assay, with beta parameters calculated using the *prevalence* package *betaExpert* function (Devleesschauwer *et al.* 2014). For the assays for which diagnostic performance has been assessed, priors were assigned based on results from from Wiltshire *et al.* (2019b) using the lowest (most conservative) estimate in each case. For the remaining assays, the estimate for *U. pinnatifida*, which had the lowest DSe of the assays assessed by Wiltshire *et al.* (2019b), was used. All assessed assays had DSp ~ 1; the same was assumed for the remaining assays. The DSe and DSp priors are described in Table 5. An

uninformative *Beta*(1,1) prior was used for true prevalence of each species; this prior allows equal probability for any prevalence between 0 and 1.

The prevalence analysis was run in JAGS (Plummer 2017) using *R* (R Core Team 2020) and package *R2jags* (Su and Yajima 2015) with 10 000 MCMC iterations thinned at a rate of 10, following 50 000 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). Highest density intervals (HDIs) demonstrating 95% of the probability mass for posterior estimates were calculated using the *HDIinterval* package (Meredith and Kruschke 2018).

Table 5. Description of priors used for DSe and DSp of each assay for estimation of true prevalence. Prior estimate shows most likely value and 95% credible range. Parameters were assigned a beta prior: beta(a,b), with a and b values calculated using *betaExpert* (Devleesschauwer *et al.* 2014).

Species	Parameter	Prior estimate	a	b
Asterias amurensis	DSe	0.89 (0.83 – 0.94)	110	13.4
	DSp	1.00 (0.99 – 1.00)	273	0.625
Carcinus maenas	DSe	0.79 (0.62 – 0.91)	21.9	5.68
	DSp	1.00 (0.99 – 1.00)	273	0.625
Crassostrea gigas	DSe	0.91 (0.83 – 0.97)	65.0	6.42
	DSp	1.00 (0.99 – 1.00)	273	0.625
Sabella spallanzanii	DSe	0.86 (0.79 – 0.93)	90.2	14.9
	DSp	1.00 (0.99 – 1.00)	273	0.625
***Undaria pinnatifida* and all other species**	DSe	0.73 (0.59 – 0.88)	25.8	9.50
	DSp	1.00 (0.99 – 1.00)	273	0.625

2.6.3. Temporal patterns of molecular detections and relative DNA

Patterns in likelihood of qPCR detection and relative DNA across sample sets were analysed for species with detections in both summer and autumn sample sets at a location. Port was included as a factor in the analysis where detections occurred in multiple ports. Bayesian zero-altered lognormal (ZALN) models were run following Zuur and Ieno (2016) and included predictors for both the binary component, i.e. likelihood of detection, and continuous component, i.e. DNA, which was calculated from the standard curve.

Two models were run, 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 sample DNA yield. The relative DNA yield in a sample (relDNA) as calculated from the C_T value may be less than the actual DNA present where PCR inhibition, as measured by the scale factor (SF), is present. To correct for inhibition, the DNA in a sample (sampDNA) is taken to be given by: sampDNA = relDNA x SF. The scale factor, however, is calculated based on differences in DNA yield of the internal control between plankton and control samples (see section 2.5). Because it is possible that the pest and internal control assays are not identically impacted by inhibition, the multiplicative effect of scale factor may not be constant between species. The total sample DNA relates to the relative field DNA (fieldDNA) concentration as: sampDNA = fieldDNA x sample volume. Since all samples were collected with the same specification plankton net, the flow-meter distance (FMdist) can be used as a measure of relative sample volume. 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(SF)

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

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 (mean 0, precision 0.0001) were used for coefficients for sample set, port and port x sample set for both model components. An informative normal prior with a mean of one and standard deviation of 0.5 was used for β_{SF}, indicating that the same response for pest DNA to inhibition as for the internal control is expected, but allowing the multiplier to vary slightly. Specifically, this prior indicates 95% probability that the scale factor multiplier is between 0.02 and 1.98.

These analyses were run and outputs assessed as per the prevalence models (see section 2.6.2) except that a larger number of iterations were needed for convergence: 50 000, thinned at a rate of 50, following 100 000 iterations for burn-in.

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. In contrast to some recent surveys where a subset of collected samples were processed and analysed (Wiltshire *et al.* 2019a; Wiltshire *et al.* 2019c), all collected samples from the current surveys were analysed. Summer sampling took place in late February in Geraldton and Bunbury, but access and logistical issues meant that sampling in Fremantle and Kwinana did not take place until mid-March (Table 6). March is still considered "summer" from the perspective of molecular surveillance, given that water temperature lags behind seasonal air temperature changes, and that "summer" sampling used to assess seasonal patterns (Wiltshire *et al.* 2019a) was also conducted in early autumn (March – April).

Table 6. Number of plankton samples collected and analysed and sample dates for each sampling event.

Sample set	Port	No. samples	Start date	End date
Summer	Bunbury	23	26-Feb-20	26-Feb-20
	Kwinana	60	18-Mar-20	19-Mar-20
	Fremantle	33	17-Mar-20	17-Mar-20
	Geraldton	15	25-Feb-20	25-Feb-20
Autumn	Bunbury	23	14-May-20	14-May-20
	Kwinana	60	11-May-20	13-May-20
	Fremantle	33	12-May-20	12-May-20
	Geraldton	15	19-May-20	19-May-20

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

Maps of sample locations and detections are shown in Figures 3 – 6. Figures are grouped by location and sampling season. All maps show the port locations at 1:25 000. In each map, port location maps show results 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 concentration are shown by location in Figures 7 – 9. Note that maps of relative DNA were only generated where at least five detections of a species occurred in a sampling event and that DNA concentration shown is the relative concentration within each species, i.e., the scale is not comparable across species, but, for *Ar. senhousia*, is comparable

across sample sets. The rasters show the full extent of each area surveyed, but values outside the immediate area of sample collection should be interpreted with caution.

3.3. Species detections by qPCR and patterns in relative DNA

Arcuatula senhousia was widely detected in Kwinana and Fremantle in both sample sets (Table 7; Figures 3 – 4). *Sabella spallanzanii* was detected in Kwinana (in 13 of 60 samples) and Bunbury (in a single sample) in autumn (Figures 4, 6), *Crassostrea gigas* was detected in 7 of 15 samples from Geraldton in autumn (Figure 6), and *Rhithropanopeus harrisi* was detected in a single sample from Fremantle in summer (Figure 3). There were no pest detections in the summer samples for Geraldton or Bunbury (Figure 5).

Highest DNA concentrations for *Ar. senhousia* were recorded in the central area of Fremantle port and around the bulk terminal of Kwinana in summer (Figure 7), and near the oil refinery and bulk jetty at Kwinana in autumn (Figure 8). For *Cr. gigas* in Geraldton in autumn, highest DNA concentrations occurred on the northern side of the harbour, although variation in DNA concentration between samples with detections was minor (Figure 9). Highest DNA concentrations of *S. spallanzanii* in Kwinana in autumn occurred around the bulk jetty and oil refinery (Figure 9).

For *Cr. gigas*, 6 of the 7 recorded C_T values were above the low-level criterion (Table 7), and all were within the highest 6% of values from detections in ports and other regions where this species is established (Deveney *et al.* 2017; Wiltshire *et al.* 2019a; Wiltshire *et al.* 2019c; Deveney *et al.* 2020). Figure 10 shows the C_T values recorded in Geraldton overlaid on a histogram of C_T values of detections from surveillance of other areas.

Figure 3. Map of plankton sample locations and molecular detections for Fremantle and Kwinana in summer 2020.

Figure 4. Map of plankton sample locations and molecular detections for Fremantle and Kwinana in autumn 2020.

Figure 5. Map of plankton sample locations and molecular detections for Geraldton and Bunbury in summer 2020.

Figure 6. Map of plankton sample locations and molecular detections for Geraldton and Bunbury in autumn 2020.

