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Validation of molecular surveillance: parallel traditional and molecular surveys for priority marine pests in the ports of Gladstone, Brisbane, Melbourne and Hobart

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

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SARDI Aquatics Sciences
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June 2019

Report to Department of Agriculture and Water Resources

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Field sampling and data collection was performed by Jacobs Group (Australia) Pty Ltd and identification of traditional sampling specimens by Benthic Australia. At SARDI Sustainable Systems: the Molecular Diagnostics Root Disease Testing Laboratory undertook the extractions, testing and automated reporting, Drs Alan McKay and Kathy Ophel-Keller provided guidance and support. At SARDI Aquatic Sciences: Mandee Theil conducted sample preparation and processing for the molecular survey method with assistance from Ian Moody, Leonardo Mantilla and Nhu Y Lieu; and A/Prof. Jason Tanner, A/Prof. Tim Ward and Prof. Gavin Begg provided guidance and support. The project aims and design were informed by useful discussions with staff from DAWR and Drs Rupert Summerson and Tony Arthur of the Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES). Valuable comments on the manuscript were provided by SARDI Aquatic Sciences staff A/Prof Jason Tanner and Dr Penny Ezzy and by two anonymous reviewers from ABARES.

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EXECUTIVE SUMMARY

Shipping is a major vector for marine pest introductions, and hence port areas are likely sites for marine pest incursions. A national monitoring strategy was devised as part of Australia's *National System for the Prevention and Management of Marine Pest Incursions* (the National System), but few surveys for marine pests have been completed, largely due to the high cost of implementing the surveys, which involve traditional sampling techniques such as dredge or trawl tows, trapping, and diver visual surveys. Molecular techniques for marine pest surveillance have potential to offer cost and time savings over traditional techniques, but their performance relative to traditional methods has not been formally assessed. The South Australian Research and Development Institute (SARDI) has developed qPCR assays for the detection of marine pests, including seven pests that inform domestic ballast water arrangements managed by the Department of Agriculture and Water Resources. SARDI has also developed a method for plankton collection, preservation and extraction suitable for testing with the pest qPCR assays. Molecular surveillance field and laboratory methods were tested and refined by SARDI using samples from six ports around Australia (Darwin, Cairns, Sydney, Melbourne, Hobart, and Adelaide) in 2015-16, with all pests expected to be at each location detected, and a new record of *Crassostrea gigas* in Adelaide. Traditional samples were not collected in that study, therefore a robust comparison of the performance of molecular methods with the National System traditional surveillance methods was not possible.

New international and domestic regulations for ballast water management came into force in September 2018. To provide updated information on the pest status of four Australian ports and compare the performance of molecular surveillance methods and traditional techniques, this project employed parallel molecular and traditional sampling, with a survey sensitivity estimated for each method using the National System monitoring design excel template (MDeT). Surveys were conducted at the ports of Gladstone, Brisbane, Melbourne and Hobart, and targeted six pests with established populations in Australia that are of concern for ballast water management: *Asterias amurensis*, *Arcuatula senhousia* (formerly *Musculista senhousia*), *Crassostrea gigas* (also known as *Magallana gigas*), *Carcinus maenas*, *Undaria pinnatifida*, and *Sabella spallanzanii*. Plankton samples for molecular testing were collected seasonally to obtain data on the best times to sample each of the target pests, while traditional surveys were conducted in winter and summer to account for known seasonal variability in the detection of some species, particularly *Undaria*, which senesces over summer.

The molecular approach is fit-for-purpose for marine pest surveillance. All six target pests were detected widely both spatially and temporally by the molecular method in Melbourne, where traditional sampling did not detect *Crassostrea* or *Carcinus.* In Hobart, *Asterias, Carcinus* and *Crassostrea* were detected by both methods. These pests are all known to occur in these areas, although published records of *Crassostrea* from Port Philip Bay were not from the port areas. *Undaria* was detected in one sample in Brisbane and *Arcuatula* was detected widely in Gladstone and in one sample from Brisbane. None of the target pests were detected in traditional sampling at these ports. The isolated *Undaria* detection is likely to be from transient material from a vessel. The *Arcuatula* results warrant further investigation as this species has not been recorded from these areas, and the *Arcuatula* assay has not been thoroughly assessed for specificity against native tropical relatives due to a lack of suitable material. Protocols to confirm results of qPCR assays using secondary molecular tests are being developed.

Our results demonstrate that molecular sampling could be conducted with less than half the person-hours of traditional sampling and for lower cost while achieving higher survey sensitivity than traditional methods. Additional cost and time savings are also likely to be achieved by the molecular method in comparison to traditional surveys due to lesser requirements for specific expertise and field equipment, and fast, simple post-collection processing and analysis. The molecular surveys achieved much higher sensitivity in ports with pests present (Melbourne and Hobart) than predicted by the MDeT, demonstrating that the number of samples required to achieve a given survey sensitivity for the molecular method is less than estimated by the MDeT, hence, additional time and cost savings are possible. Consideration should be given to updating the assumptions in the MDeT or developing an improved survey design tool. The MDeT underestimates sensitivity of the molecular method relative to traditional sampling due to assumptions about the number of detectable units in plankton generated by each adult pest. The MDeT calculations also lack consideration of seasonality, in particular how sampling in different seasons can affect survey sensitivity. Our results show that survey sensitivity of the molecular method varied between seasonal sampling sets, and sensitivity can be maximised by sampling in appropriate seasons for the target pests. A combination of summer and autumn sampling provides the best likelihood of detection across the six target species in temperate ports for the fewest required samples. Further work is required, however, to determine optimal sampling times in tropical ports or for any new target species that establish in Australia or for which new molecular assays are developed or implemented.

The molecular detection system including internal controls operated well; PCR inhibition occurred in some samples, which may have impacted detection in individual samples, but overall did not prevent detection of established pests. Molecular methods are more likely to provide an indication of ballast water risk than traditional methods by detecting pest propagules or tissue within the vicinity of wharves and in ballast water uptake zones. Where such pest material originates outside the survey area, source populations will not be detected by traditional methods. Molecular methods may detect new incursions or range expansions of the target pests, but due to the sensitivity of the method, detections may sometimes occur that do not represent an established pest population, e.g. caused by transient material from ballast water release or hull-fouling. Molecular detections also do not provide specific information on the location or abundance of established pests, although the number of detections and relative pest DNA concentration in samples are likely to increase with adult pest range, proximity and abundance. We provide guidelines on the interpretation of molecular results, based on the number of detections and relative pest DNA, to aid in distinguishing between new incursion and transient presence of a previously unrecorded pest. We also suggest some management responses that could assist in determining whether a pest population is present, and which escalate with increasing likelihood of an incursion.

RECOMMENDATIONS

Based on the outcomes of this project, we recommend that:

1. Molecular survey methods are fit for purpose for marine pest surveillance to support ballast water arrangements.
2. For surveillance of temperate ports, sampling twice yearly in autumn and summer will provide the best overall survey sensitivity for a given number of samples for detection of 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*) .
3. Timing and optimisation of surveillance in tropical ports or for additional species requires further investigation.
4. Molecular surveillance provides better survey sensitivity with reduced required effort and substantially lower costs compared to traditional methods.
5. MDeT is problematic for designing molecular surveillance because it:

   a. overestimates sample numbers required to achieve acceptable survey sensitivity
   b. assumes too few detectable units are produced per adult pest
   c. assumes sampling occurs randomly through time and that season does not influence detection likelihood
   d. is complex and not user-friendly.
6. Survey design could be improved, and predicted sample numbers made more realistic by:
   a. Applying MDeT at a lower target survey sensitivity than the default setting, and using best available knowledge of seasonality in survey design and/or
   b. Parameterising MDeT, or a new design tool, with more realistic assumptions and including consideration of sample timing and seasonal effects
   c. Adapting the MDeT user interface or designing a new tool that is more intuitive and user-friendly.
7. Validated assays are vital for reliable molecular surveillance results, including understanding seasonality in new environments and providing information on confidence of absence.
8. Hydrodynamic data and modelling could inform better optimised spatial sampling and is likely to be useful for investigating new detections.

Keywords: marine pest, molecular surveillance, survey sensitivity.

1. INTRODUCTION

1.1. Background

Reducing the risk of marine pest incursions and spread has economic, environmental and social benefits. 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). Not all species introduced to new locations establish or become pests, but in an environment of increased global trade and connectivity, the rate of species introductions is rising, with a concomitant increase in the risk of pest establishment and impacts (Bax *et al.* 2003; Williams *et al.* 2013). Shipping transports marine species in ballast water, as fouling on ships hulls, including niches such as sea chests, and on equipment (e.g. fishing nets), (Bax *et al.* 2003; Molnar *et al.* 2008). Port areas are therefore at high risk of marine species introductions.

Surveillance is a key component of managing incursion risk. Early and reliable detection maximises the likelihood of responses to incursions being successful and assists 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 2018, and surveillance is also required to support port status for the assessment of Australian Sourced Ballast Applications (ASBA) in the Marine Arrivals Reporting System (MARS), which focuses on seven species that have established populations within Australia (Arthur *et al.* 2015; Department of Agriculture and Water Resources 2018). This need for marine pest surveillance is recognised under *Australia's National System for the Prevention and Management of Marine Pest Incursions* (the National System) (Department of Agriculture and Water Resources 2018). A surveillance strategy was established in the 2000s, based on a statistical survey design tool for surveys using 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 the National System monitoring strategy identified that lack of surveillance was largely due to the expense of traditional surveillance methods and some lack of clarity around the purpose of the surveillance strategy (Arthur *et al.* 2015). The review noted that other monitoring strategies may be more appropriate for management of ballast water risks than the National System survey methods (Arthur *et al.* 2015). Molecular methods for pest detection provide results rapidly and are

considerably cheaper than traditional surveillance, but require further validation for use in management systems (Bott *et al.* 2010b; Deveney *et al.* 2017; Department of Agriculture and Water Resources 2018).

The South Australian Research and Development Institute (SARDI) began development of a system using molecular methods for monitoring marine pests from plankton in 2004 (Giblot-Ducray and Bott 2013). SARDI has developed 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). The qPCR assays were assessed for analytical sensitivity and laboratory validated for specificity during development (Ophel-Keller *et al.* 2007; Bott *et al.* 2010a; Bott and Giblot-Ducray 2011a, b, 2012). They were applied to plankton samples collected in Port Adelaide (Wiltshire and Deveney 2011) and also assessed broadly for field specificity and fitness for purpose in the National Biosecurity Committee (NBC) funded project Australian Testing Centre for Marine Pests (2014-2016) (the ATCMP project, Deveney *et al.* 2017). The ATCMP project involved application of the assays to plankton samples collected from six ports around Australia in two seasons each, and all target pests that are established were reliably detected in Adelaide, Melbourne, Hobart and Sydney. No target pests are known to occur in Cairns and Darwin, the two other ports surveyed. The *Corbula gibba* assay 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). This indicated that the assay required re-design, and reinforced that field validation of molecular assays is necessary for understanding assay performance and specificity in the biodiverse systems where they are likely to be applied. While the majority of assays performed well and known established pests were detected successfully during the ATCMP project, the molecular method had not been performed in parallel with traditional techniques in a manner that permits robust comparison of the performance of the two surveillance methods.

1.1. Objectives

This project aimed to compare traditional and molecular field methods for marine pest detection and assess fitness for purpose of the molecular methods. The studies were designed to generate data to provide an understanding of relative survey sensitivity and specificity of molecular assays, which would in turn inform sample sizes, survey design, and cost. Plankton samples for molecular testing were collected seasonally to obtain data on the best times to sample each of the target

pests. The traditional surveys were also planned to provide pest samples for further molecular analysis and assay refinement. The project focused on six pests of current concern for ballast water management which have established populations in Australia and for which qPCR assays are available: *Asterias amurensis*, *Arcuatula senhousia*, *Crassostrea gigas*, *Carcinus maenas*, *Undaria pinnatifida* and *Sabella spallanzanii*. We designed both traditional and molecular surveys for marine pests for Ports at Hobart, Melbourne, Brisbane and Gladstone using the Monitoring Design Excel Template (MDeT) (Wiltshire and Deveney 2017). Both survey designs were constructed to provide a theoretically known detection sensitivity and sampling was conducted following the National System protocols for traditional sampling (National System for the Prevention and Management of Marine Pest Incursions 2010a, b), and the SARDI developed methods of molecular surveillance using plankton tows (Deveney *et al.* 2017).

This study was designed to provide an understanding of fitness for purpose of molecular assays as a method for marine pest surveillance, particularly for surveillance to support domestic ballast water arrangements. The data also provided updated information on the status of six priority pest species for the ports of Gladstone, Brisbane, Melbourne and Hobart.

2. METHODS

2.1. Sample collection

Survey designs were generated for the four ports of interest: Gladstone and Brisbane, Queensland; Melbourne, Victoria; and Hobart, Tasmania, using the Monitoring Design excel Template (MDeT) version 2.5 as described in Wiltshire and Deveney (2017). The MDeT was developed to facilitate design of surveys undertaken as part of the National System monitoring strategy and ensure these were conducted with adequate sensitivity (National System for the Prevention and Management of Marine Pest Incursions 2010a, b). The survey designs for the current project focused on seven marine pest species that have established populations in Australia and are of interest for the ASBA in MARS:

- 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*)

The ATCMP project, however, found that the *Corbula gibba* assay produced false positive results in northern Australian ports, where temperature is above the upper thermal limit for this species (Deveney *et al.* 2017). The assay for *Corbula gibba* is therefore being redesigned, and, while this species was still targeted and recorded in traditional sampling, the assessment of parallel molecular and traditional survey methods for the current project is based on results for the other six species of interest only.

Sample numbers were generated by the MDeT for survey sensitivities of 0.6 and 0.8 for each port, with the required numbers split evenly over four sampling sets for molecular methods and two sets for traditional methods (Wiltshire and Deveney 2017). Within each port a number of sublocations, defined to include wharves where ballast water uptake and discharge occurs, and surrounding areas of suitable substrate for the pests of interest, were surveyed. Maps of proposed sampling locations were originally generated based on the sample numbers calculated by the MDeT for a survey sensitivity of 0.6, but it was subsequently decided to collect samples to the numbers predicted by MDeT to provide survey sensitivity of 0.8, and revised proposed sampling

locations were generated reflecting these higher sample numbers (Appendix 1), which therefore differ from those shown in Wiltshire and Deveney (2017). Proposed sampling locations were determined based on systematic sampling as recommended by the National System monitoring manual, with a focus on sites where the target species are most likely to occur, and with a wide geographical spread of samples both within and across sampling events (National System for the Prevention and Management of Marine Pest Incursions 2010b). For trap and visual samples two options were generated, so that the total number of proposed locations was double that required. For trap and visual samples the proposed locations were intended only as a guide to potential sites and as a demonstration of suitable sample spacing. Identification of the locations to target using these methods is best done with local knowledge or initial assessment of suitability of locations for the species of interest. For dredge samples and plankton tows, the proposed locations could also be altered if necessary due to field conditions, or access or logistical constraints. The final selection of sample locations was, therefore, at the discretion of the third-party field sampling organisation. All field sampling and related data collection was conducted by Jacobs Group (Australia) Pty Ltd (Jacobs).

Four sets of samples were collected approximately seasonally at each port for the molecular survey: winter (July – August) 2017, spring (October – November) 2017, summer (February – March) 2018 and autumn (May – June) 2018. Traditional samples were collected from each port in winter (July – August) 2017 and summer–autumn (February – May) 2018. All sublocations at each port were sampled during each sampling event. The number of samples collected in some sublocations varied from those proposed. Sample numbers for each event and sampling dates are shown in Table 2. Maps of sampling locations are shown, with detection results, in section 3.2.

2.1.1. Plankton tows for molecular analysis

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) 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. During the summer 2018 sampling in Melbourne, the volume of material in the tows conducted within the Yarra River sublocation was noted to be low by the Jacobs field team. The field team therefore targeted a length of ~140 m for the tows conducted in that set and sublocation only. 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). Plankton 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 2.2).

2.1.2. Traditional sampling

Traditional sampling methods used were: dredges, traps and diver visual surveys; these methods were chosen based on their appropriateness for the target species and feasibility (Wiltshire and Deveney 2017). Dredge samples of ~80 m length were taken with a benthic sled with a mouth 0.7 m wide by 0.15 m high and a mesh size of either 5 mm (Gladstone and Brisbane) or 4 mm (Melbourne and Hobart). After retrieval of the sled, the entire sled contents were coarsely sorted and inspected for larger target species. Voucher specimens of any potential targets detected were collected and placed in analytical grade ethanol absolute (AR ethanol). Sled contents were then homogenized and a subsample of approximately 1 L collected and preserved with AR ethanol for further sorting and analysis. Trap sampling used opera-house style traps baited with sardine (*Sardinops sagax*), and deployed for ~18–24 hours. On retrieval, trap contents were inspected for the presence of target species, with voucher specimens of any targets placed in AR ethanol. Visual surveys were conducted by divers using SCUBA and targeting a transect length of 100 m. Counts of target species per 10 m section were recorded, and voucher specimens of any potential target species observed were collected and placed into AR ethanol. Counts were not made for *Crassostrea*, however, since this species is difficult to distinguish from native oyster species in the field. Voucher specimens were not collected from every sample, voucher specimens were collected for each species from each sublocation where a detection occurred in traditional sampling. All voucher specimens collected by the traditional survey methods were sent to Benthic Australia Pty Ltd (Benthic Australia) for confirmation of identification. Dredge subsamples were also sorted and identified at Benthic Australia, and counts were recorded for any targets. Only target pest species were identified to species level; once ruled out as being potential targets, other specimens were not identified to lower taxonomic levels. In addition to the seven established pests that were the primary target of the survey, the following pests that are currently exotic to Australia were also targeted:

- Chesapeake blue crab (*Callinectes sapidus*)
- Chinese mitten crab (*Eriocheir sinensis*)
- Zuiderzee crab (*Rhithropanopeus harrisii*)

- Asian green mussel (*Perna viridis*)
- Brown mussel (*Perna perna*)
- New Zealand green mussel (*Perna canaliculus*)
- Black striped false mussel (*Mytilopsis sallei*)

Field data, comprising sampling waypoints or waypoint identifiers, plankton tow flowmeter readings, and notes pertaining to field conditions and individual samples, were recorded by the field team using an iPad with proprietary software or on hard copy data sheets. Waypoints were recorded either directly by the iPad or using a separate handheld or the vessel GPS. All field data were provided to SARDI for compilation.

2.2. Processing and analysis of molecular samples

A subset of the collected plankton samples, representing sampling numbers for a calculated survey sensitivity of 0.6 from the MDeT, were processed for analysis. The subset was chosen based on sample locations, using recorded waypoints, to provide as even spatial coverage of each sublocation as possible. The selected set of plankton samples 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 Molecular Diagnostics Root Disease Testing Laboratory (RDTS). DNA was extracted from samples using 20 mL of DNA extraction buffer 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 of the DNA was done in 160 µL 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 (see Table 1) plus two exogenous organisms that were added to samples as controls. The target pests were the six established pests as described in section 2.1 plus two pests currently exotic to Australia that were also targeted in the traditional survey and for which SARDI-developed qPCR assays are available: *Mytilopsis sallei* and *Perna canaliculus.* Assays are referred to hereafter by the genus name of the target. 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 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. 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 C_t value for the effects of inhibition (Ophel-Keller *et al.* 2008).

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

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

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, decontaminated using LookOut® DNA Erase between sample sets.

2.3. Comparison of sampling logistics and costs

Molecular surveys are expected to be faster and cheaper to perform than traditional surveys (Bott *et al.* 2010b; Deveney *et al.* 2017). The field data provided by Jacobs was used to estimate the average time taken per sample for each method and hence estimate the total person-hours per

method. For all samples, the date of collection was recorded, and, in most cases, the time of each sample commencement was also recorded. Analysis did not include samples for which the time of sample commencement was not recorded. The number of samples of each method collected per day was determined and the per sample time calculated as the time difference between first and last samples, divided by one fewer than the number of samples, since the time for completion of the last sample in each day is not included. On some sampling days different methods were interspersed, e.g. dredges or traps were deployed between dives. Data from these days were included in the sampling time analysis only where blocks of time for each method could be clearly separated. The overall average sampling time was calculated as the weighted average of the per sample time over all applicable days for that method. Since traps are deployed on one day and collected the next but time estimates could only be made for deployments, the time taken for collection was assumed to be the same as deployment, although collection could be expected to take slightly longer where target species are present that need to be processed. Total sample time in person-hours was calculated for each sampling method as:

Total person-hours = average sample time x required number of staff x number of samples

The methods applied typically require different staffing levels. Not including crew for operating the vessel, plankton sampling typically requires two persons, dredge and visual dive surveys three persons, and a single person for deployment and collection of traps. Required staff number in the person-hours calculation was therefore assumed to be two for plankton, three for dredge and visual, and one for traps. The number of samples used in the calculation was the number processed and analysed for each method at each port, which, for molecular samples, was fewer than the number collected (Table 2). These calculations do not include mobilisation and demobilisation times at the start and end of each or travel time from boat launching facilities or marinas to the sampling locations; these times will vary depending on the site but will be the same for either type of sampling.

An estimate of relative operating costs for each of the survey methods was made based on costings for work carried out by SARDI. Costs considered were: vessel hire for the required vessel size and number of days for each method, costs of primary sampling equipment and consumables (plankton net, dredge, traps, SCUBA diving gear including air), accommodation for the required number of staff and total number of days for each method. Costs considered are only for field sampling, and not for subsequent sample processing and identification. As with the comparison of required time for sampling, mobilisation and demobilisation were not considered. Actual costs

are confidential, and in any case will vary over time due to inflation, therefore, we report the ratio between total costs of each method at each port.

2.4. Mapping and statistical methods

Field data collected by Jacobs, traditional identification data from Benthic Australia, and molecular analysis results from SARDI Molecular diagnostics were compiled in a database. Traditional method detections were linked to recorded field locations based on the sample location codes that were used by the Jacobs field team to label samples sent to Benthic Australia. Molecular results were linked to field sampling locations using the sample identifier that was recorded by Jacobs with associated field location data. During field sampling, the length of plankton and dredge tows was determined based on GPS; however, reliability of coordinates marking the end point of tows was variable. For 27 plankton samples, end coordinates were not recorded, including all 15 samples from Hobart in summer due to GPS failure. In other cases, coordinates were taken after the vessel had moved away from the end point of the dredge or plankton tow, leading to apparent tow distances of > 150 m and up to > 600 m, or of < 75 m, for 87 plankton samples (*c.f.* nominal distance of 100 m), and of > 120 m and up to > 300 m, or of < 60 m, for 34 dredge tows (*c.f.* nominal distance of 80 m). Some additional issues with accuracy of the waypoints recorded using the iPad were noted, hence the use of this device for directly recording location data was discontinued subsequent to the spring 2017 sampling and a separate GPS device (either hand held or the vessel GPS) was used instead. The locations recorded for the first two sample sets in Melbourne and Hobart, therefore, may not be accurate due to use of the iPad to record waypoints for these sample sets. All waypoints for Gladstone and Brisbane were recorded using a separate GPS for all sample sets. Actual tow length could therefore not be calculated in some cases and was inaccurate in others, so tow length was not considered as a factor in analyses. For results presented in this report, sample locations of dredge and plankton samples were mapped based on sample start point. A single GPS waypoint was recorded by the field team for dive and trap samples.

The relative effectiveness of molecular and traditional sampling was assessed, and additionally, for each port where a target pest was detected by the molecular method in multiple samples across sampling events, analyses of seasonal patterns and assessment of factors influencing molecular results were performed as described below.

Table 2. Number of samples collected and analysed and sample dates for each sampling event.

Season	Port	Sample type	Collected	Analysed	Start date	End date
Winter	Gladstone	Plankton(Molecular)	107	65	30-Jul-17	03-Aug-17
		Visual (Traditional)	8	8	27-Jul-17	29-Jul-17
		Trap (Traditional)	16	16	03-Aug-17	06-Aug-17
		Dredge (Traditional)	32	32	04-Aug-17	06-Aug-17
	Brisbane	Plankton	65	37	14-Aug-17	16-Aug-17
		Visual	6	6	30-Aug-17	31-Aug-17
		Trap	12	12	17-Aug-17	30-Aug-17
		Dredge	19	19	17-Aug-17	18-Aug-17
	Melbourne	Plankton	124	72	29-Jul-17	01-Aug-17
		Visual	4	4	24-Jul-17	25-Jul-17
		Trap	14	14	30-Jul-17	30-Jul-17
		Dredge	26	26	26-Jul-17	28-Jul-17
	Hobart	Plankton	25	15	07-Aug-17	14-Mar-18
		Visual	3	3	05-Aug-17	05-Aug-17
		Trap	5	5	05-Aug-17	05-Aug-17
		Dredge	8	8	06-Aug-17	06-Aug-17
Spring	Gladstone	Plankton	111	65	31-Oct-17	02-Nov-17
	Brisbane	Plankton	65	37	15-Nov-17	16-Nov-17
	Melbourne	Plankton	126	72	08-Nov-17	12-Nov-17
	Hobart	Plankton	24	15	06-Nov-17	06-Nov-17
Summer	Gladstone	Plankton	111	65	27-Feb-18	02-Mar-18
		Visual	7	7	22-Feb-18	24-Feb-18
		Trap	19	19	22-Feb-18	25-Feb-18
		Dredge	32	32	24-Feb-18	26-Feb-18
	Brisbane	Plankton	65	37	03-Apr-18	04-Apr-18
		Visual	6	6	03-May-18	04-May-18
		Trap	12	12	05-Apr-18	03-May-18
		Dredge	19	19	05-Apr-18	05-Apr-18
	Melbourne	Plankton	126	72	22-Mar-18	27-Mar-18
		Visual	4	4	19-Mar-18	20-Mar-18
		Trap	14	14	20-Mar-18	27-Mar-18
		Dredge	26	26	20-Mar-18	22-Mar-18
	Hobart	Plankton	23	15	14-Mar-18	14-Mar-18
		Visual	3	3	13-Mar-18	13-Mar-18
		Trap	4	4	14-Mar-18	14-Mar-18
		Dredge	8	8	15-Mar-18	15-Mar-18
Autumn	Gladstone	Plankton	111	65	26-Jun-18	28-Jun-18
	Brisbane	Plankton	65	37	13-Jun-18	21-Jun-18
	Melbourne	Plankton	126	72	25-Jun-18	28-Jun-18
	Hobart	Plankton	21	15	08-Jun-18	15-Jun-18

Table 3. Description of sublocation codes and number of samples analysed from each sublocation for each method. All traditional samples were processed, while a subset of molecular samples were analysed (see Table 2).

	Sublocation		Molecular	Traditional		
Port	**Code**	**Description**	**Plankton**	**Dredge**	**Visual**	**Trap**
Brisbane	BBT	Bulk Terminal	8	2	2	2
	BFI	Fisherman Islands	56	10	4	12
	BHW	Hamilton Wharves	32	8	3	4
	BOR	Oil Refinery	20	6	2	2
	BPB	Pinkenba-Bulwer	32	12	2	4
		TOTAL	148	38	13	24
Gladstone	GBA	Barney-Auckland	36	8	10	6
	GCE	Curtis East	12	4	2	2
	GCW	Curtis West	20	8	3	6
	GCT	Coal Terminal	96	11	2	8
	GFL	Fishermans Landing	44	10	2	4
	GST	South Trees	28	10	2	4
	GWI	Wiggins Island	24	10	2	7
		TOTAL	260	61	23	37
Hobart	HNW	Nyrstar Wharf	8	2	2	2
	HSC	Sullivan Cove	32	10	2	6
	HSP	Selfs Point	20	4	2	2
		TOTAL	60	16	6	10
Melbourne	MSP	Station Pier	24	6	2	6
	MWD	Webb Dock	39	10	2	6
	MWW	Willliamstown Wharves	65	12	2	6
	MYR	Yarra River	160	24	2	10
		TOTAL	288	52	8	28

2.4.1. Relative performance of sampling methods

To compare the relative effectiveness of the molecular and traditional sampling methods, a Bayesian probabilistic model was developed, following the approach of Furlan *et al.* (2016). The probability that a target will be present within a sample (P_{pres}), depends on the mean density or concentration of the target (λ) and the volume of the sample (V_s) as:

$$P_{pres(species,\ method,\ sublocation,\ time)} = 1 - \exp(-1 \times \lambda \times V_s)$$

For the surveys in the current project, the target for traditional surveys was the adult pests, while molecular methods target planktonic propagules (gametes, larvae, spores) but may also detect environmental DNA from adult or other life history stages of pests. The actual concentration of adult pests in each sampled sublocation (Conc) is unknown, but was included as an unobserved latent variable in the model and the relative concentration was thereby estimated from the data. The target concentration, λ, at each time is given by:

$$\lambda_{(species,sublocation,\ time)} = z_{(species,\ sublocation)} \times Conc_{(species,\ sublocation)} \times N_{prop(species,\ time)}$$

Where z is an indicator variable taking the value of either 0 or 1 for pest absence or presence, thereby allowing complete absence of a pest from a location, and N_{prop}, which was included only for molecular methods, is the multiplier between adult and planktonic pest concentration for each sample time. The multiplier indicates a combination of the number of propagules (or other units of detectable DNA) per adult pest and dilution in the water column of the planktonic phase, so was allowed to be a positive real number by assigning a truncated diffuse normal prior. For traditional methods, λ was assumed to be the same for the two times in which these methods were applied, and results therefore represent average performance of each traditional method across the two sample times. The indicator z was given a Bernouli prior with probability given by a Beta(0.1,0.1) prior. Log(Conc) for each species in each sublocation was modelled with a normal prior with mean and variance estimated from the data using a diffuse normal prior for the mean and uniform(0,10) prior for the variance.