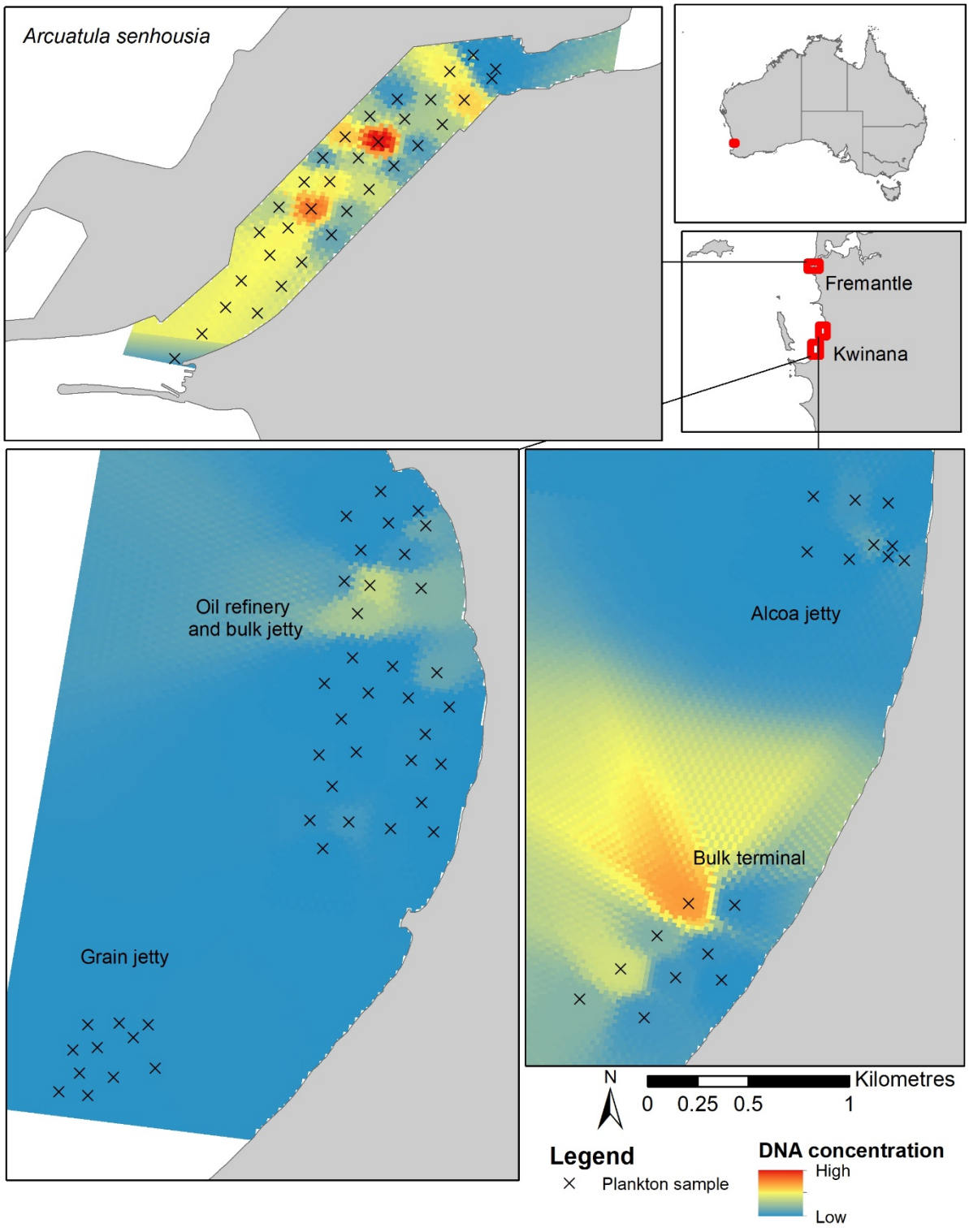

Figure 7. Map of relative DNA concentration for *Arcuatula senhousia* in Fremantle and Kwinana in summer sampling.

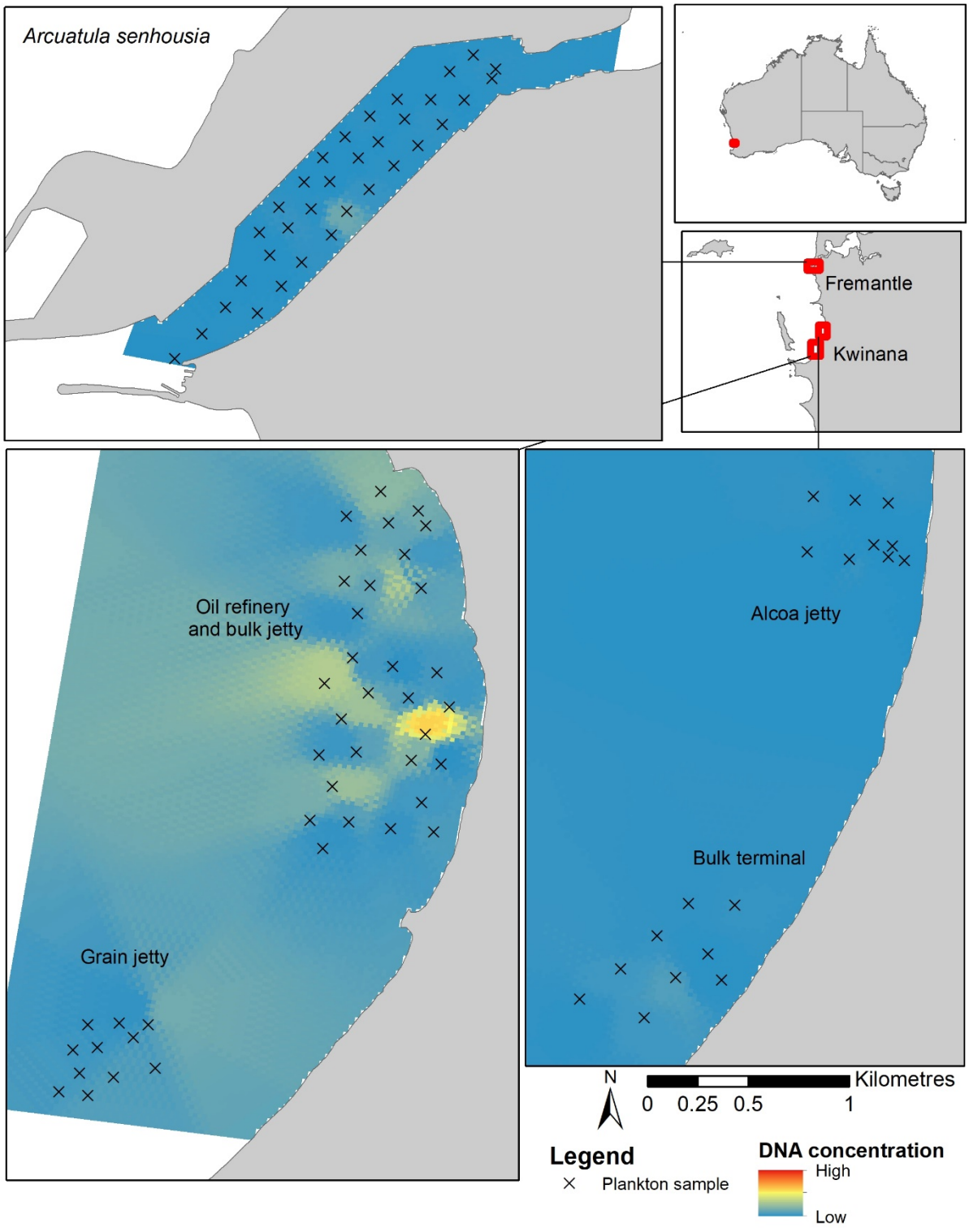

Figure 8. Map of relative DNA concentration for *Arcuatula senhousia* in Fremantle and Kwinana in autumn sampling.