A target may not always be captured or detected when present in a sample. Overall probability of detection (P_{det}) therefore also depends on efficiency of each method at detecting the target (Eff):

$$P_{det(species,\ method,\ sublocation,\ time)} = Eff_{(species,\ method)} \times P_{pres(species,\ method,\ sublocation,\ time)}$$

Priors for method efficiency were chosen to reflect known suitability of each method for each species. Some methods are clearly inappropriate for some species, e.g. traps will not detect non-motile species, and dredge tows will only detect benthic species in soft sediment. Inappropriate species/method combinations were given a Beta(1,50) prior to reflect 99% belief that likelihood of detection of relevant species by these methods was <0.1 with the most likely value being <0.02. Methods which are considered valid by the MDeT for a species but were not used to target that particular species in the current survey were given a Beta(1,10) prior to indicate detections by these methods were also unlikely to occur (most likely value <0.1) but were plausible. This also ensured that non-detections by these methods did not imply pest absence. All other

species/methods combinations were given a Beta(10,2) prior, reflecting 99% belief of detection likelihood >0.5 with mean of 0.8; note that this is detection likelihood contingent on the species being present within the sampling unit (volume or area). JAGS code for the model is provided in Appendix 2. Modelled efficiency of each method is not the same as the diagnostic sensitivity, which refers to the probability of a positive detection where a target is successfully captured in the sample. The efficiency is a function of the likelihood of a sample capturing the target and also the diagnostic sensitivity, but these two components cannot be separated with current data. A high efficiency, however, can only be achieved with high diagnostic sensitivity, while low efficiency may result from ineffective capture, low diagnostic sensitivity or a combination of both.

Seasonal effects were not compared between methods given traditional surveys were conducted only twice over the year, the number of samples for most traditional sample types was too low to permit any formal analysis, and traditional samples were also generally not taken at the same sites within each sublocation across sample sets with the aim of increasing spatial coverage, not of replicating the surveys for comparison. Sampling across four seasons was undertaken for molecular methods because the best times to sample were unclear for most of the target species. Molecular sampling is likely to be most effective during reproductive periods when gametes, larvae or spores are present in the water column, but reproductive seasons for some of the considered pests have not been well studied, and molecular detections for at least some species may also occur due to sources of pest DNA other than reproductive material. Sampling across all seasons was not necessary for the traditional survey because adult stages are typically present year-round, or, for species in which there is a best season to sample, this is well defined.

Markov Chain Monte Carlo (MCMC) simulations were obtained by running the model in JAGS v. 4.3.0 (Plummer 2017) using three chains for 10,000 iterations, thinned at a rate of 10, following 2,000 iterations for adaptation and 10,000 iterations for burn-in. JAGS was run using the 'R2jags' package (Su and Yajima 2015) in R (R Core Team 2017). Convergence was assessed by the Gelman-Rubin convergence statistic, and confirmed by visual inspection of trace, density and autocorrelation plots generated using the 'MCMCplots' package (McKay Curtis 2015).

Based on the MDeT, the number of samples required to achieve equivalent survey sensitivity (i.e. likelihood of detecting a target in at least one sample taken from an area) varied widely between methods (Wiltshire and Deveney 2017), which is to be expected given that the methods have different sampling volumes, and target different substrates and species life stages. Posterior predictions of efficiency from the model, which incorporate these differences between methods,

were used to calculate the probability of detection for each species, and the required number of samples for each method to achieve a survey sensitivity (S_{SE}) of 0.6 and 0.8, for a range of adult pest concentrations, which were based on the predicted concentrations for each sublocation from the model. The relationship between the modelled concentration and true pest population size is unknown, but these predictions show the relative performance of each method for equivalent pest concentration. The number of samples (n) required to achieve a given S_{SE}, is calculated as:

$$n = \log(1 - S_{SE})/\log(1 - P_{det})$$

Where P_{det} is calculated as per the probabilistic model. This calculation was performed for the molecular method for each species and sampling time, and for the most appropriate traditional method for each species, with the required traditional sampling number assumed not to vary between sampling times. The person-hours required for sampling to these predicted numbers was calculated using the average sample time and required personnel for each method as described in section 2.3.

The modelled relative concentration for each pest in each sublocation is also presented.

For ports where there were multiple detections of a species, Bayesian logistic regression was used to compare the frequency of detections between methods and to determine the effective S_{SE} of the surveys as applied to each sublocation. This analysis could only be performed for ports with detected species, since S_{SE} is undefined where a species is truly absent. For a given number of samples (n) and detection likelihood (P_{det}), S_{SE} is given by:

$$S_{SE} = 1 - (1 - P_{det})^n$$

In addition to calculating achieved S_{SE} at the sample numbers collected, S_{SE} was calculated using the sample numbers predicted by the MDeT to give survey sensitivity = 0.6 and 0.8. The number of samples collected and processed by traditional methods was based on the MDeT predicted 0.8 numbers, while molecular samples were processed only to MDeT predicted 0.6 sensitivity numbers (section 2.2). For both methods, the actual sample number collected or processed differed in some cases from the respective MDeT predicted numbers. Firstly, sample numbers generated by the MDeT were rounded up to the next multiple of two or four as needed to provide equal sample numbers across sampling events, and secondly, sample numbers collected by the field team sometimes varied slightly from those proposed.

Some species were either not detected or were perfectly detected (in 100% of samples) for some combinations of method and sublocation, resulting in complete separation in the data (i.e. a covariate or combination of covariates providing perfect prediction). The model could therefore not use diffuse normal priors, such as are typically used in Bayesian analysis to give parameter estimates that are driven by the data and match those of frequentist maximum likelihood methods (Zuur and Ieno 2016). Maximum likelihood, however, does not give finite parameter estimates in the case of separation (Gelman *et al.* 2008). Bayesian logistic analysis can be performed for data showing complete separation, but will fail if the priors used are too vague, since the parameter estimates tend to $\pm\infty$, so suitable weakly informative priors need to be applied (Gelman *et al.* 2008). The use of informative priors in general leads to parameter estimates that are more influenced by prior information, with the degree of influence depending on how informative the priors used are in comparison to the strength of the data (Zuur and Ieno 2016). In the case of separation, the priors should be centered on zero, and be just informative enough to prevent the parameter estimates from becoming implausibly large (Rainey 2016). Gelman *et al.* (2008) suggests using scaled-*t* distribution priors, and specifically Cauchy priors as a minimally informative option; the Cauchy being a special case of the scaled-*t* distribution with one degree of freedom. In some cases of separation, however, Cauchy priors are still too vague, leading to a lack of convergence, very slow convergence, or parameter estimates with excessively large variance (Abrahantes and Aerts 2012; Rainey 2016; Ghosh *et al.* 2018), hence Ghosh *et al.* (2018) recommend scaled-*t* priors with 7 degrees of freedom, hereafter referred to as t_7 priors, which are still a relatively non-informative option and match the tail-heaviness of the logistic distribution (Gelman *et al.* 2008; Ghosh *et al.* 2018). We found that models using Cauchy priors did not converge, and therefore applied t_7 priors, with a scale of 10 for the intercept and 2.5 for other parameters. In effect, this reflects a prior belief that the baseline category has a probability of between 10^{-9} and $1 - 10^{-9}$, and that covariates in the model may shift the probability by as much as ±0.997. Models with and without an interaction term (sublocation x method) were compared using the deviance information criterion (DIC) to determine whether detection likelihood by method varied between sublocations. These models were run and assessed using the same methods as the probabilistic model but with 20,000 iterations, thinned at a rate of 20, following 2,000 iterations for adaptation and 40,000 iterations for burn-in.

2.4.2. Maps of detections and 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 a standard curve has not been developed for *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 spatially and seasonally. A measure of relative DNA (relDNA) for each sample was therefore 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 at which any pest detection occurred.

Rasters of interpolated relative DNA were generated for each port/season in which pests with multiple detections were detected. 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 unlikely that the scaling response is linear for scaling factors above ~2. See section 2.2 for details of the derivation of the scale factor for each sample. To relate DNA present in a sample to field DNA concentration, the volume of water filtered by the plankton net needs to be considered. As the same specification net was used for all samples, the water volume will be directly proportional to the flow meter distance. Relative field DNA was therefore calculated as scaled relative DNA divided by flow meter distance. Flow meter readings were missing from a small number of samples, which were excluded from the analysis. Overall rasters of relative DNA were generated by summing seasonal rasters to show areas where DNA was consistently high. Interpolation was based on inverse distance weighting accounting for barriers (coastline) using the R package ipdw (Stachelek 2018). Maps of interpolated DNA and summarising detections per method were generated using ArcGIS 10.6 (Esri Inc).

2.4.3. Seasonality of molecular detections and relative DNA

Seasonal patterns in molecular detections and relative DNA were analysed to inform the best season for molecular sampling of each detected species. Seasonality of traditional methods was not formally analysed since, for most species, adult stages are expected to be present year-round, and result maps for traditional methods show detections over both surveys. For the analysis of molecular results, Bayesian zero-altered lognormal (ZALN) models were run following Zuur and Ieno (2016). The ZALN model consists of two components: a binary or logisitc component reflecting the likelihood of detection; and a continuous component to model the relative DNA

concentration (relDNA, calculated as in section 2.4.2) in samples where a detection occurred. A log-normal distribution was chosen for the continuous component based on initial data exploration which indicated that this distribution provided a good fit to the data. The log-normal distribution is appropriate for strictly positive continuous data, and is very flexible and allows for varying degrees of skew in the data. The ZALN included predictors for both the binary and continuous components as described below.

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 scale factor calculated for each sample is a measure of inhibition and shows the ratio of the DNA yield of the exogenous control organism, which was added to all samples at the time of extraction, in the plankton sample to the yield in a sample known to be free of inhibition (see section 2.2). The scale factor is used as a multiplier to correct apparent pest DNA yield for the effect of inhibition, therefore, the total relative DNA in a sample is taken to be given by: sampDNA = relDNA x Scale Factor. The Scale Factor is calculated based on DNA yield of the internal control, and so it is possible that the response of pest DNA to inhibition varies, and that the Scale Factor may not be a perfect multiplier for pest DNA.

The total sample DNA relates to the relative field DNA concentration as: sampDNA = fieldDNA x sample volume. All samples were collected with the same specification plankton net and the flow meter distance can therefore be used as a measure of relative sample volume.

For the continuous component of the ZALN, the log-normal distribution used means that logarithm of the response (here, relDNA) is taken to be normally distributed, with mean η dependent on predictors (see below), and variance σ. Two models were run for each species, one to predict DNA yield in samples (sampDNA) and one to predict relative field DNA concentration (field DNA), i.e. correcting for the effect of varying sample volume. The models used were therefore:

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

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

Where β_{SF} is a coefficient allowing for variation in the response of measured DNA to inhibition as measured by the Scale Factor.

For species recorded in only one port, predictors for the binary component were: sampling event (season), scale factor, sample dry weight and flow meter distance. As sampling events were conducted approximately seasonally, and since seasonal influences are likely to be the primary driver of differences in rate of detection between sample sets, we refer to the sample set effect as a season effect. Sampling was conducted at a single time point within each season, however, and sampling took place over a single year, so we note that the data are insufficient to fully characterise seasonality or completely ascribe differences between sample sets to seasonal effects. Scale factor was included as a predictor to assess the effect of inhibition on likelihood of detection. High inhibition is likely to lead to a decrease in likelihood of detection for samples with low pest DNA concentration, but the scale factor at which inhibition becomes problematic for detection is unclear (Deveney *et al.* 2017). Sample volume, as measured by flow meter distance, and sample dry weight, i.e. mass of the retained sample after filtering and freeze-drying, may also affect likelihood of detection, since a sample of greater volume or mass may contain more DNA. These relationships may be complicated however, since net-clogging may occur in areas with high plankton concentration, leading to low effective tow distance but with a relatively large volume of plankton retained. High sample mass may not always relate to a large volume of plankton in a sample, since inorganic material may also be captured. For the continuous component, sampling event (Season) and dry weight were included as predictors in addition to the scale factor offset, as well as flowmeter offset for the field DNA model, as described above.

For species recorded in more than one port, models were run also including Port and Port x Season as additional predictors in both binary and continuous components for models both with and without the flowmeter offset. We did not perform model selection to determine whether the interaction terms should be included, i.e. whether seasonal patterns were different between ports, since the available methods to compare models such as the deviance information criterion are not valid for comparing mixture models such as ZALN (Zuur and Ieno 2016).

For the continuous component, diffuse normal priors were used for coefficients for season and for Port, and for Port x Season where applicable. Variance of the log-normal distribution was also estimated, using a uniform (0,10) prior, i.e. allowing the log-normal variance (σ) to take any value up to 10 with equal probability. An informative normal prior with 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 inhibition 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. Some species were either not detected or were perfectly detected (in 100% of samples) for some sample

sets, leading to separation in the data. As with the logistic regression described in section 2.4.1, the binary component of the ZALN models could therefore not use diffuse normal priors, and t_7 priors were applied, with a scale of 10 for the intercept and 2.5 for other parameters.

Both models with and without the flow meter offset, to model sample DNA and field DNA respectively, were run as 2-component 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 in the results.

These analyses followed the method used in section 2.4.1, except that package 'rjags' (Plummer 2018) was used, and a larger number of MCMC iterations was required for convergence: 50,000 with thinning rate of 50, following 10,000 for adaptation, and 50,000 for burn-in. Convergence was assessed as described in section 2.4.1. To visualise results, predictions for the models were made using mean flow meter distance and sample dry weight for each port and season combination and scale factor = 1 (no inhibition). For the continuous component of each model, the mean expected value (E) of the response variable (relDNA) was calculated from the modelled log-normal mean (η) and variance (σ) as: $E = \exp(\eta + 0.5\sigma)$ (Zuur and Ieno 2016). The variance (Var) of a log-normally distributed variable (here, relDNA) increases with the mean, and is given by: $Var = \exp(2\eta + \sigma^2) \times (\exp(\sigma^2) - 1)$ (Zuur and Ieno 2016). The effect of this variance is difficult to visualise given that the log-normal distribution may be highly skewed, therefore, in the results we present the mean predicted relative DNA (either per sample or field concentration) and the 95 percentile range of the log-normal distribution based on the model predictions for η and σ.

3. RESULTS AND DISCUSSION

3.1. Species detections

Molecular methods detected the primary target species at all ports surveyed, and all six target species were detected by both methods across the ports, but not all species detected by molecular methods were found in traditional sampling at the same ports (Table 4). The locations of samples and detections are shown in section 3.2 (Figures 1 – 35). None of the pests exotic to Australia were found by traditional sampling and there were no molecular detections for *Mytilopsis sallei* or *Perna canaliculus.* The target species with coincident detection by both molecular and traditional methods were: *Arcuatula, Asterias, Sabella* and *Undaria* in Melbourne, and *Asterias, Carcinus* and *Crassostrea* in Hobart. All of these species have known established populations in these ports. *Carcinus* and *Crassostrea* were also detected by molecular methods in Melbourne but were not found in traditional sampling. *Carcinus* is recorded from Melbourne and believed to be established there, while *Crassostrea* was not recorded in the port of Melbourne, although it occurs elsewhere in Port Philip Bay (ALA 2019). No problems with specificity have been identified with this assay. Specificity issues occur when the assay provides a positive result to a non-target species (low specificity) or, less commonly, where the assay malfunctions (false positive). *Crassostrea* was, furthermore, detected in a high proportion of molecular samples across multiple sample sets (112 of 288 plankton samples in total; Table 4) suggesting that an established population is present, despite the lack of detection in traditional sampling. Multiple plankton samples across sample sets from Gladstone had molecular detections of *Arcuatula* (116 of 260 samples; Table 4). This species is not recorded from Gladstone and was not detected in traditional sampling for the current project or by surveys conducted around the port of Gladstone between 2009 and 2017 (GHD 2009; Vision Environment 2015; Environment 2017; Vision Environment 2017). With the exception of GHD (2009), however, these surveys did not target the port area or did not use relevant sampling methods for detection of *Arcuatula*; in particular, a 2015 biosecurity survey (Vision Environment 2015) collected benthic samples only in dredge disposal areas and not within the port area. Specificity of the *Arcuatula* assay, however, has not been assessed as thoroughly as the other assays applied in this project due to a lack of availability of biological specimens and sequences of related taxa. The assay is known to be able to detect the target species, i.e. *A. senhousia*, and the *Arcuatula* detections in Melbourne are believed to be of this species, since there is an existing population in this port, and multiple specimens were found in the traditional survey. The assay may, however, also cross-react with DNA from other taxa, most likely close relatives of *A. senhousia* that did not have sequences or specimens available for

testing during assay development, e.g. native *Arcuatula* spp. and other closely related bivalves that occur in tropical Australia. Few bivalves were captured in the traditional samples from Gladstone (Benthic Australia data), so it is difficult to ascertain what species are present to cause the detections. Given the number and wide geographic spread of detections, there is likely a relatively high abundance of the organism responsible within the Gladstone port area, but with populations occurring outside the immediate berth areas that were targeted by traditional sampling in this project. Recent surveillance of the wider Gladstone port is lacking, and the occurrence of *Arcuatula senhousia* cannot be confidently ruled out. Further investigation is needed to determine if the molecular detections are *A. senhousia* or the result of low assay specificity. These investigations are under way. If the assay lacks specificity, it will be redesigned. Collected bivalve material from Gladstone will be reviewed to identify whether native *Arcuatula* spp. or close relatives occur, and PCR product from suitable molecular samples will be sequenced to obtain more data on the specific cause of the detections. Results from these investigations were not available at the time of writing this report. Given the uncertainty around the Gladstone *Arcuatula* detections, these data were not included in the probabilistic model for method assessment.

In Brisbane, a single sample from the spring 2017 sample set returned a low-level (high C_t) detection for *Undaria* and a single sample from summer 2018 returned a low level detection for *Arcuatula.* The *Undaria* detection occurred in the primary port area and may have been due to material in a ballast discharge or hull fouling. As no other molecular detections occurred, and since the area is unlikely to be suitable for *Undaria* growth due to high turbidity and marginally suitable water temperatures, it is likely this detection was an isolated occurrence and does not indicate an established population. As with the Gladstone detections of *Arcuatula*, it is unclear whether the Brisbane detection actually is *A. senhousia*, given no detection of this species in traditional sampling and no records, although there is a lack of surveillance for Brisbane. This *Arcuatula* detection, however, as with that of *Undaria*, appears to be an isolated occurrence. This species has been found as hull-fouling on vessels entering Australia, so it is also feasible that the detection came from hull fouling, or in ballast water advected from nearby wharves.

Table 4. Number of samples with a detection for each species and sampling method by sublocation. Abbreviations are: v – visual, t – trap, d – dredge, Trad – traditional, Mol – molecular. See Table 3 for explanation of sublocation codes. For molecular results, the number of samples with a low level detection (C_t >40) is shown in brackets. For traditional samples, the number of detections in each relevant method is shown, with the total in brackets. Blue shading indicates species detected at a port by both traditional and molecular sampling. Green shading indicates molecular detections where the species is not confirmed to occur at that port, and orange shading indicates molecular detections where traditional sampling failed to detect the species, but the species is known to be present.

Port	Number of samples					*Arcuatula*		*Asterias*		*Carcinus*		*Crassostrea*		*Sabella*		*Undaria*	
Sublocation	v	t	d	Trad	Mol	Trad	Mol	Trad	Mol	Trad	Mol	Trad	Mol	Trad	Mol	Trad	Mol
Brisbane	**12**	**24**	**38**	**74**	**148**	**0**	**1 (1)**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1 (1)**
BBT	2	2	2	**6**	**8**	0	0	0	0	0	0	0	0	0	0	0	0
BFI	4	12	10	**26**	**56**	0	0	0	0	0	0	0	0	0	0	0	1 (1)
BHW	2	4	8	**14**	**32**	0	0	0	0	0	0	0	0	0	0	0	0
BOR	2	2	6	**10**	**20**	0	1 (1)	0	0	0	0	0	0	0	0	0	0
BPB	2	4	12	**18**	**32**	0	0	0	0	0	0	0	0	0	0	0	0
Gladstone	**17**	**37**	**64**	**118**	**260**	**0**	**116 (63)**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
GBA	4	6	8	**19**	**36**	0	17 (12)	0	0	0	0	0	0	0	0	0	0
GCE	2	4	4	**10**	**12**	0	5 (2)	0	0	0	0	0	0	0	0	0	0
GCT	2	10	14	**26**	**96**	0	47 (26)	0	0	0	0	0	0	0	0	0	0
GCW	2	4	8	**14**	**20**	0	7 (4)	0	0	0	0	0	0	0	0	0	0
GFL	2	4	10	**16**	**44**	0	19 (8)	0	0	0	0	0	0	0	0	0	0
GST	2	4	10	**16**	**28**	0	8 (3)	0	0	0	0	0	0	0	0	0	0
GWI	2	5	10	**17**	**24**	0	13 (8)	0	0	0	0	0	0	0	0	0	0
Hobart	**6**	**10**	**16**	**32**	**60**	**0**	**0**	**6v 5d (11)**	**43**	**1t**	**20**	**3v**	**21 (1)**	**0**	**0**	**0**	**0**
HNW	2	2	2	**6**	**8**	0	0	2 v	5	1t	4	1v	3	0	0	0	0
HSC	2	6	10	**18**	**32**	0	0	2v 3d (5)	25	0	4	1v	12 (1)	0	0	0	0
HSP	2	2	4	**8**	**20**	0	0	2v 2d (4)	13	0	12	1v	6	0	0	0	0
Melbourne	**8**	**28**	**52**	**88**	**288**	**13d**	**102 (102)**	**7v 27d (34)**	**192**	**0**	**23**	**0**	**112 (2)**	**8v 6d (14)**	**252 (17)**	**3v**	**103 (4)**
MSP	2	6	6	**14**	**24**	1d	8 (8)	2v 5d (7)	18	0	4	0	7 (1)	2v 1d (3)	21 (1)	1v	16
MWD	2	6	10	**18**	**39**	4d	13 (13)	1v 7d (8)	30	0	3	0	11	2v	36 (2)	1v	30
MWW	2	6	12	**20**	**65**	3d	24 (24)	2v 5d (7)	50	0	9	0	23 (1)	2v 1d (3)	59 (1)	1v	51
MYR	2	10	24	**36**	**160**	5d	57 (57)	2v 10d (12)	94	0	7	0	71	2v 4d (6)	136 (13)	0	6 (4)

Artemia yield from field samples containing the quality assurance control was lower than that of control jars for Gladstone in summer sampling only, suggesting some degradation may have occurred in that sample set, which would have been the set exposed to the highest field temperatures. Multiple detections of *Arcuatula* still occurred in this sample set (Figure 3). No other sample set showed evidence of degradation.

Molecular methods were successful at detecting all target species that are known to be established in each port. *Undaria* and *Arcuatula* have been recorded in southern Tasmania, but not in the immediate vicinity of Hobart or the wharf areas surveyed in this project. The nearest known population of *Undaria* to Hobart occurs near the entrance to the Derwent estuary, approximately 10 km from the location sampled, while records of *Arcuatula* are from Oyster Cove, near Kettering, over 25 km from the Hobart port area (ALA 2019).

3.2. Maps of sampling locations and detections

Maps of sample locations and detections are shown in Figures 1 – 35. Figures are grouped by location with molecular results followed by traditional results within each port. For each port, molecular detections are shown seasonally (Gladstone: Figures 1 – 4; Brisbane: Figures 8 – 11 and 15 – 18; Melbourne: Figures 22 – 25; Hobart; Figures 29 - 32). A single map showing sample locations and detections from both traditional survey times combined is presented for each traditional sample type (dredge, trap, visual) at each port (Gladstone; Figures 5 – 7; Brisbane: Figures 12 – 14 and 19 – 21; Melbourne: Figures 26 – 28; Hobart: Figures 33 – 35). Traditional results are not shown seasonally as there were fewer samples in total, and seasonal variation is not expected in adult populations of most species. All maps show sublocations at the same scale (1:25 000). There are additional maps for Brisbane in comparison to other ports since not all Brisbane sublocations could be displayed on a single map at this scale; hence, Brisbane maps are split into the eastern sublocations (Fisherman Island and Oil refinery) at the mouth of the Brisbane River, and the western or upstream sublocations (Pinkenba-Bulwer, Bulk Terminal, Hamilton Wharves). All sublocations for the remaining ports are presented on a single map for each set of results. In each map, sample locations are shown in the overall port index map, while sublocation 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. Note that some sample points may appear to be on land due to the resolution of the base map used, or because these represent samples (trap or visual) taken underneath wharves.

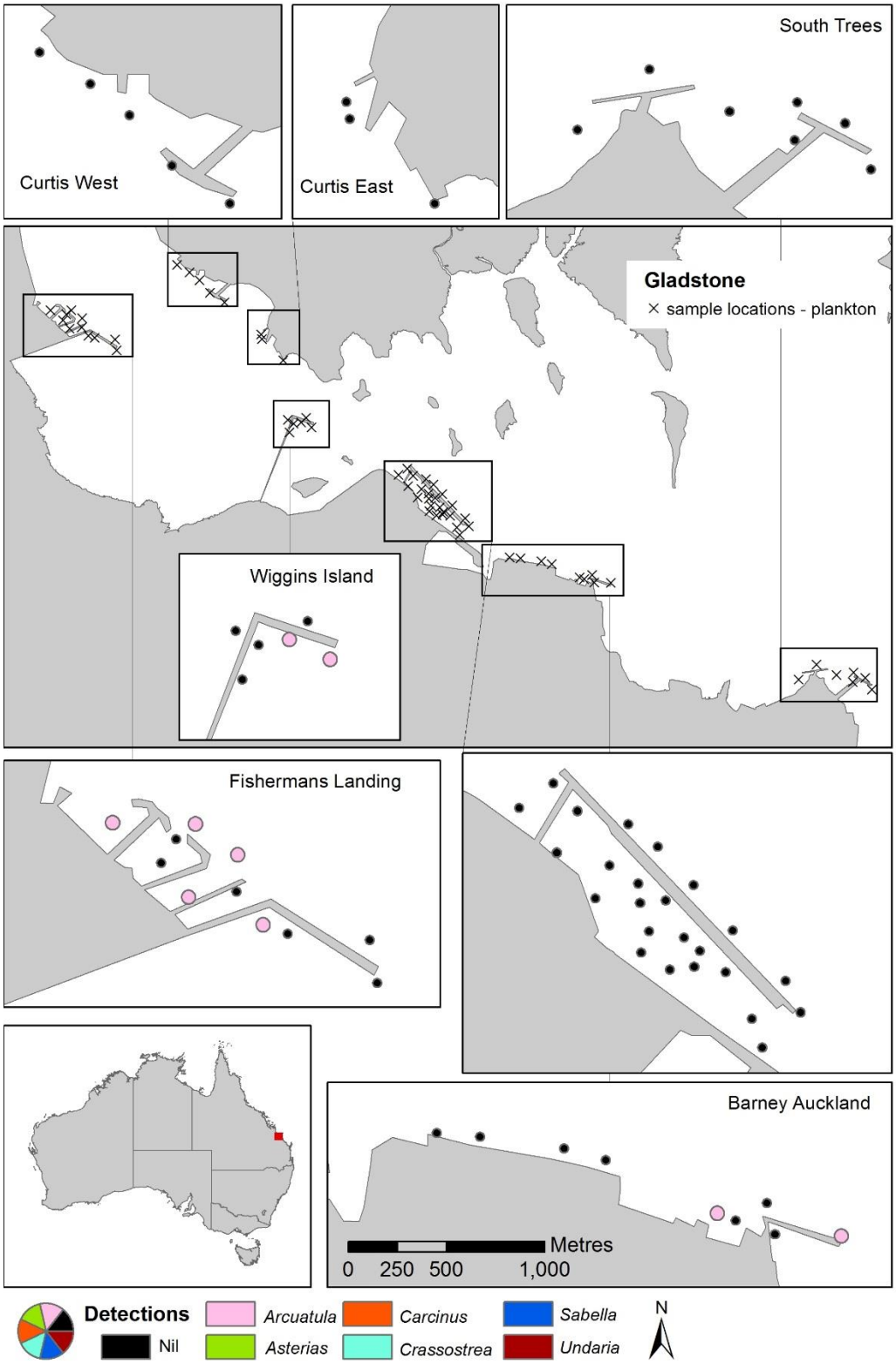

Figure 1. Map of plankton tow locations and molecular detections for Gladstone in winter 2017.

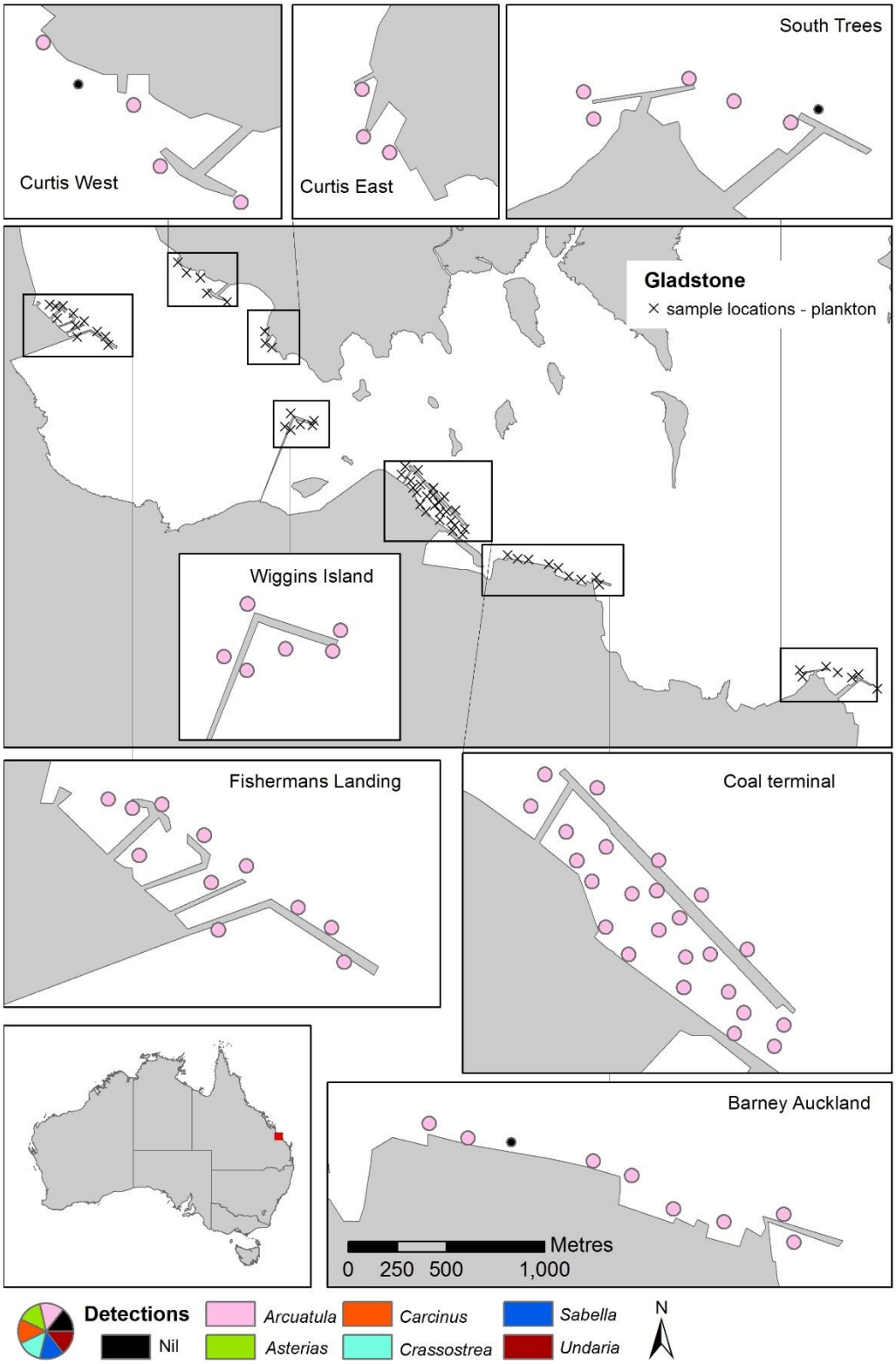

Figure 2. Map of plankton tow locations and molecular detections for Gladstone in spring 2017.