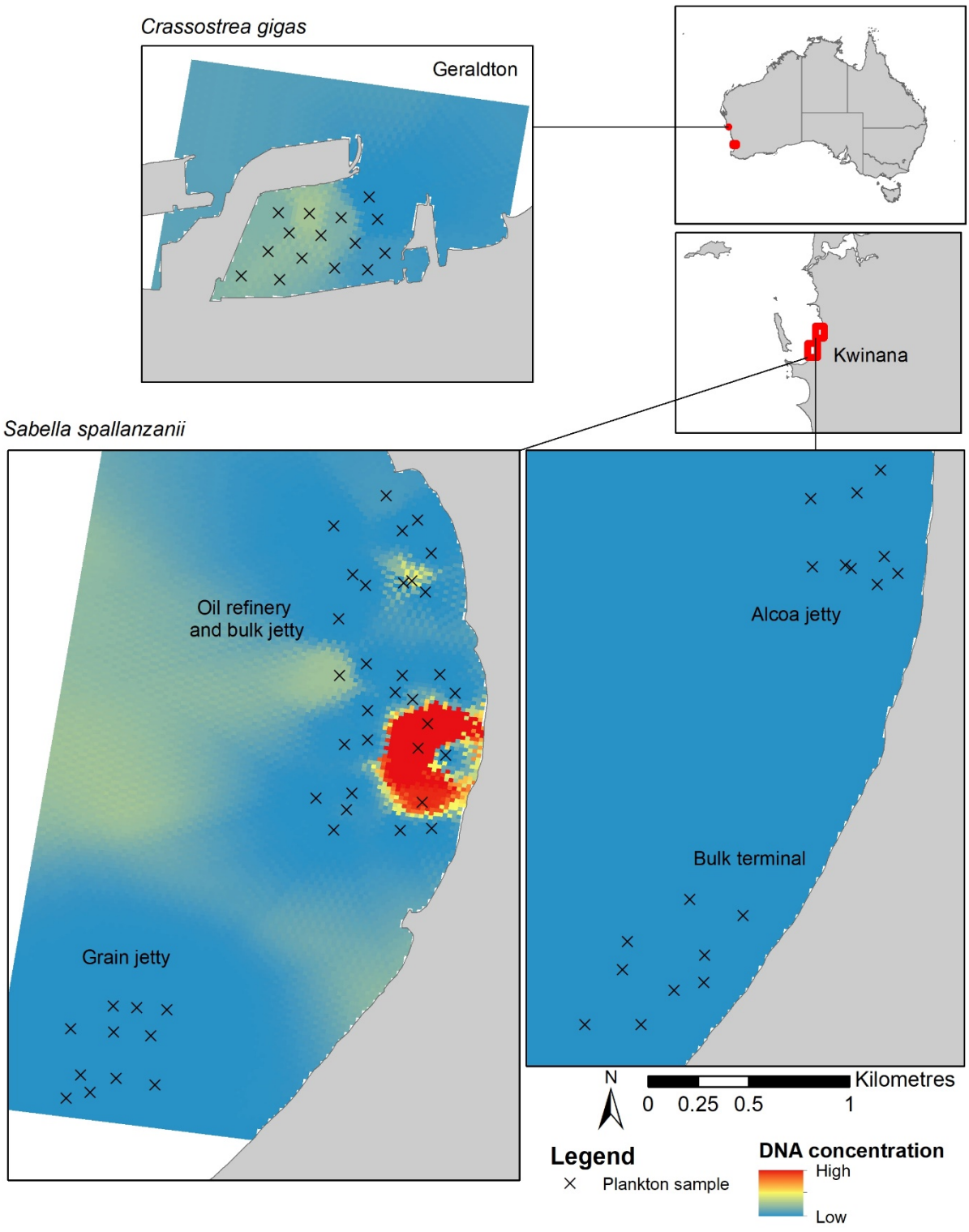

Figure 9. Map of relative DNA concentration for *Crassostrea gigas* in Geraldton (top), and *Sabella spallanzanii* in Kwinana (bottom) in autumn sampling.

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 > low-level criterion; see Table 4) is shown in brackets where > 0.

Sample Set Port	No. of Samples (no. with high SF)	No. of detections (number low level)			
		Ar. senhousia	*Cr. gigas*	*S. spallanzanii*	*R. harrisii*
Summer					
Bunbury	23 (1)	0	0	0	0
Kwinana	60 (0)	57	0	0	0
Fremantle	33 (0)	32	0	0	1 (1)
Geraldton	15 (0)	0	0	0	0
Autumn					
Bunbury	23 (2)	0	0	1 (1)	0
Kwinana	60 (0)	58	0	13 (7)	0
Fremantle	33 (0)	33	0	0	0
Geraldton	15 (0)	0	7 (6)	0	0

3.4. Estimated true prevalence

Arcuatula senhousia was the species detected most frequently in the survey, occurring in nearly every sample from both Kwinana and Fremantle in both sample sets, but it was not detected at Bunbury or Geraldton. Estimated true prevalence was close to 1 for this species in Kwinana and Fremantle (Table 8). Overall estimated prevalence of *S. spallanzanii,* which was detected only in autumn, was 0.132 (95% HDI 0.070 – 0.199) in Kwinana, and 0.047 (95% HDI 0.000 – 0.112) in Bunbury, where a single detection occurred. Overall estimated prevalence of *Cr. gigas* in Geraldton was 0.277 (95% HDI 0.120 – 0.442).

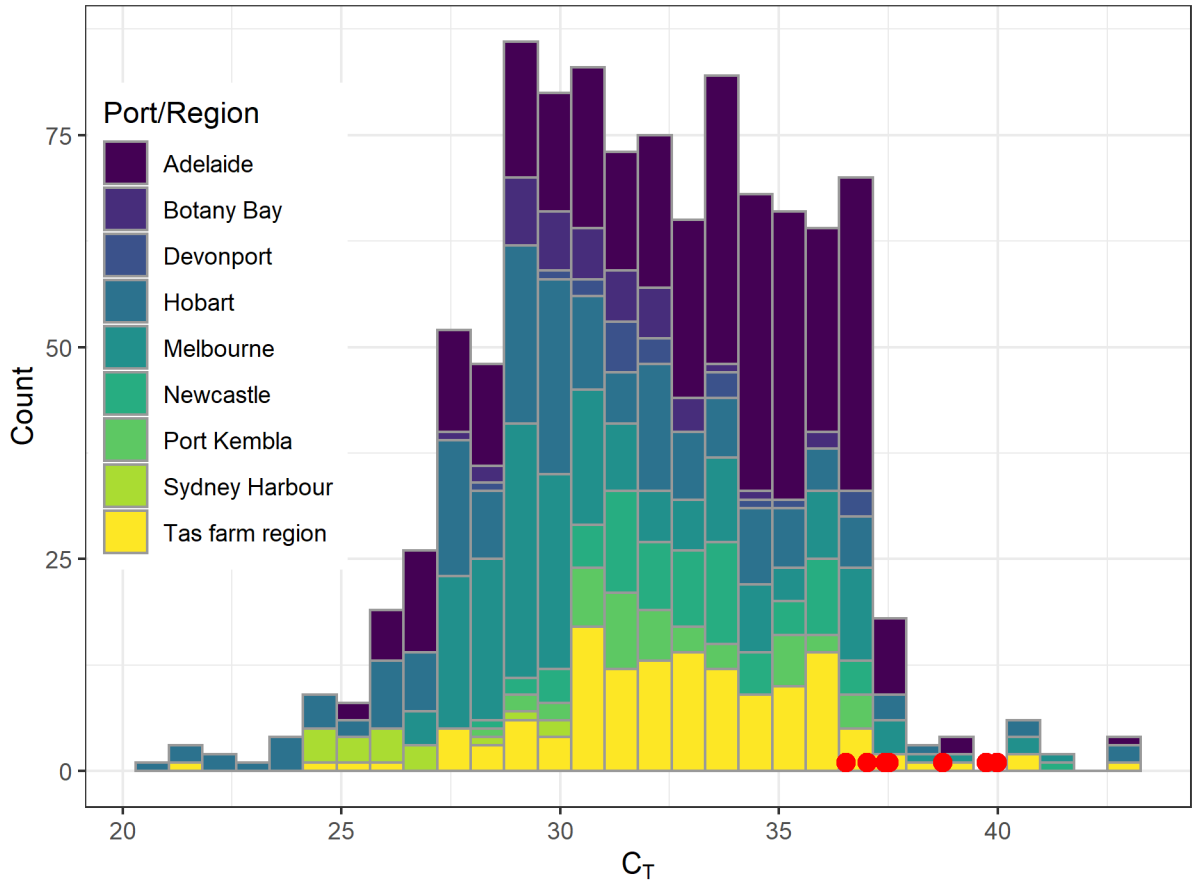

Figure 10. Histogram of C_T values for *Crassostrea gigas* detections from previous surveillance of areas where the species occurs, overlaid with C_T values from Geraldton detections in red. Note that high C_T is indicative of low sample DNA yield (see section 2.5).

For species without detections, or where a detection is potentially of transient (i.e. temporarily present) material, the upper limit of the 95% HDI shows the maximum plausible prevalence, given the number of samples analysed and assay performance, which is the basis for Bayesian proof-of-freedom approaches (Low-Choy 2013; Stanaway 2015). Note that performance has not been assessed for some assays, but we applied a conservatively low estimate of DSe, which will lead to higher maximum plausible prevalence than assuming higher DSe. For *Rhithropanopeus harrisii*, which returned a single detection in Fremantle, maximum plausible prevalence was 0.092. The maximum plausible prevalence for undetected species varied across locations due to the differing sampling effort, being highest in Geraldton where a total of 30 samples were analysed, compared with 46 – 120 samples at other ports. The maximum plausible prevalence for a species with no detections was 0.135 (Table 8).

Table 8. Overall estimated prevalence cross sample sets for each species accounting for diagnostic test performance (* indicates actual performance of assay has been assessed; for other species, a conservative estimate was used). Shaded cells indicate ports with species detections, with darker shading and bold text showing species with multiple detections and light shading showing single detections.

	Estimated true prevalence (95% highest density interval)			
Port	**Bunbury**	**Kwinana**	**Fremantle**	**Geraldton**
Arcuatula senhousia	0.023 (0.000 – 0.064)	**0.984 (0.955 – 1.000)**	**0.983 (0.951 – 1.000)**	0.033 (0.000 – 0.098)
*Asterias amurensis**	0.024 (0.000 – 0.072)	0.009 (0.000 – 0.027)	0.016 (0.000 – 0.048)	0.034 (0.000 – 0.104)
*Carcinus maenas**	0.027 (0.000 – 0.079)	0.010 (0.000 – 0.032)	0.019 (0.000 – 0.056)	0.041 (0.000 – 0.121)
*Crassostrea gigas**	0.023 (0.000 – 0.066)	0.009 (0.000 – 0.025)	0.016 (0.000 – 0.047)	**0.277 (0.120 – 0.442)**
Rhithropanopeus harrisii	0.030 (0.000 – 0.090)	0.012 (0.000 – 0.034)	0.039 (0.001 – 0.092)	0.044 (0.000 – 0.135)
*Sabella spallanzanii**	0.047 (0.000 – 0.112)	**0.132 (0.070 – 0.199)**	0.017 (0.000 – 0.052)	0.036 (0.000 – 0.107)
All other species	0.029 (0.000 – 0.084)	0.011 (0.000 – 0.034)	0.021 (0.000 – 0.064)	0.044 (0.000 – 0.128)

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

PCR inhibition, as measured by the scale factor, occurred in some samples from most sampling events, but predominantly at a low level (scale factor < 2) and without consistent patterns. Inhibition was more common in summer than autumn for Geraldton and Fremantle, while Kwinana and Bunbury both had more samples with inhibition in autumn than summer (Figure 11). Across both sample sets, only three samples showed high inhibition (scale factor >5), all of which were from Bunbury, one in summer (scale factor 5.1) and two in autumn (maximum scale factor 8.6). Moderate inhibition (scale factor 2 – 5) occurred in three summer samples from each of the ports and, in 28 and 13 autumn samples from Kwinana and Bunbury, respectively.

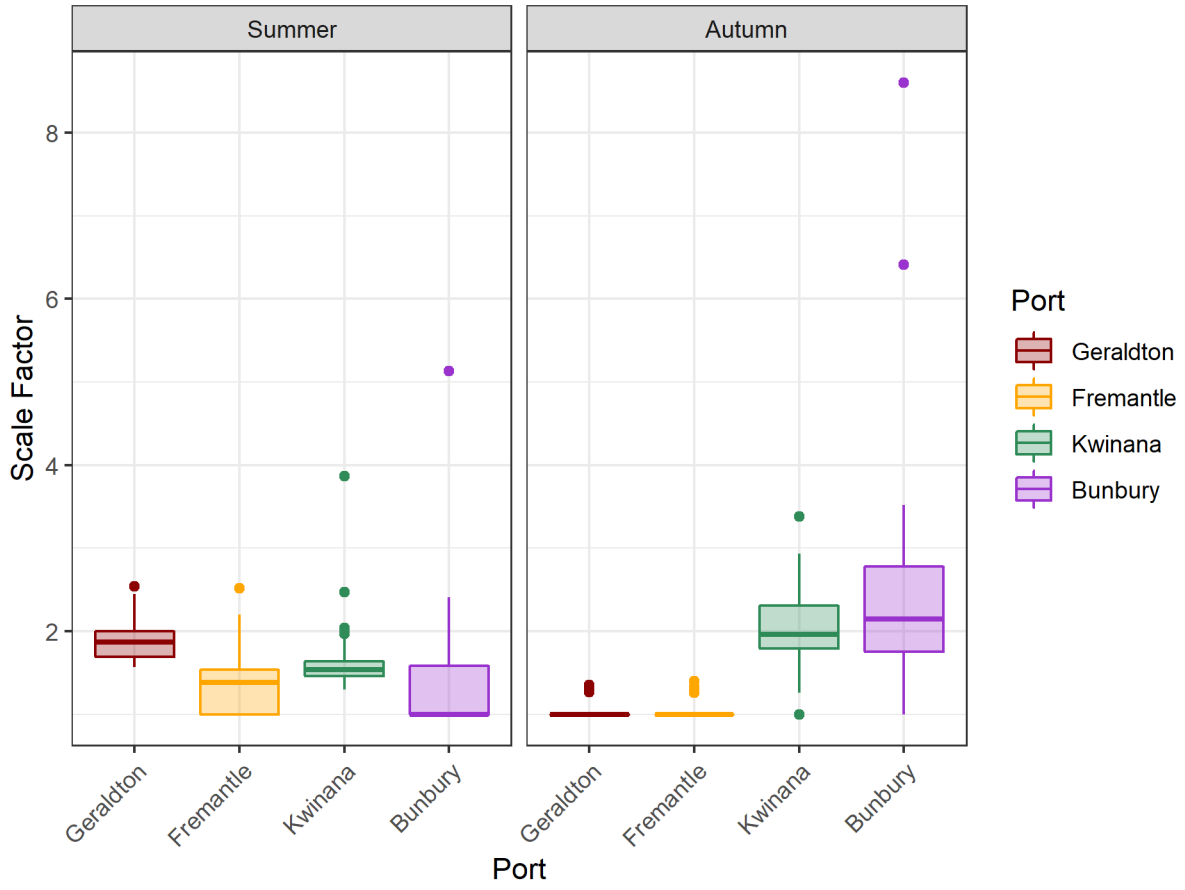

Figure 11. Boxplot of scale factor by port and collecting event.

Flow-meter distance, which provides the effective tow length and is directly proportional to sample volume given that the same dimension and mesh size net was used for all sampling, was generally similar across ports and sample sets, with the exception of Fremantle in summer, where effective tow length was relatively low (Figure 12). A sample length of 100 m, as measured by GPS, was targeted for the sampling, but flow-meter distances were typically less than 50 m, indicating that 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.