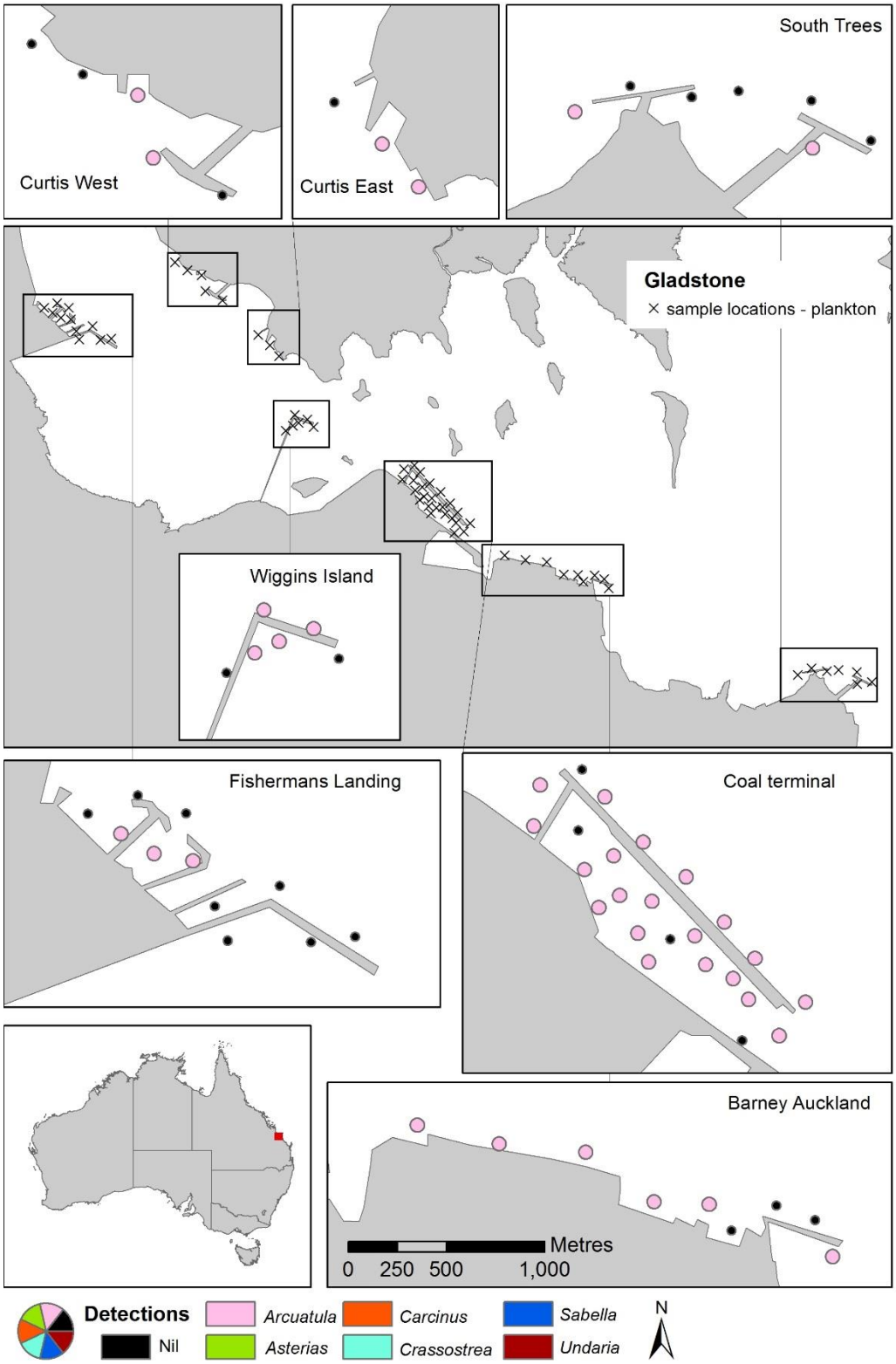

Figure 3. Map of plankton tow locations and molecular detections for Gladstone in summer 2018.

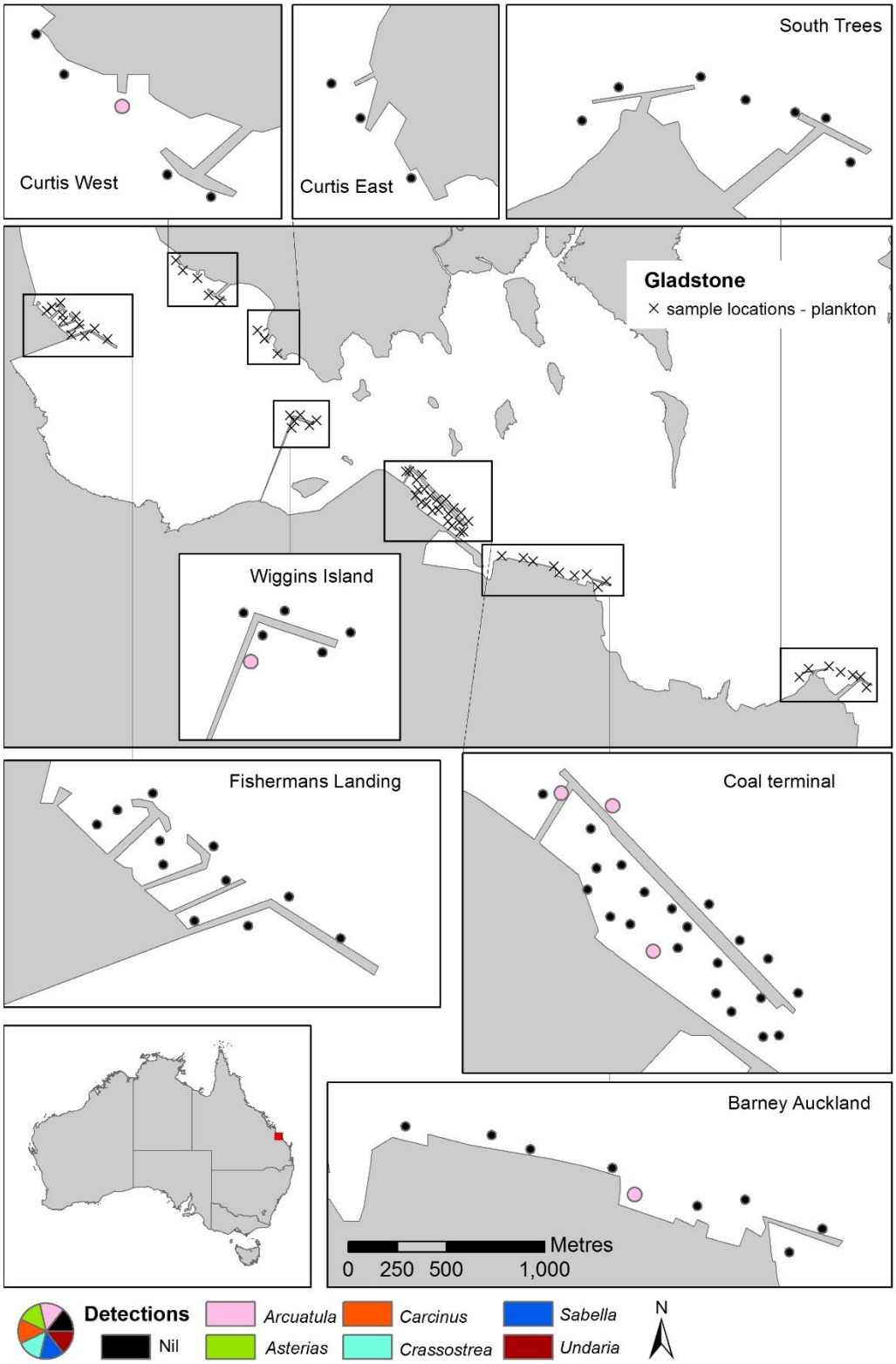

Figure 4. Map of plankton tow locations and molecular detections for Gladstone in autumn 2018.

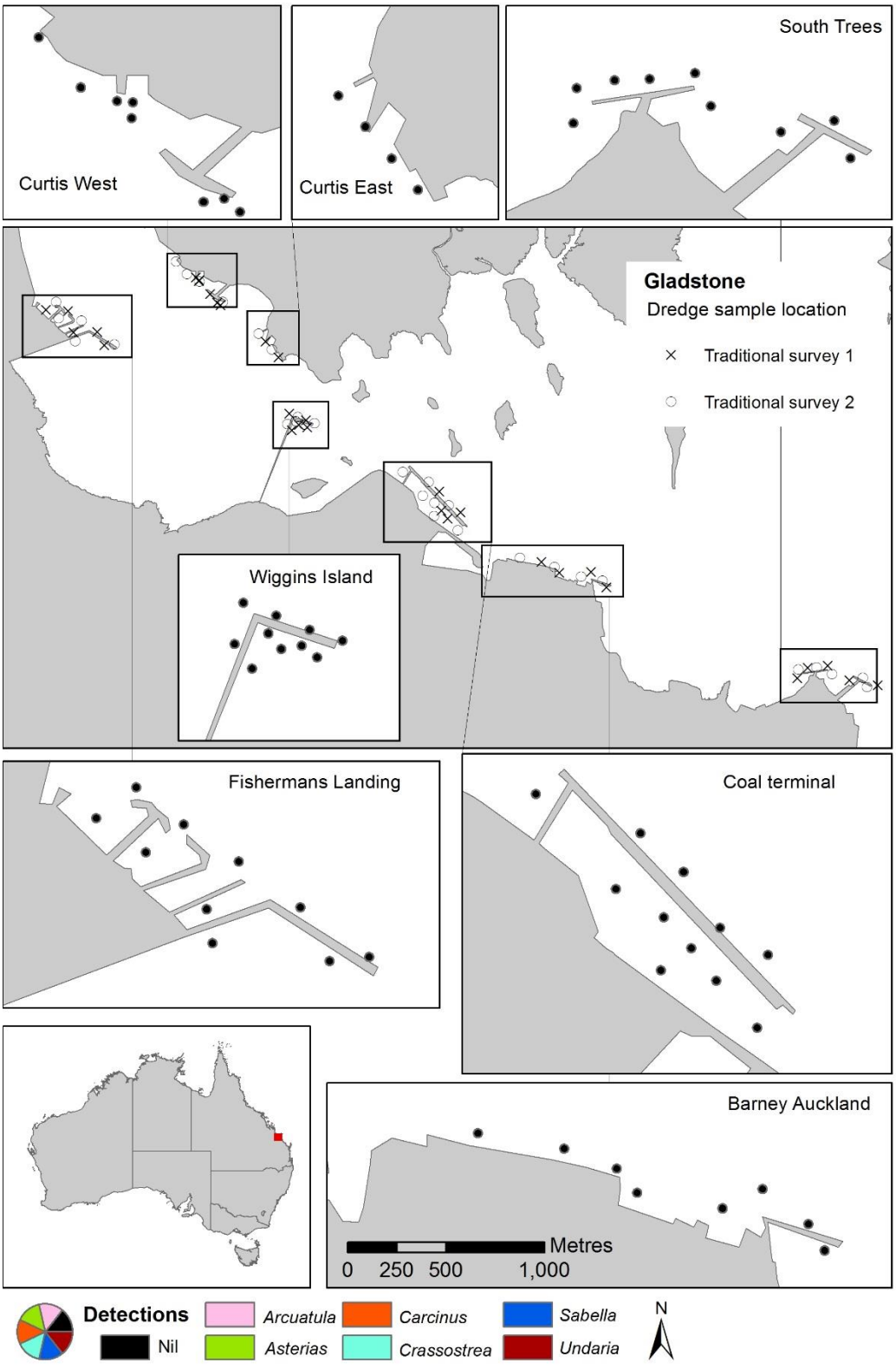

Figure 5. Map of dredge tow locations and detections for both traditional surveys in Gladstone.