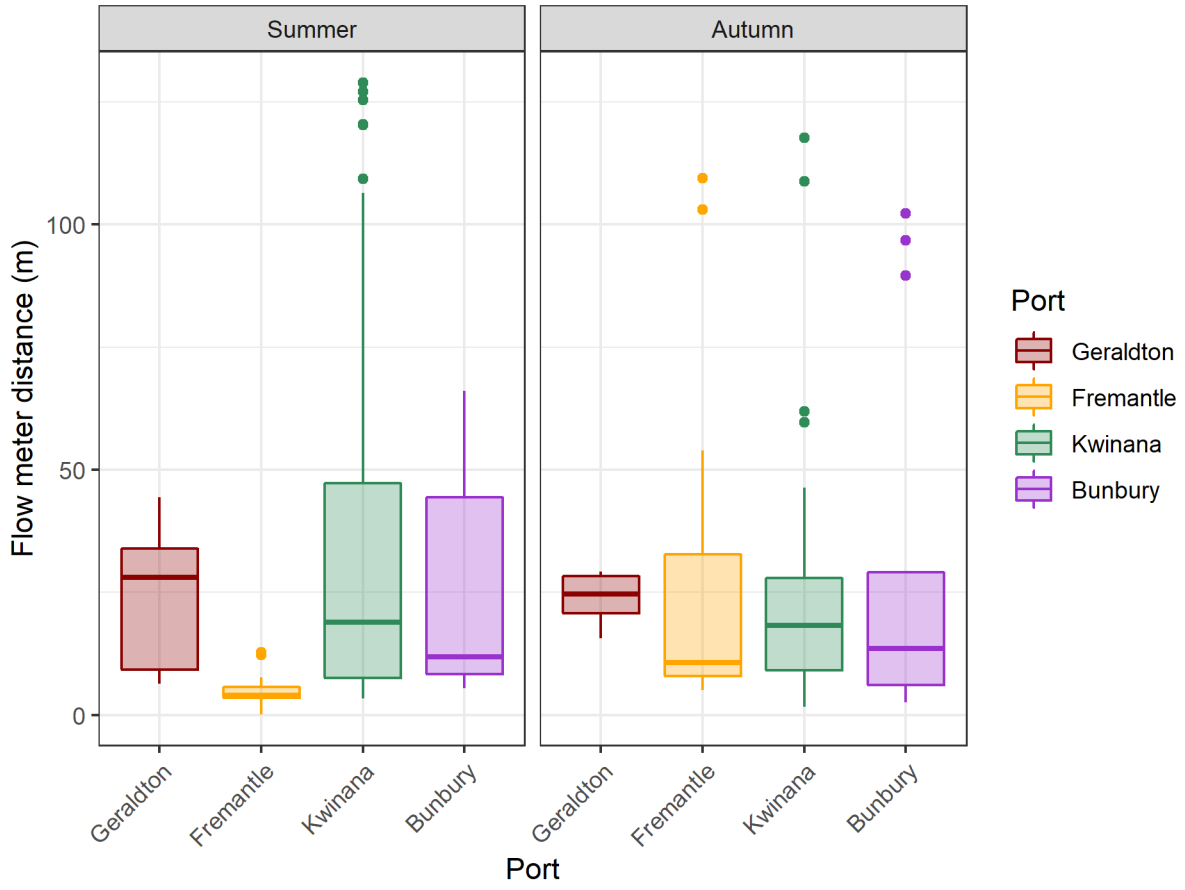

Figure 12. Boxplot of flow-meter distance, as a proxy for sample water volume, by port and collection season.

Sample dry weight was typically 1 – 2 g, similar to that recorded in most ports and sample sets in Wiltshire *et al.* (2019a); autumn samples from Fremantle and Bunbury, however, typically had dry weights <1 g (Figure 13).

Flow-meter distance and sample dry weights were within typical ranges of previous sampling (Wiltshire *et al.* 2019a; Wiltshire *et al.* 2019c). *Artemia* yield from field samples was similar to that of controls in all cases indicating that there was no evidence of sample degradation in any sample set. Normal *Artemia* yield and low PCR inhibition indicate that there were no issues with sampling that compromised detections.

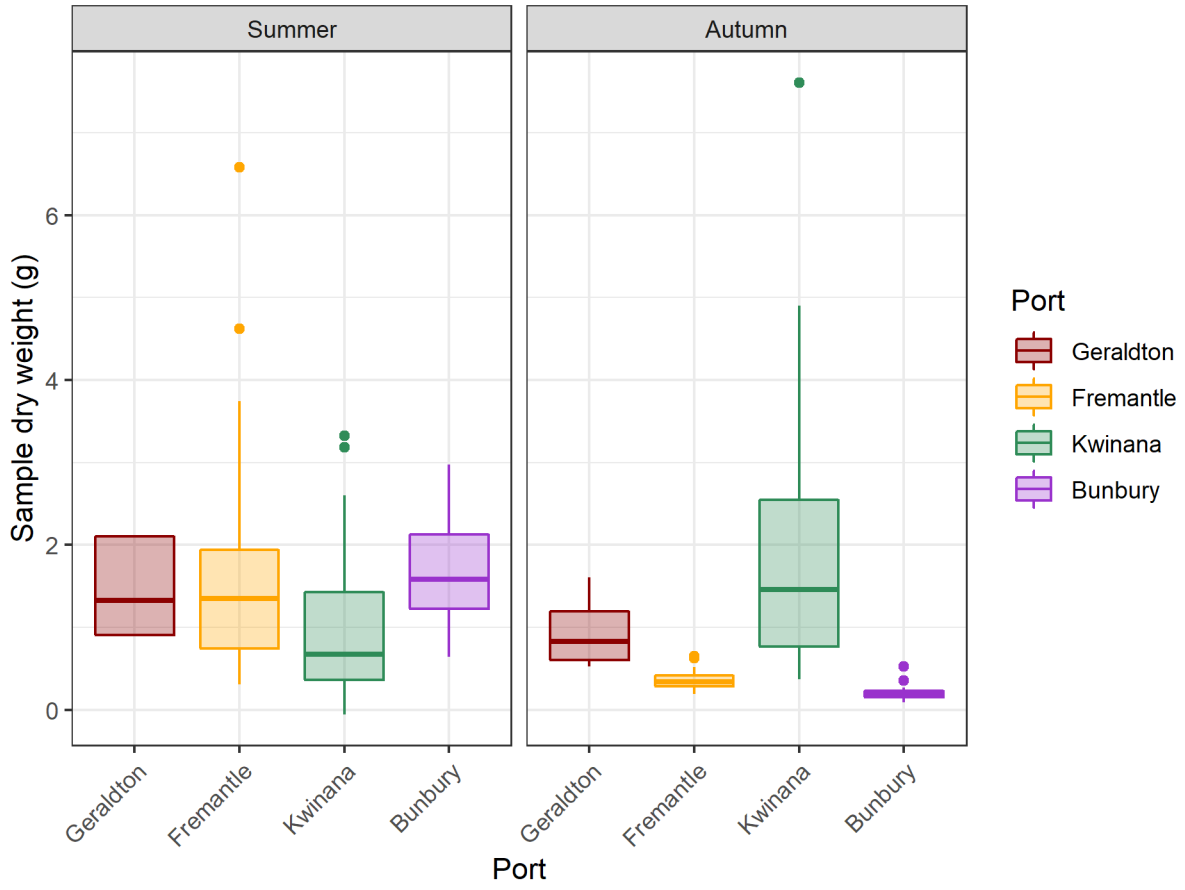

Figure 13. Boxplot of filtered sample dry weight by port and collecting season.

3.6. Temporal variation in *Arcuatula senhousia* detections and relative DNA

Arcuatula senhousia was the only species with detections across sample sets; these detections were at two ports, Fremantle and Kwinana. There was no difference in likelihood of detection between sample sets and ports, with detection likelihood predicted by the zero added log normal (ZALN) model being > 0.975 for both sample sets in each port (Figure 14). Relative DNA yield in samples displayed an apparently different seasonal pattern between ports: Fremantle had higher DNA yields in autumn than summer, and Kwinana had higher DNA yield in summer than autumn, (Figure 15). Field DNA concentration adjusted for sample volume was lower overall in Kwinana than Fremantle and lower in summer than autumn, but the difference in seasonal patterns between ports remained clear (Figure 16).

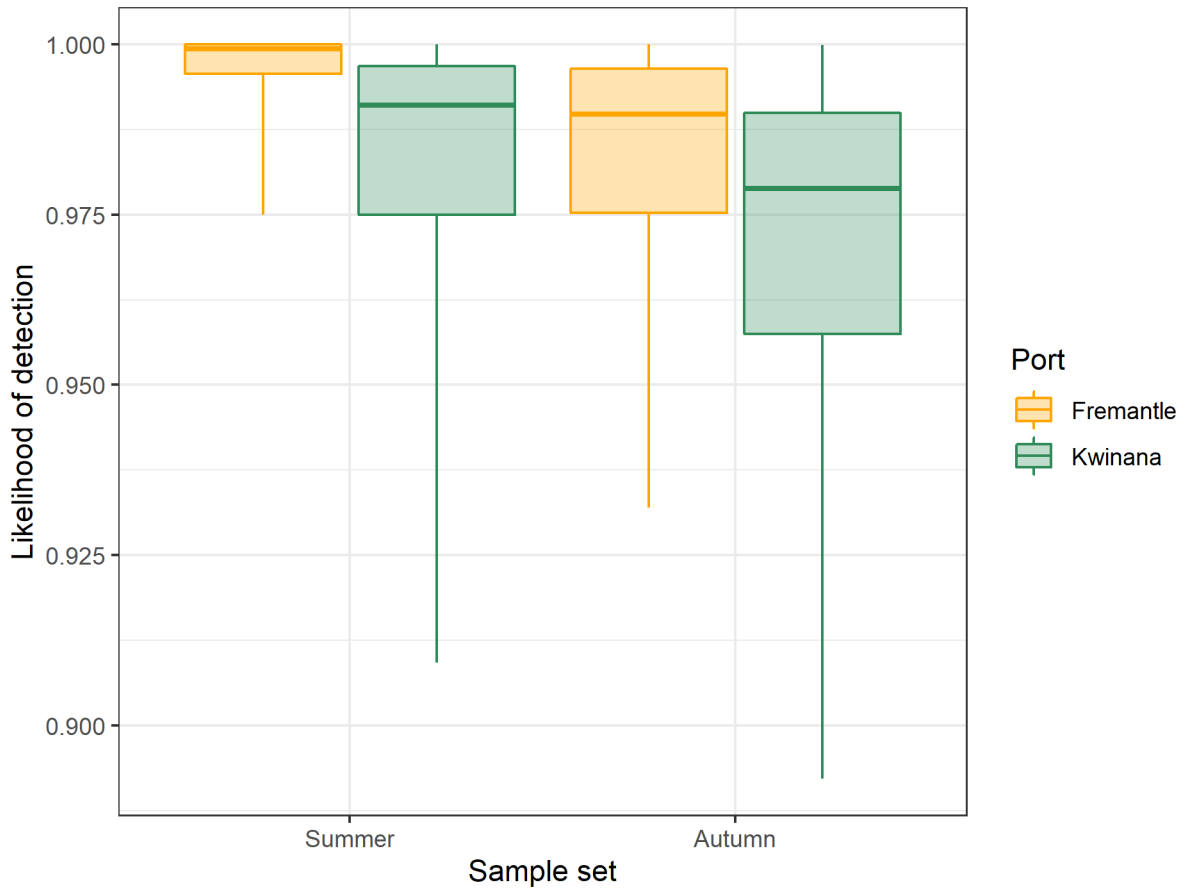

Figure 14. Posterior predictions of mean detection frequency for *Arcuatula senhousia* in Fremantle and Kwinana samples by sample set. Boxes show mean and interquartile range, with whiskers showing 95% HDIs.

PCR inhibition, as measured by scale factor, did not affect detection likelihood for *Ar. senhousia* (posterior mean coefficient −0.89, 95% HDI −3.03 – 1.22), but affected relative DNA concentration, with the scale factor multiplier predicted to be 0.81 (95% HDI 0.20 – 1.41). It should be noted, however, that there was little variation in scale factor between samples, and no samples with high inhibition included in this analysis.

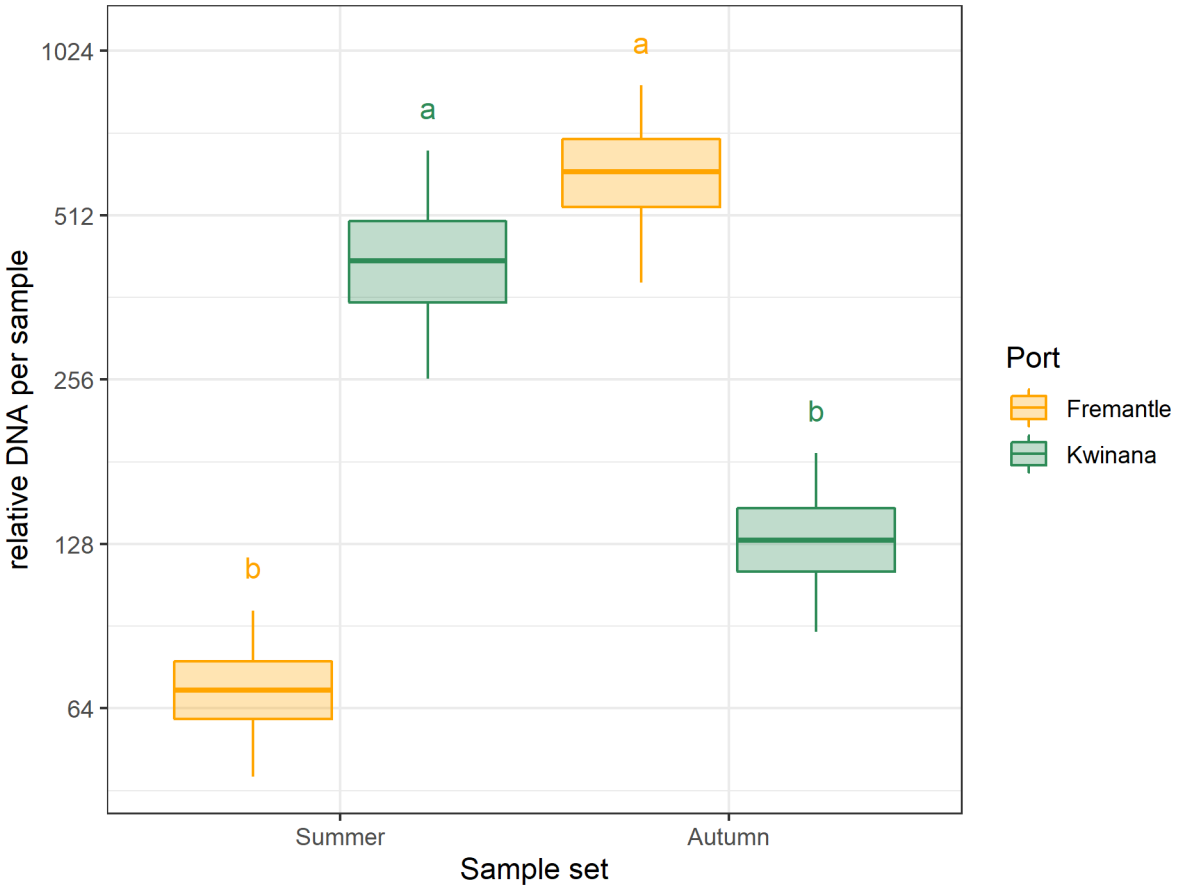

Figure 15. Posterior predictions of mean relative DNA yield for *Arcuatula senhousia* in Fremantle and Kwinana samples by sample set. Boxes show mean and interquartile range, with whiskers showing 95% HDIs. Different letters indicate 95% HDIs that do not overlap.

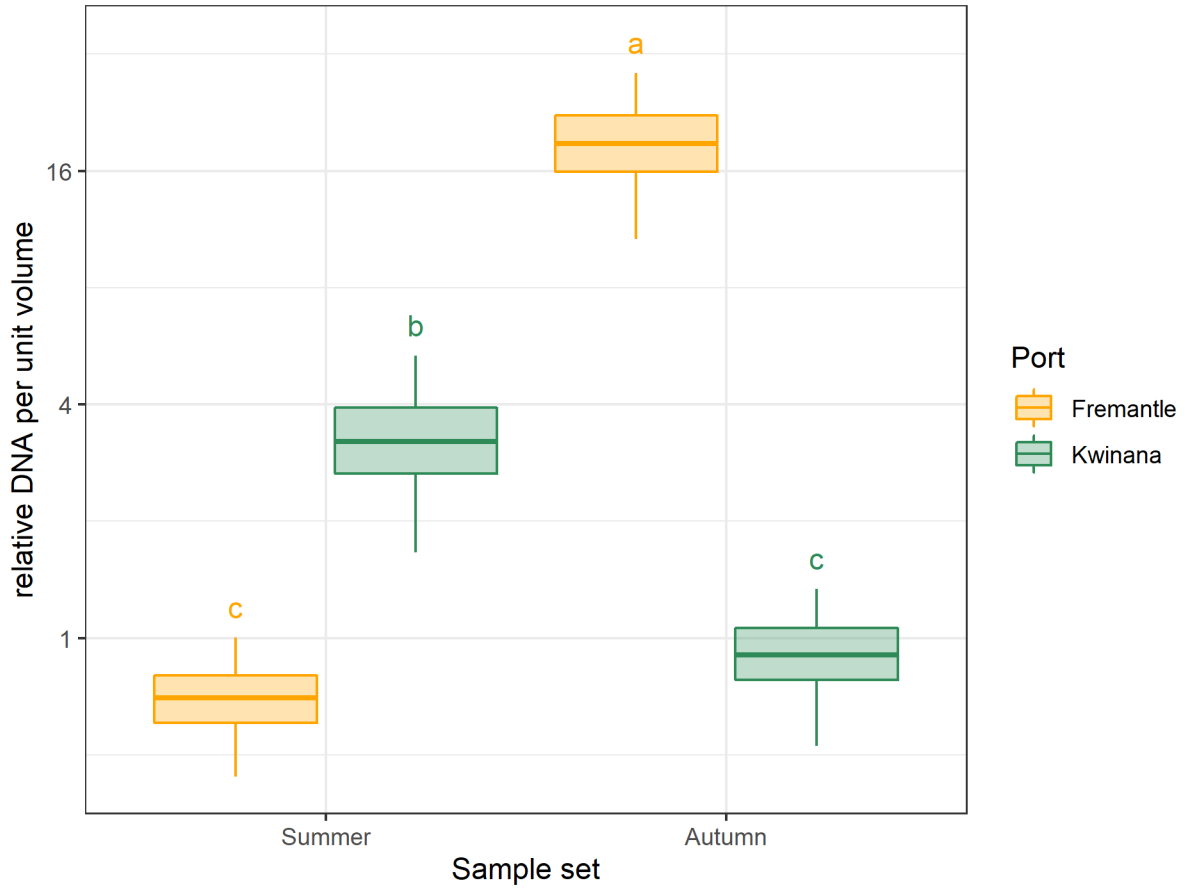

Figure 16. Posterior predictions of mean relative DNA field concentration for *Arcuatula senhousia* in Fremantle and Kwinana by sample set. Boxes show mean and interquartile range, with whiskers showing 95% HDIs. Different letters indicate 95% HDIs that do not overlap.