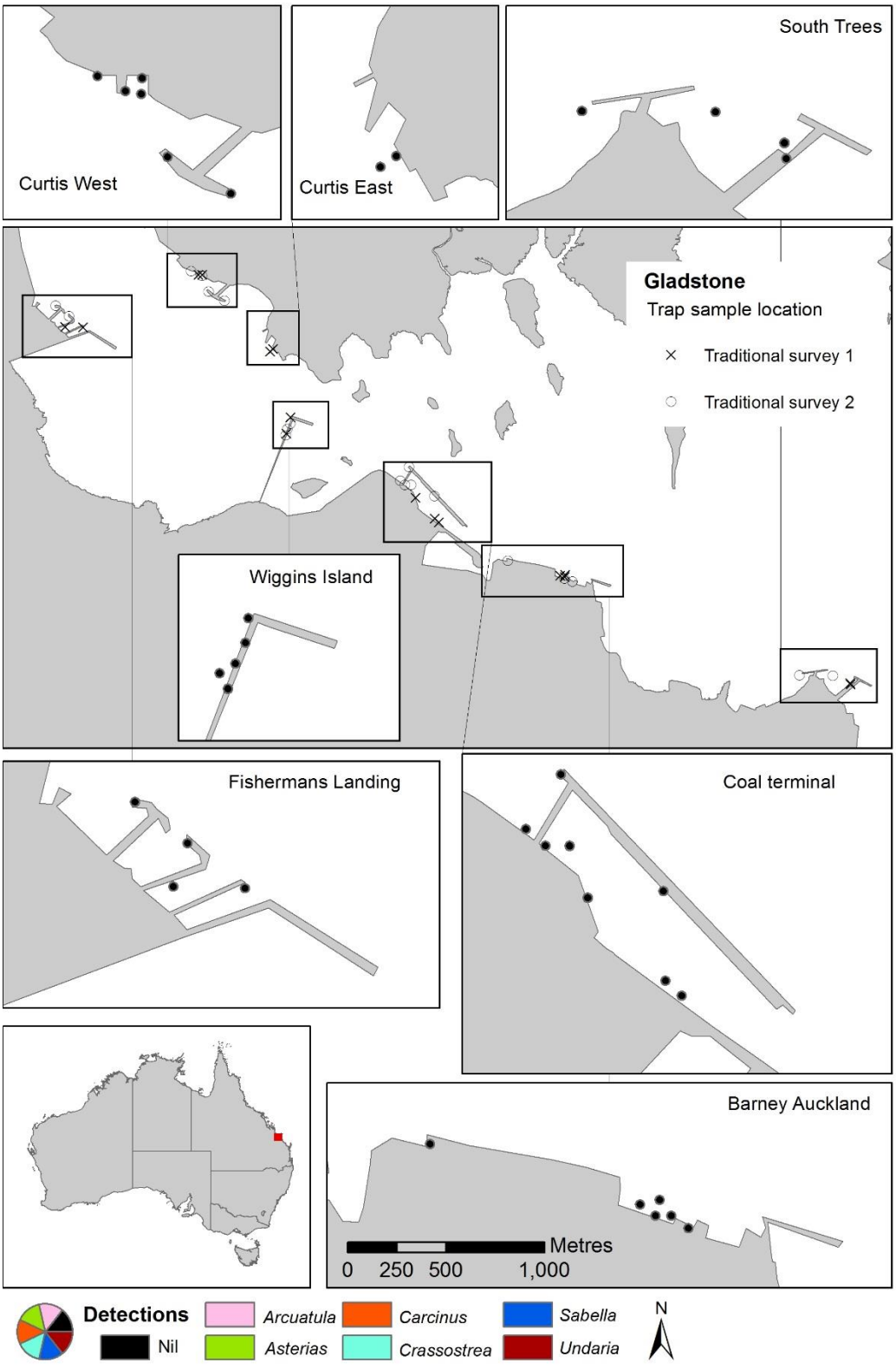

Figure 6. Map of trap locations and detections for both traditional surveys in Gladstone.

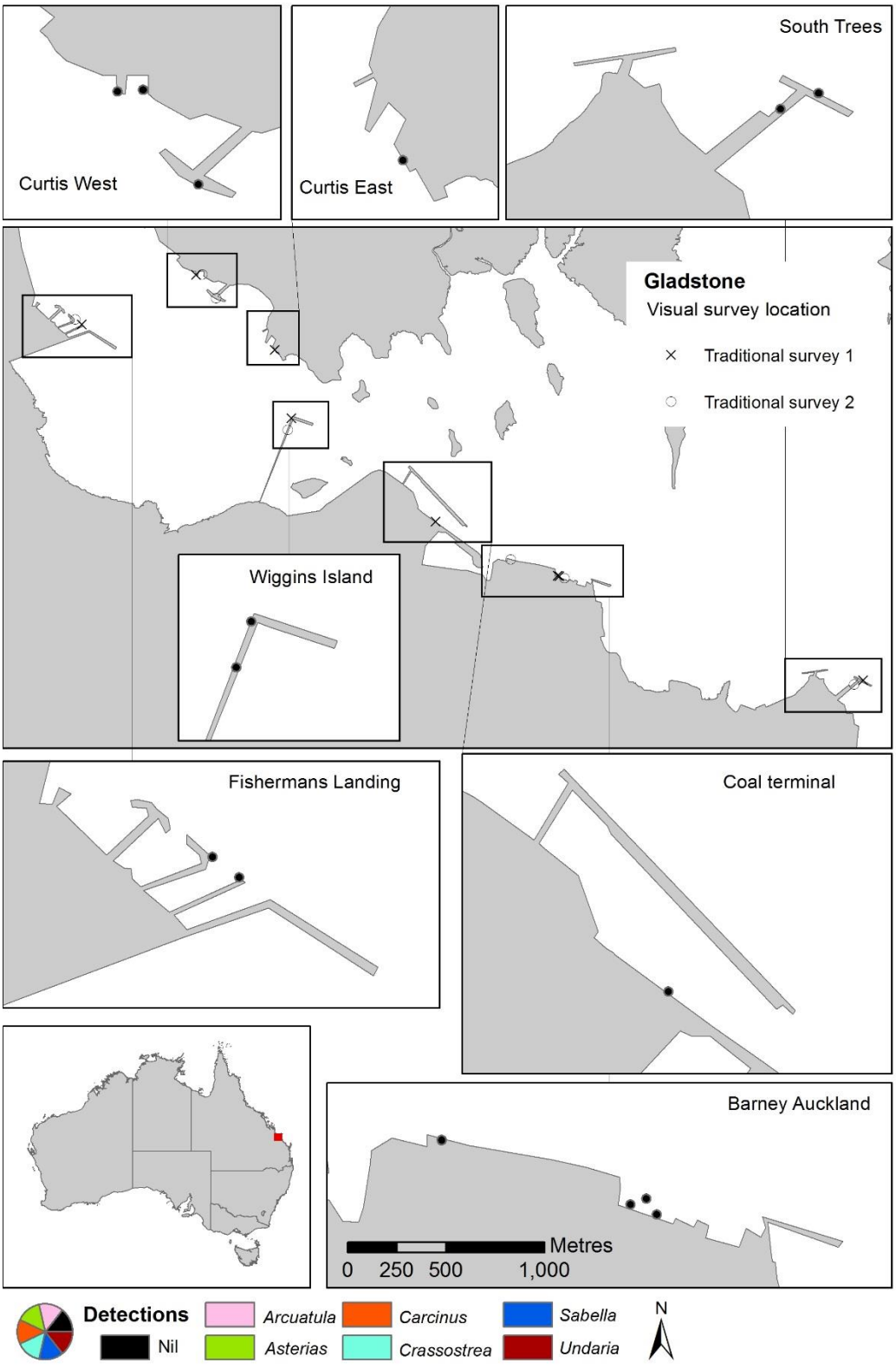

Figure 7. Map of visual survey locations and detections for both traditional surveys in Gladstone.

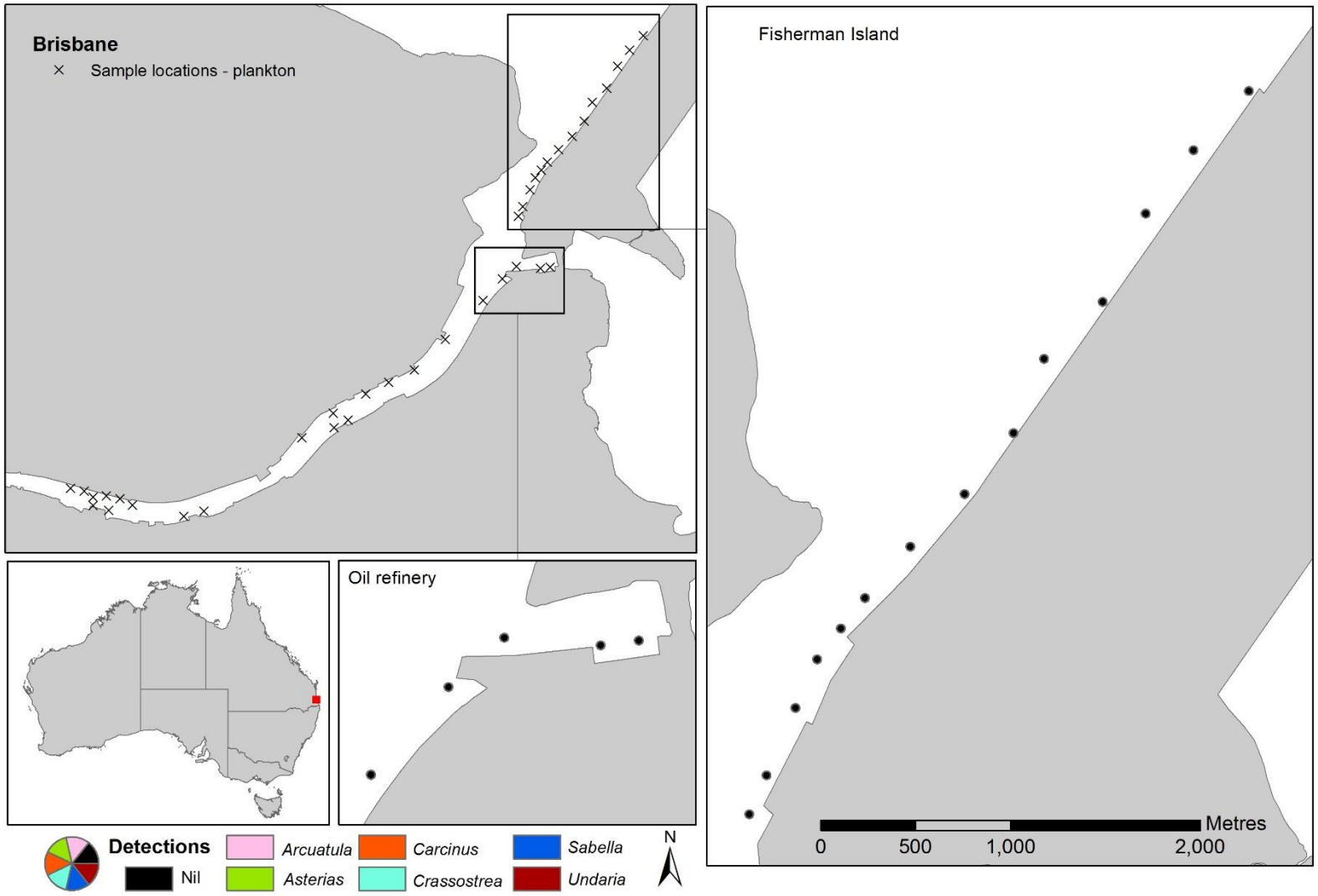

Figure 8. Map of plankton tow locations and molecular detections for Fisherman Island and Oil refinery sublocations in Brisbane in winter 2017.

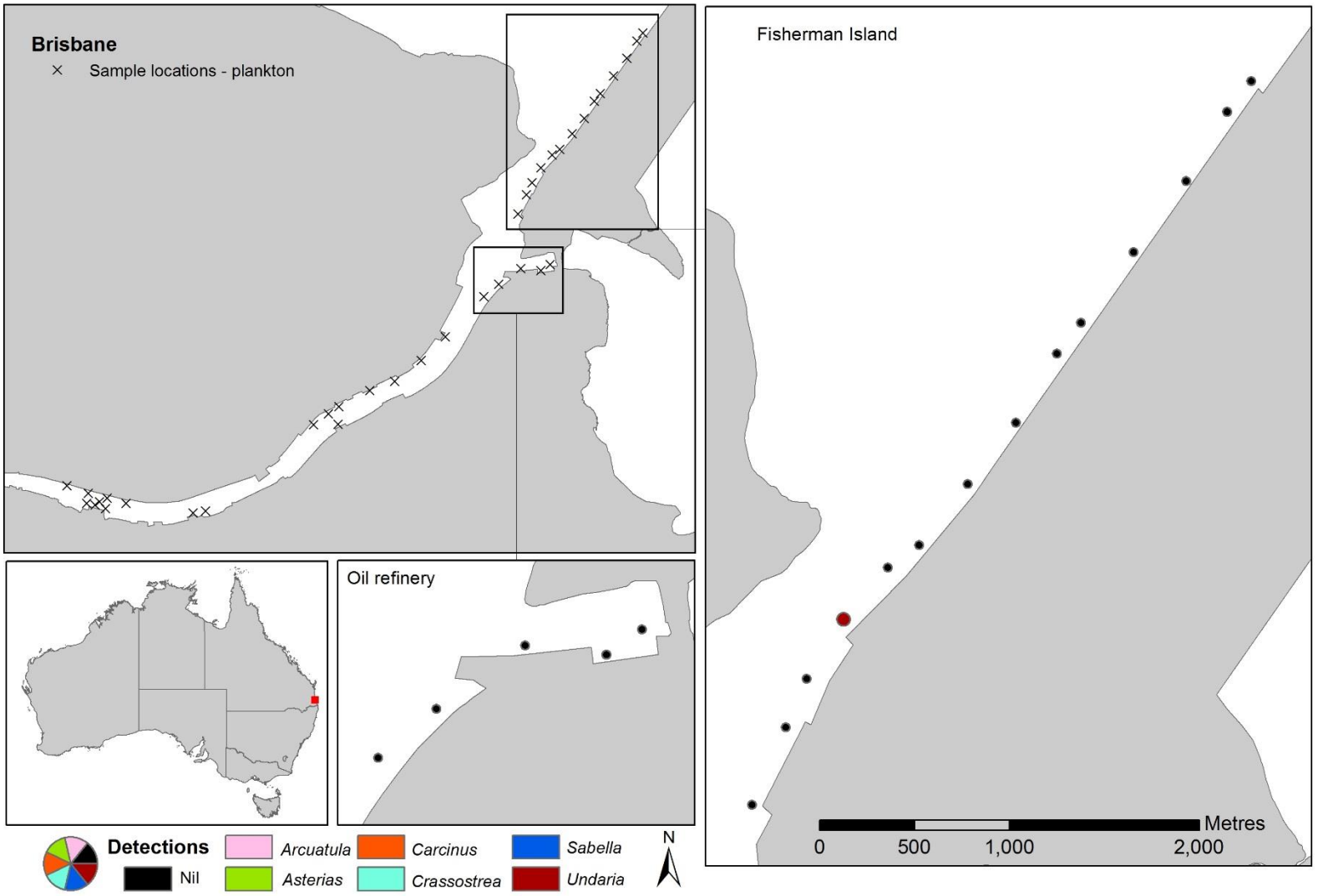

Figure 9. Map of plankton tow locations and molecular detections for Fisherman Island and Oil refinery sublocations in Brisbane in spring 2017.