4. DISCUSSION

These molecular surveys provide updated information on the status of six pests of concern for domestic ballast water management in the ports of Bunbury, Kwinana, Fremantle and Geraldton, and also assessed these ports for the occurrence of six pests that are exotic to Australia but are at risk of introduction by international shipping. Molecular surveillance detected pests known to occur at each location: *Arcuatula senhousia* in Fremantle and Kwinana, and *Sabella spallanzanii* in Kwinana and Bunbury.

Arcuatula senhousia was discovered in Fremantle in 1983, and, by the 1990s, had formed extensive populations in the region (Slack-Smith and Brearley 1987; McDonald and Wells 2009a). Following a record summer rainfall event and subsequent toxic algal bloom in 2000, *Ar. senhousia* appeared to have become locally extinct, with 2007 surveillance for this mussel not detecting any specimens in the Fremantle and Kwinana region (McDonald and Wells 2009a). This mussel has re-occurred, however (Bridgwood and McDonald 2014), and several specimens were collected in the Fremantle region in 2013 (ALA 2020).

In samples from several tropical regions, the *Ar. senhousia* assay has returned detections that are probably cross-reactions with non-target DNA, most likely from a native relative present in those regions (Wiltshire *et al.* 2019a, c). In temperate regions, as in the present study, molecular detections have been recorded only in ports where *Ar. senhousia* is known to occur (Deveney *et al.* 2017; Wiltshire *et al.* 2019a). The widespread molecular detections of this mussel in Kwinana and Fremantle suggest that there is a population of *Ar. senhousia* in this region.

Fremantle and Kwinana had the same estimated true prevalence of close to 1 for *Ar. senhousia*, with detection in nearly all samples in each sample set, but displayed different seasonal patterns in relative DNA yield. Given that these ports are in close proximity (10 km apart), it is unlikely that the seasonal pattern in DNA differs. Rather, the difference likely reflects spatio-temporal variation in DNA concentration that may have been driven by hydrodynamics, or by the timing of surveys relative to local spawning events, e.g. a spawning event in Kwinana coincident with summer sampling and one in Fremantle coincident with autumn sampling. Across the two ports, average DNA concentration was similar between summer and autumn, being marginally higher in autumn after accounting for sample volume (Figure 16). The high number of detections for both ports and sample sets suggests that the reproductive period spanned both summer and autumn.

Data from Melbourne suggested that summer was the best season for *Ar. senhousia* detection, with autumn providing low detection likelihood and DNA concentration for this species (Wiltshire

et al. 2019a). The Melbourne analysis was based on samples collected in late March ("summer") and late June ("autumn"), however, in contrast to collection dates in mid-March and mid-May for the current survey. Autumn spawning may therefore have been missed in the Melbourne survey due to sampling later in the year. It is also plausible that the WA ports show a different seasonal pattern to Melbourne because, in addition to being located at a lower latitude (~32°S for Kwinana and Fremantle *c.f.* 38°S for Melbourne), water temperature along the WA coast is influenced by the Leeuwin current. This leads to warmer temperatures, especially in autumn, at these ports than other locations at comparable latitudes (Caputi *et al.* 2009). These results demonstrate that seasonal patterns for all species should continue to be assessed as more data is obtained.

Sabella spallanzanii was recorded in WA in 1994, when dense beds of this fanworm were found in the Kwinana region (Clapin and Evans 1995). Follow-up surveys found *S. spallanzanii* within the port of Fremantle, and also at Bunbury and Albany (Clapin and Evans 1995). Surveillance in 2007 confirmed the ongoing occurrence of *S. spallanzanii* in the Fremantle and Kwinana region (McDonald and Wells 2009b), marine pest surveys of Bunbury port in 2009 did not detect this species (Parsons Brinckerhoff 2013), but it was detected at that port by 2018 – 2019 surveillance (pers. comm. J. McDonald, Department of Primary Industries and Regional Development (DPIRD)). The current molecular surveys detected *S. spallanzanii* in several samples from Kwinana, and one sample from Bunbury, with the latter being a low-level detection. The Kwinana detections confirm the ongoing presence of *S. spallanzanii* in this port region. Its status in Fremantle and Bunbury is less clear. A single, low-level, detection such as that recorded in Bunbury could be caused by transient material, e.g. DNA that is present temporarily from a ballast water release or hull-fouling, but given the recent detection by traditional surveillance, it is likely that this species is present at low abundance in Bunbury. Given records of established *S. spallanzanii* populations in Fremantle in the 1990s and 2007, continued presence of this species in the Fremantle region also cannot be ruled out.

Data from Melbourne, where *S. spallanzanii* is abundant, demonstrated high likelihood of detection of this species in all seasons, with a peak in summer (late March) and autumn (late June) (Wiltshire *et al.* 2019a). Samples collected in 2017 – 2019 in Adelaide, where *S. spallanzanii* also occurs, showed best detections in March – May and few detections in January and February (Wiltshire *et al.* 2020). In combination with results from this survey, where *S. spallanzanii* was not detected in samples collected in February, these data suggest that autumn is likely to be the best season for detection of this species. A combined molecular

surveillance data set is being analysed that will provide more information on detection seasonality of this species.

The crabs *Carcinus maenas* and *Charybdis japonica* have been recorded in the Fremantle area (Bridgwood and McDonald 2014), but were not detected in the current molecular survey. The records are of isolated occurrences, and it appears that populations of these crabs have not established in WA (Bridgwood and McDonald 2014). *Carcinus maenas* is known from a single specimen collected in the Swan River in 1965 (Wells *et al*. 2010). Surveillance for marine pests, including trapping targeting *Ca. maenas*, in the Fremantle area in 2000, and in both Fremantle and Kwinana ports in 2008, failed to detect this crab (Wells *et al.* 2010). Four adult *Ch. japonica* were caught in WA by recreational fishers between 2010 – 2014, three from the Swan River and one from the adjacent Peel-Harvey Estuary, but intensive trapping surveys between 2012 – 2014 (Hourston *et al.* 2015), general marine pest surveillance in 2011 – 2015 and further targeted trapping in 2017 – 2020 (pers. comm. J. McDonald, DPIRD) did not capture any further specimens of this crab. A lack of detections in the current molecular survey provides further evidence for the absence of populations of these species from WA.

Crassostrea gigas DNA was detected in 7 of 15 samples from autumn sampling in Geraldton. This species is not known to occur in WA and was not recorded in a 2012 survey of Geraldton harbour for marine pests (Bridgwood and McDonald 2014) or in subsequent marine pest monitoring by DPIRD (pers. comm. J. McDonald). The number of detections in the current survey and their wide dispersal throughout the survey area in the harbor suggests a potential presence of the species, although C_T values were near or above the low level positive criterion, indicating a low DNA concentration (Table 7; Figure 9). C_T values for *Cr. gigas* in Geraldton were in the highest 6% of those from surveillance of areas where this species is established (Figure 10), indicating sample DNA yields were low in Geraldton in comparison to samples from other areas, although the number and spatial spread of detections indicates a sizeable total DNA quantity. A large ballast release or spawning of oysters in hull fouling therefore cannot be ruled out as a source of the DNA.

The *Cr. gigas* detections are unlikely to have resulted from a cross-reaction with non-target DNA. The genus *Crassostrea* is well-studied, and a comprehensive molecular phylogeny has been constructed (Salvi *et al.* 2014; Salvi and Mariottini 2017), which demonstrates a substantial genetic distinction between *Cr. gigas* and Ostreidae that are known to occur in Australia. One native Australian species, *Crassostrea* (also known as *Magallana*) *dactylena* has not been

sequenced, but this species was found abundantly in Gladstone by traditional surveillance while no *Cr. gigas* detections occurred in any of four seasonal molecular sample sets collected in the same port areas over the same time period (Wiltshire *et al.* 2019a). Specificity of the *Cr. gigas* assay has been assessed against the most closely related species of the genus and also *Saccostrea glomerata* and *Ostrea angasi*, the two most common and widely-distributed native Australian oyster species (Patil *et al.* 2005; Bott and Giblot-Ducray 2012). Previous molecular surveillance has not provided detections in areas where *Cr. gigas* is not known to occur (Deveney *et al.* 2017; Wiltshire *et al.* 2019a; Wiltshire *et al.* 2019c).