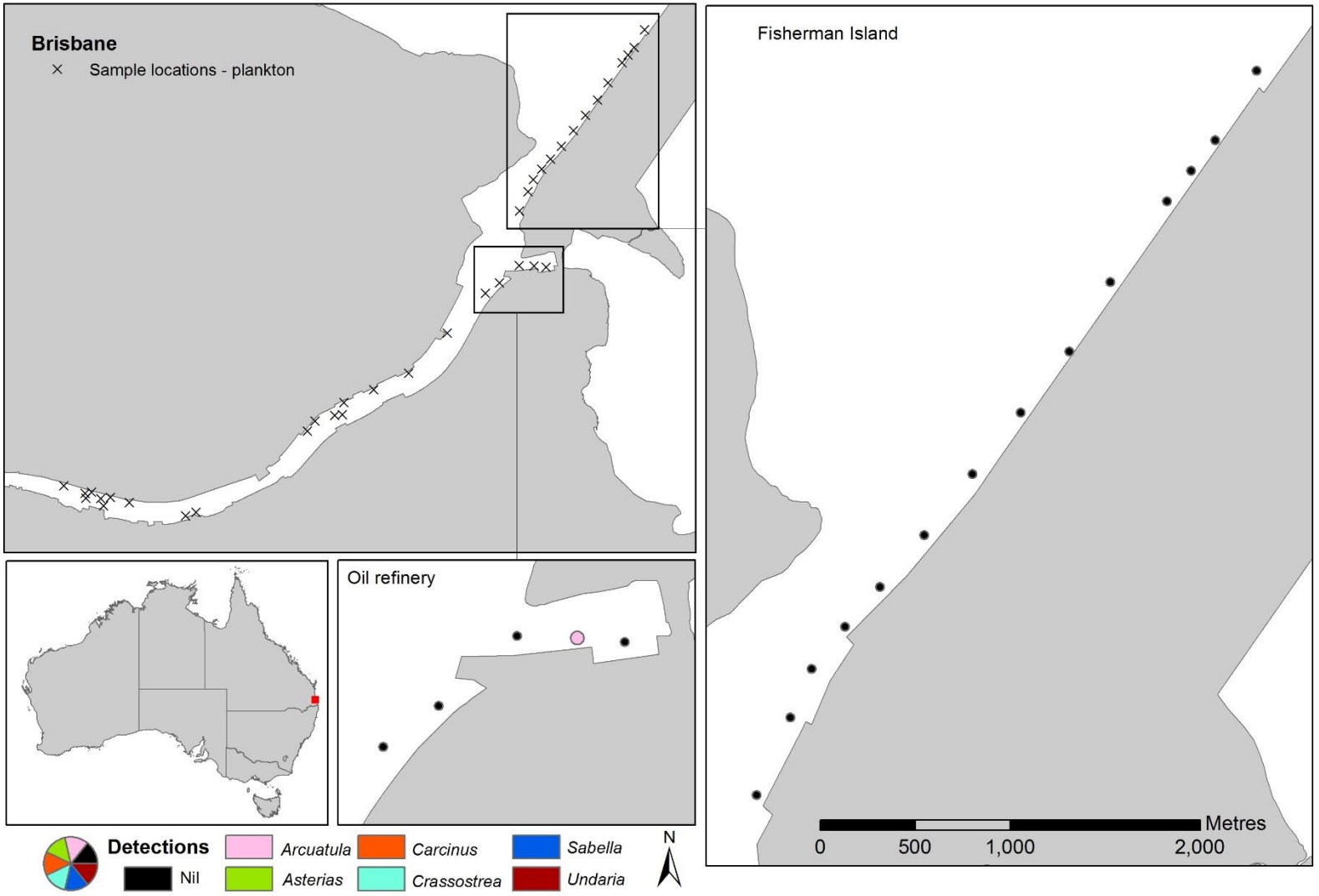

Figure 10. Map of plankton tow locations and molecular detections for Fisherman Island and Oil refinery sublocations in Brisbane in summer 2018.

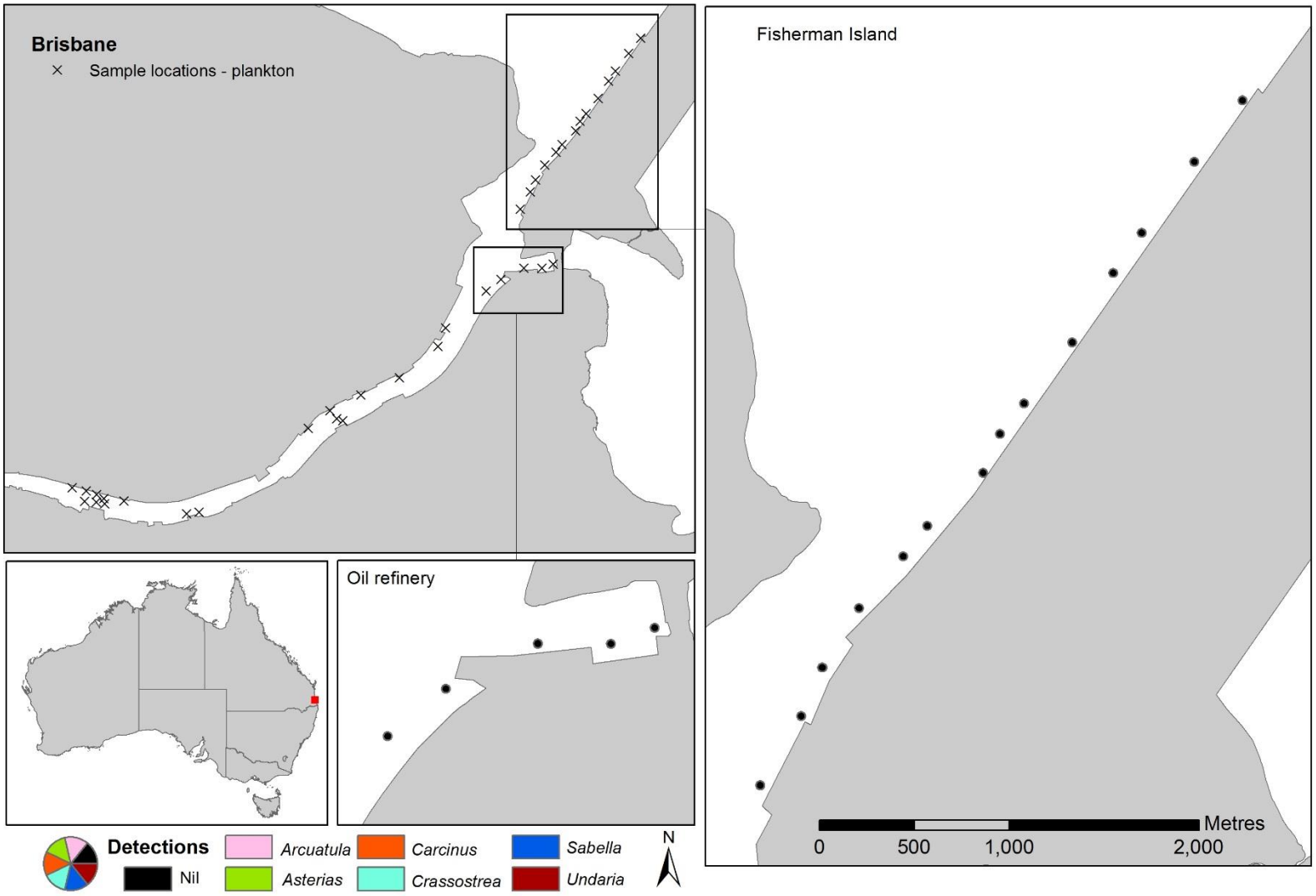

Figure 11. Map of plankton tow locations and molecular detections for Fisherman Island and Oil refinery sublocations in Brisbane in autumn 2018.

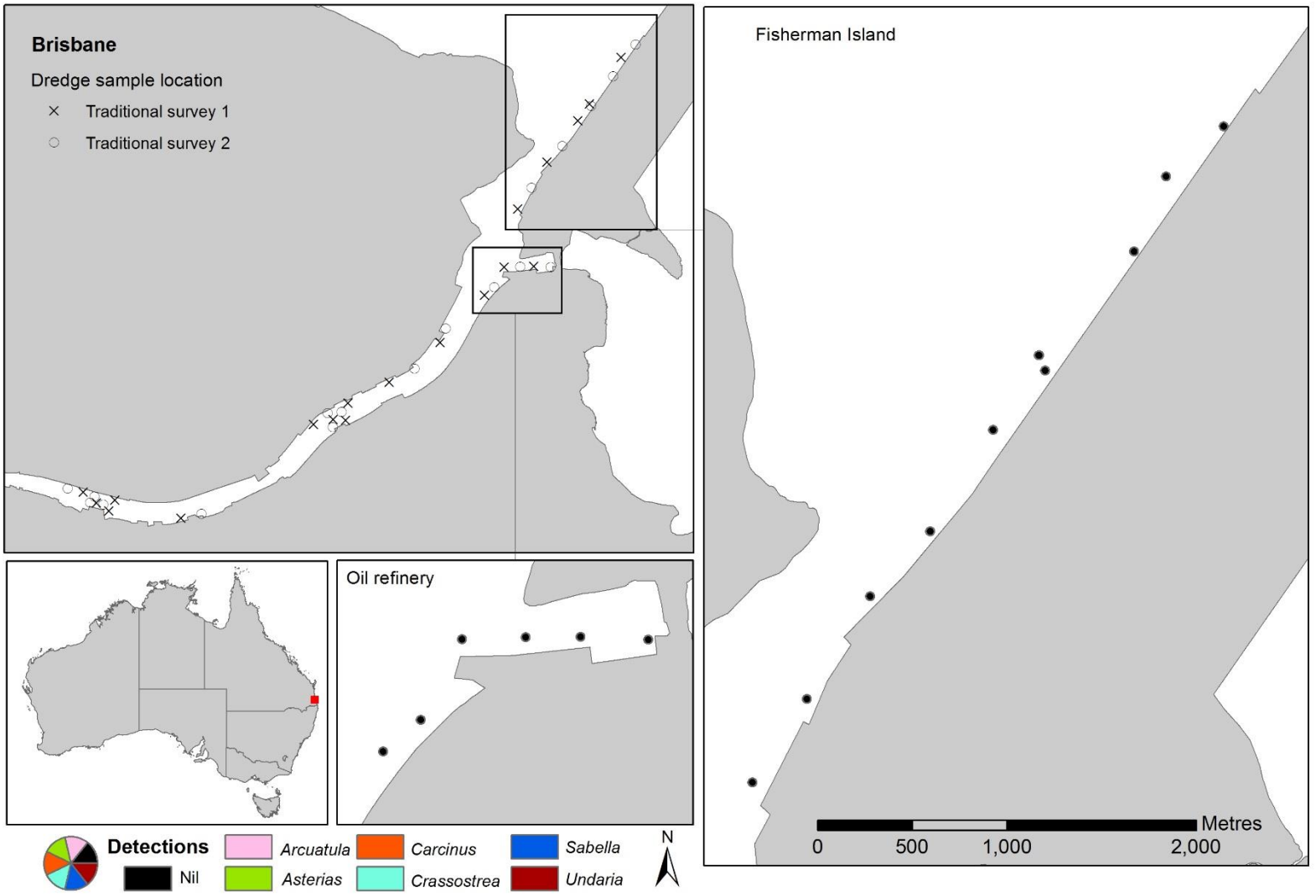

Figure 12. Map of dredge tow locations and detections for Fisherman Island and Oil refinery sublocations for both traditional surveys in Brisbane.

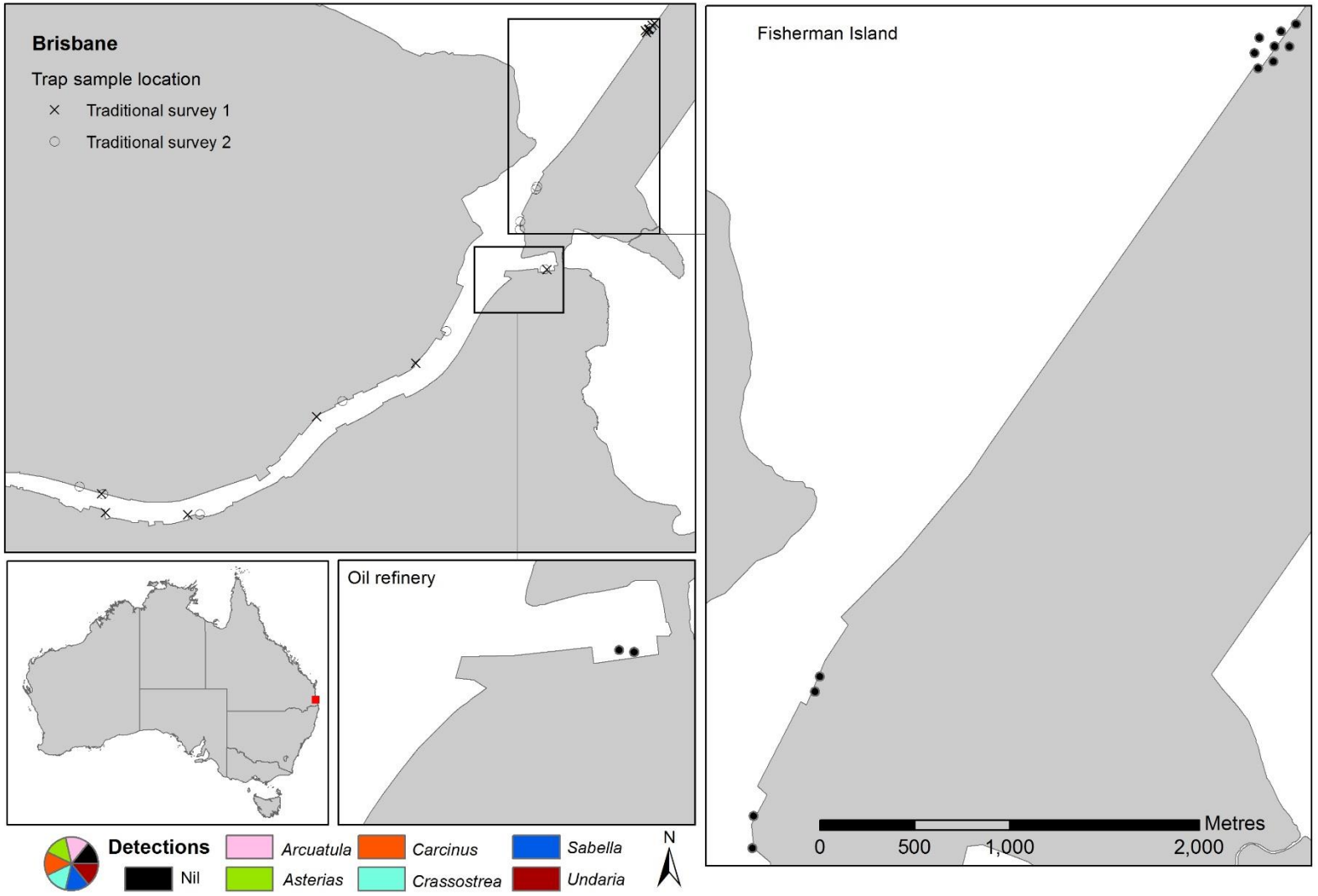

Figure 13. Map of trap locations and detections for Fisherman Island and Oil refinery sublocations for both traditional surveys in Brisbane.