Geraldton is located at 29°S. Temperate Australian regions, south of ~32°S, are regarded at highest risk of *Cr. gigas* invasion (Hayes *et al.* 2007), but *Cr. gigas* has a wide temperature tolerance, and has established populations or is successfully cultivated in tropical and sub-tropical regions globally (Wiltshire 2007; Carrasco and Barón 2009; Goulletquer 2020). Water temperature in Geraldton (range 18 - 27°C) is within the 10 - 30°C range suitable for *Cr. gigas* growth and reproduction (Wiltshire 2007; Carrasco and Barón 2009; Goulletquer 2020). Air temperature and heating due to sun exposure are also important factors controlling the distribution of intertidal species, and may restrict establishment at lower latitudes, even where water temperature is suitable (Hayes *et al.* 2007). *Crassostrea gigas*, however, is tolerant of short exposure to high temperatures (> 40°C), and can rapidly acclimate, increasing its thermal tolerance through expression of heat-shock proteins (Hamdoun *et al.* 2003; Rajagopal *et al.* 2005). *Crassostrea gigas* may have a lesser competitive advantage over native oysters (e.g. *Saccostrea glomerata*) in the high intertidal at lower latitudes, but occurs abundantly at mid to low tidal heights in these regions, including Port Stephens, New South Wales (Krassoi *et al.* 2008), where the annual water temperature range (17 – 26°C) is similar to Geraldton.

One observation that could indicate that there is not a resident population of *Cr. gigas* in Geraldton is that our detections there were only in autumn. Summer has been consistently shown to be the best season for detection of *Cr. gigas*, corresponding to the peak spawning period for this species (Deveney *et al.* 2017; Wiltshire *et al.* 2019a; Wiltshire *et al.* 2019c). Spawning and detections can however occur from spring to autumn, and some detections may occur in winter (Deveney et al. 2017; Wiltshire et al. 2019a; Wiltshire et al. 2019c; Deveney et al. 2020). Reproduction in *Cr. gigas* is strongly linked to temperature in temperate regions: gametogenesis begins at a minimum temperature of ~10°C, and spawning typically at ~18°C (Shpigel 1989; Ruiz *et al.* 1992; Fabioux *et al.* 2005; Enríquez-Díaz *et al.* 2008). When grown in warm-water ponds, however, *Cr. gigas* spawned at temperatures between 20 and 26°C, rather than at the summer maximum

temperature of ~28°C (Shpigel 1989). Spawning events may be initiated in ripe oysters by temperature increases or by periods of high food availability, e.g. phytoplankton blooms (Ruiz *et al.* 1992; Enríquez-Díaz *et al.* 2008). A lack of summer detections in Geraldton may be due to water temperature being more suitable for *Cr. gigas* reproduction in autumn than summer in this sub-tropical location, or simply due to timing of surveillance relative to spawning events. It is also possible that a vessel with mature hull-fouling oysters arrived just prior to the sampling, and conditions (temperature or food availability) in Geraldton port induced spawning.

These results demonstrated that follow-up surveillance using molecular and/or traditional methods in Geraldton was warranted to ascertain whether the species is present. DPIRD carried out surveillance, comprising 21 water samples for eDNA and 7 visual surveys, in September 2020 with no detections of *Cr. gigas* resulting (pers. comm. J. McDonald). This further suggests that a ballast water release or hull-fouling was the source of the DNA detected, although the sensitivity of the follow up surveillance is difficult to assess without details of sampling and analysis methods used. Investigation of what vessels were in the port during or immediately prior to sample collection would also assist in determining the likelihood of ballast or hull-fouling contributing to the detections.

The single low-level detection of *Rhithropanopeus harrisii* DNA in Fremantle is likely to be of transient material. This species is not known to occur in Australia but is regarded as being at risk of introduction via ballast water or vessel fouling (Hayes and Sliwa 2003; Hewitt *et al.* 2011; Crafton 2015), both of which are demonstrated invasion pathways for this crab (Brockerhoff and McLay 2011). *Rhithropanopeus harrisii* is present at some ports of origin for international vessel arrivals to Fremantle (Bridgwood and McDonald 2014).

A cross-reaction of the *R. harrisii* assay with non-target DNA, producing a false positive, is possible but unlikely. The assay for *R. harrisii* was among a group of new assays developed by Simpson *et al.* (2018), which have not been validated to the same extent as the previously developed SARDI developed assays. The SARDI assays have been applied in the field since 2011, including in two field validation projects (Deveney *et al.* 2017; Wiltshire *et al.* 2019a), and the assays for five species have undergone further laboratory validation of diagnostic performance (Wiltshire *et al.* 2019b). The present project is the first to apply the new assays to field samples. The likelihood of a cross-reaction appears low, because the family Panopeidae, which includes *R. harrisii*, has only one species that occurs in Australia, *Homoioplax haswelli* (see Davie 2002). There are no sequences for this species in GenBank (https://www.ncbi.nlm.nih.gov/)

or other published literature, however, so the specificity of the assay against this species cannot be assessed. A cross-reaction with an unrelated species cannot be completely ruled out, but is unlikely due to the nature of qPCR assays, which use a combination of primers and probe, all of which need to bind with DNA in the sample for a detection to result (Darling and Mahon 2011). Application of the *R. harrissi* assay to additional field samples will assist in determining specificity; this could include applying the assay to DNA from previously collected samples. Given the single detection, either *R. harrisii* or the species responsible for a cross-reaction is likely uncommon in the region.

Proving absence of a pest or disease is infeasible. To detect low 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). Proof-of-freedom approaches are therefore based on 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. Bayesian analysis, such as applied in this project, can provide information on the maximum plausible prevalence of an undetected pest, given the method performance and number of samples. In our case, we assumed no prior knowledge of pest presence or absence, but the Bayesian analysis can be extended to include existing information, e.g. results of earlier surveys, and incorporate data on relative risks of introduction for each species over time (Low-Choy 2013; Stanaway 2015). Using data from the current molecular surveys and uninformative priors, the upper limits of the 95% HDI for prevalence of species with no detections (and also for cases of likely transient detections) were ≤ 0.135. We did not formally incorporate prior belief of species occurrence in the analysis, but interpret results with respect to species records. The predicted prevalence for *S. spallanzanii* in Bunbury was similar to the prevalence predicted for species with no detections, but prior occurrence of *S. spallanzanii* in this location increases the likelihood that the species is present, in contrast to species that have never been detected in WA. To increase confidence in the absence of undetected species, additional samples would need to be collected (see Wiltshire *et al.* 2019b). It should be noted 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 if DNA is randomly distributed or spatially clumped (Furlan *et al.* 2016). DNA concentration and hence prevalence is also likely to vary seasonally (Wiltshire *et al.* 2019a). The relationships between adult pest abundance and DNA prevalence in

plankton are also not established. It is likely that larger pest populations provide higher DNA concentrations and thus greater prevalence, increasing the likelihood of qPCR detection with increasing pest density. The exact relationship is likely to be complicated, differ between species and vary seasonally.

5. CONCLUSIONS

This survey provides updated knowledge of six pests of concern for domestic ballast water management. Detections of *Arcuatula senhousia* in Fremantle and Kwinana, and *Sabella spallanzanii* in Kwinana and Bunbury confirm their continued presence in these ports, although *S. spallanzanii* probably occurs at a low abundance in Bunbury. *Crassostrea gigas* DNA was detected at Geraldton, where this species is not known to occur. Target DNA yield of the samples with detections was relatively low, but detection in 7/15 samples across a wide area indicates a sizeable total quantity of DNA was present, suggesting potential species occurrence. Further investigation, however, did not detect the species, suggesting that the detections were transient, with a large ballast water release or spawning of hull-fouling oysters a potential source of the DNA. Six assays for exotic pests of concern were applied, with the current project providing the first data on field performance of four new assays. A single low-level detection of *Rhithropanopeus harrisii* DNA in Fremantle is likely to be of transient material. A cross-reaction with non-target DNA is unlikely given only one related species is present in Australia, but data to confirm specificity of the assay is lacking. Additional testing and validation of the new assays for exotic pests will provide greater confidence in their field and analytical performance. Results from the current project provide further data on seasonality of detections, and suggest that seasonal patterns for some species vary between locations. The best times to sample for each species should continue to be assessed as more data is obtained.

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