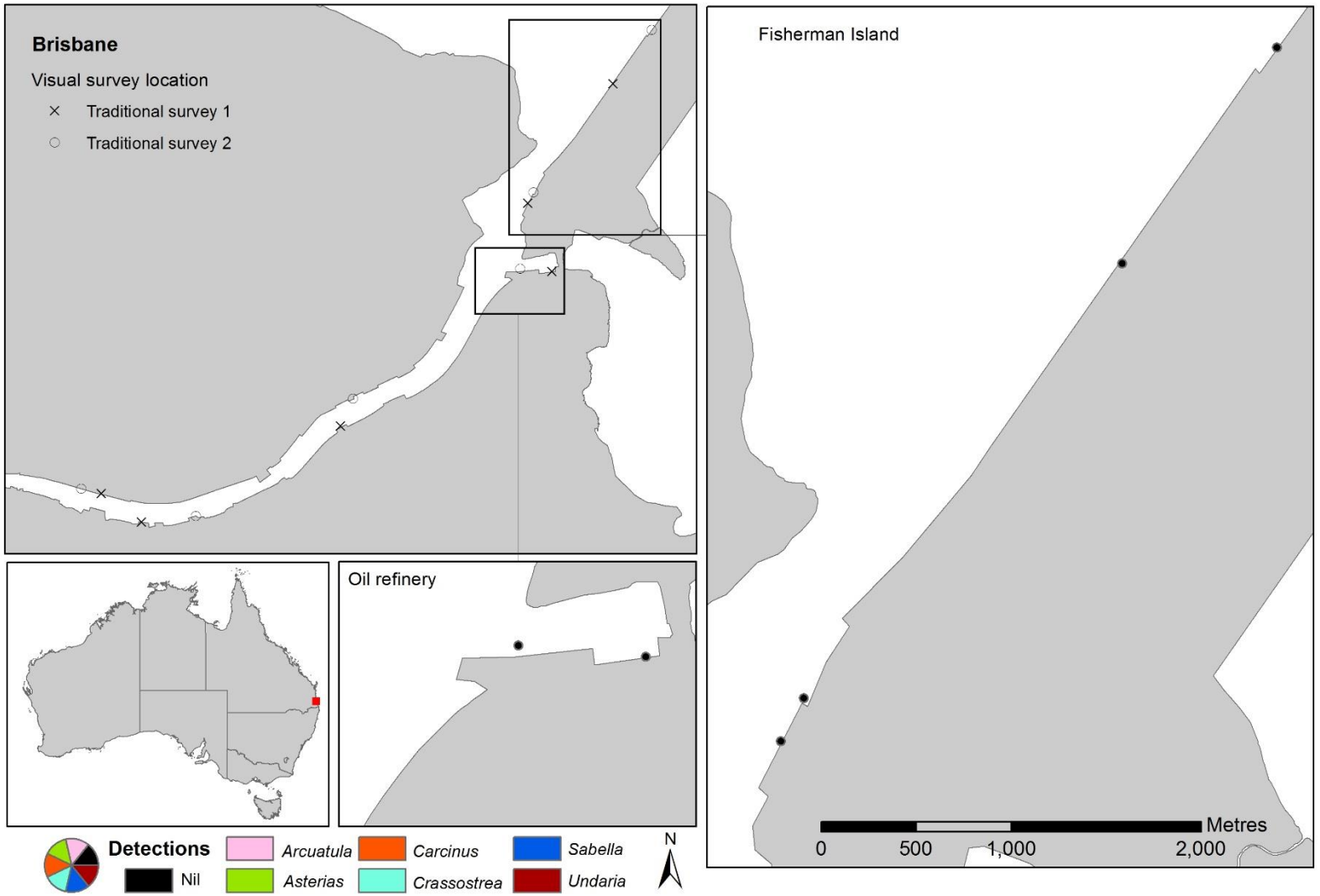

Figure 14. Map of visual survey locations and detections for Fisherman Island and Oil refinery sublocations for both traditional surveys in Brisbane.

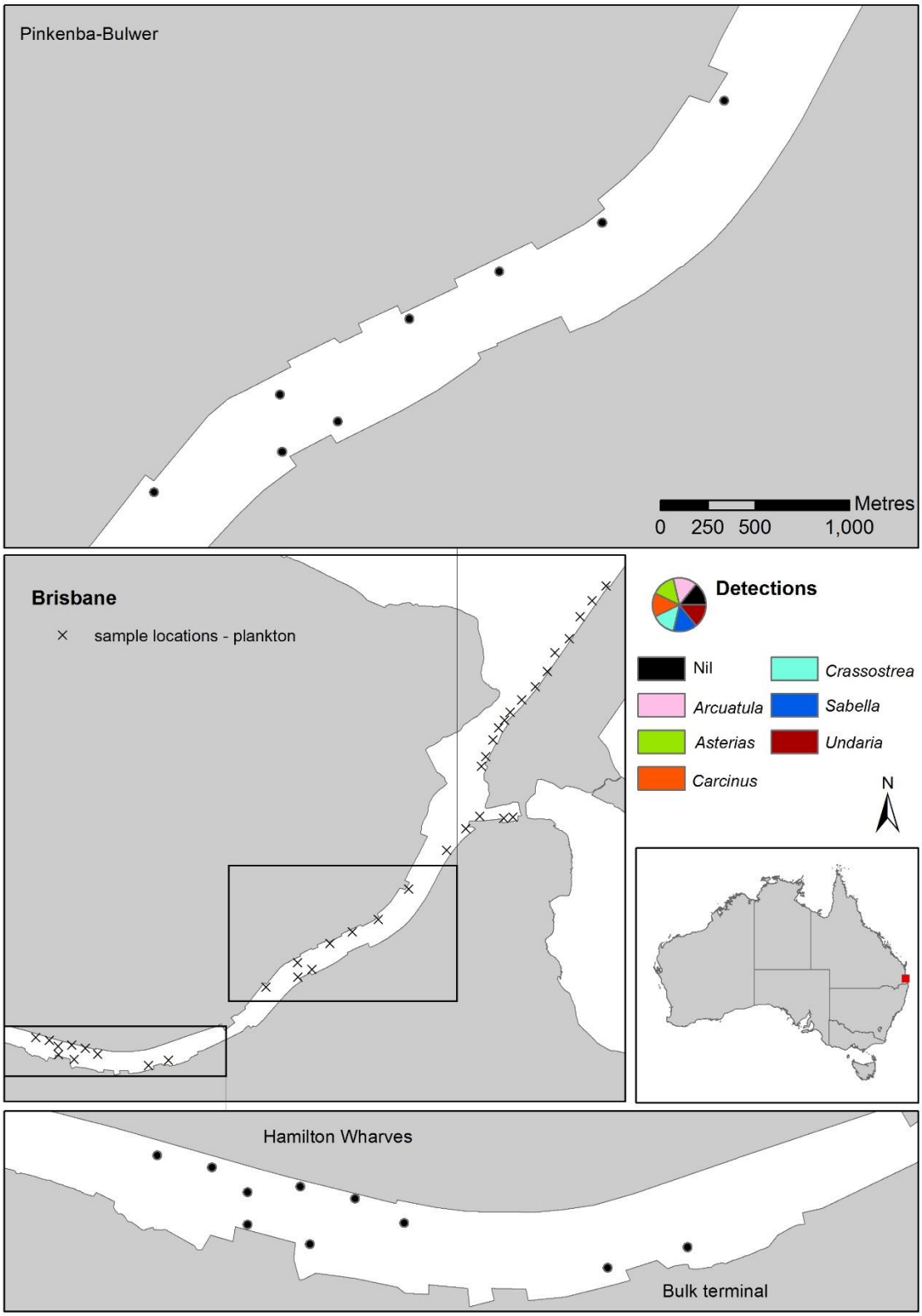

Figure 15. Map of plankton tow locations and molecular detections for Pinkenba-Bulwer, Bulk terminal and Hamilton wharves sublocations in Brisbane in winter 2017.

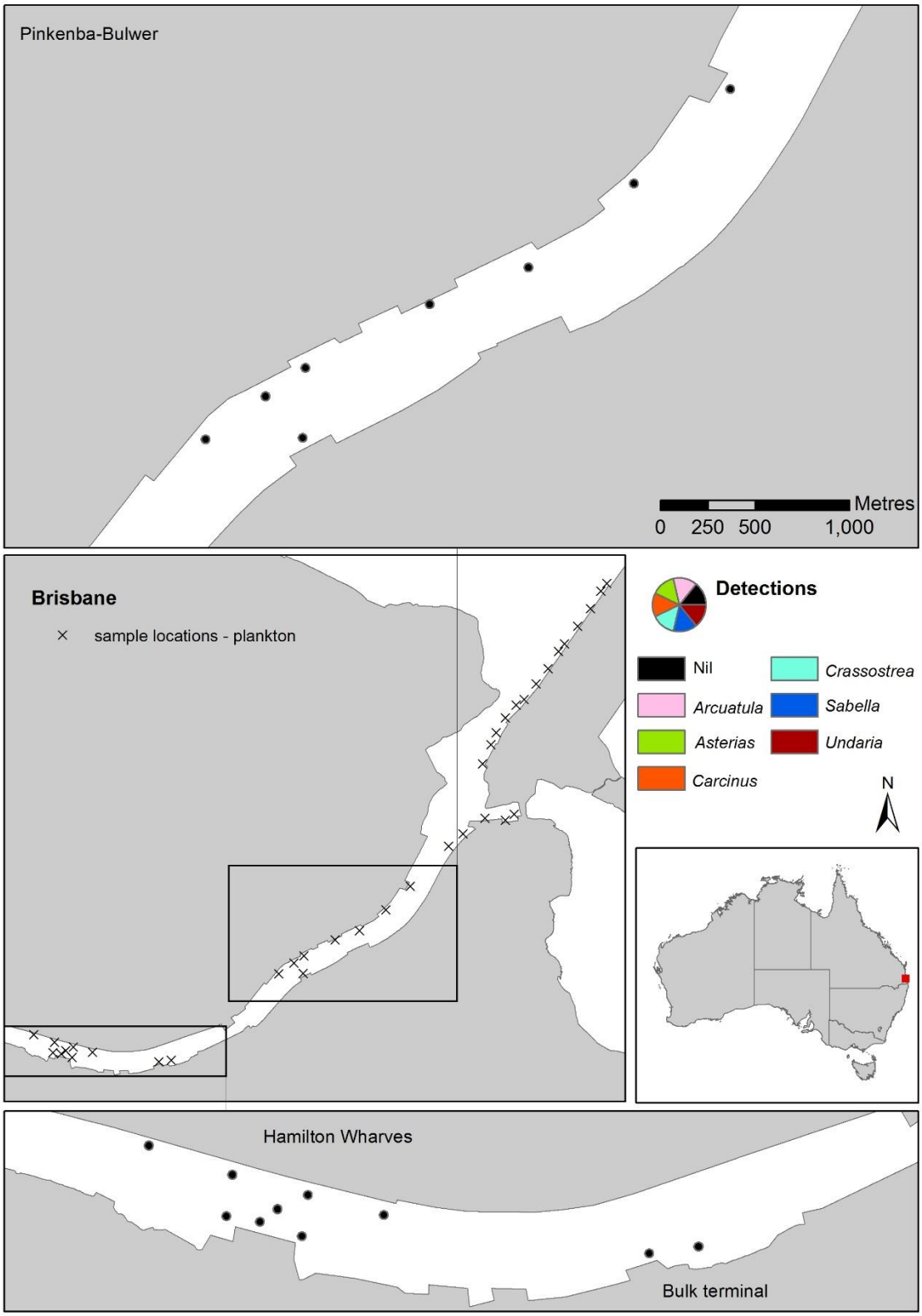

Figure 16. Map of plankton tow locations and molecular detections for Pinkenba-Bulwer, Bulk terminal and Hamilton wharves sublocations in Brisbane in spring 2017.

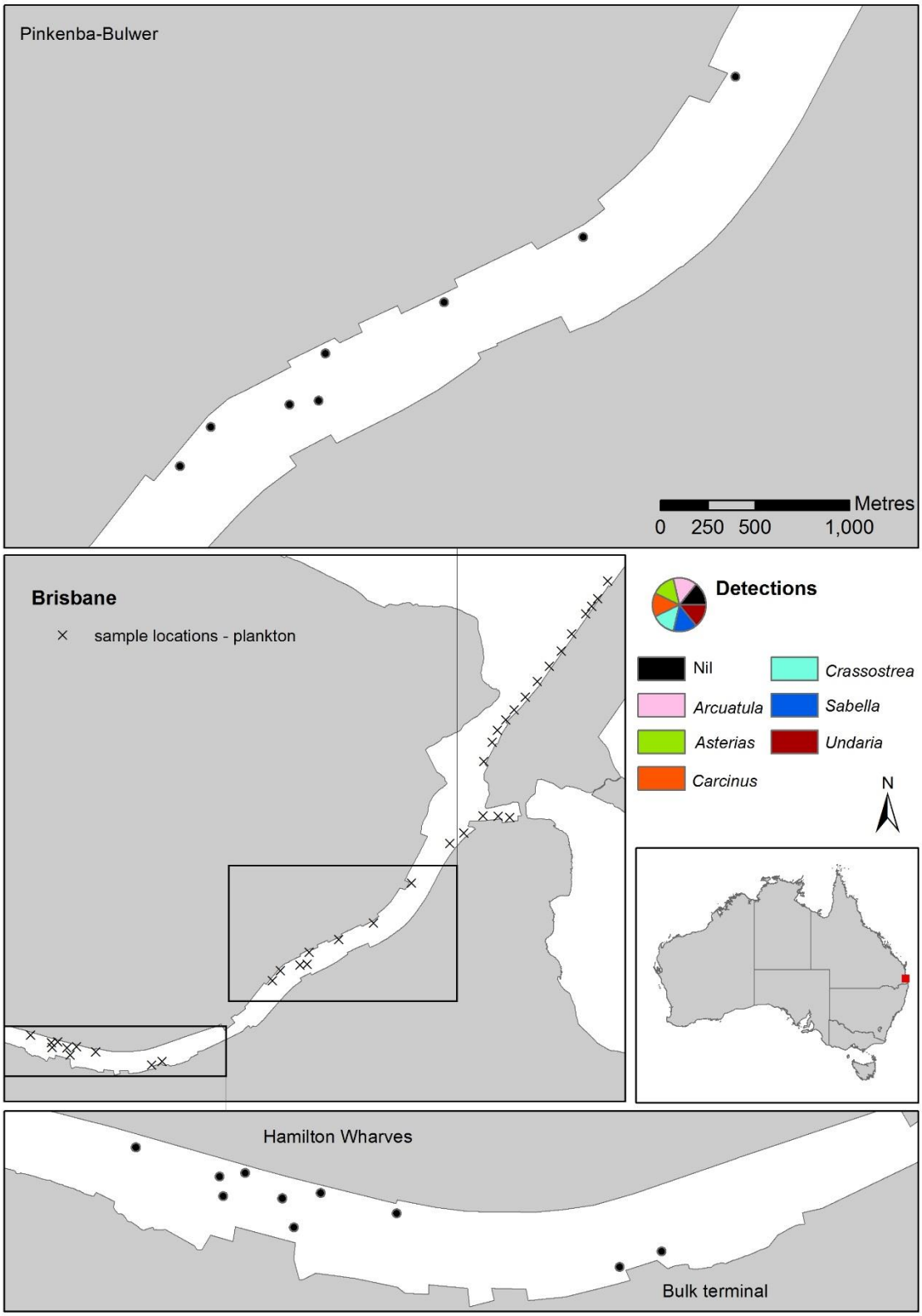

Figure 17. Map of plankton tow locations and molecular detections for Pinkenba-Bulwer, Bulk terminal and Hamilton wharves sublocations in Brisbane in summer 2018.

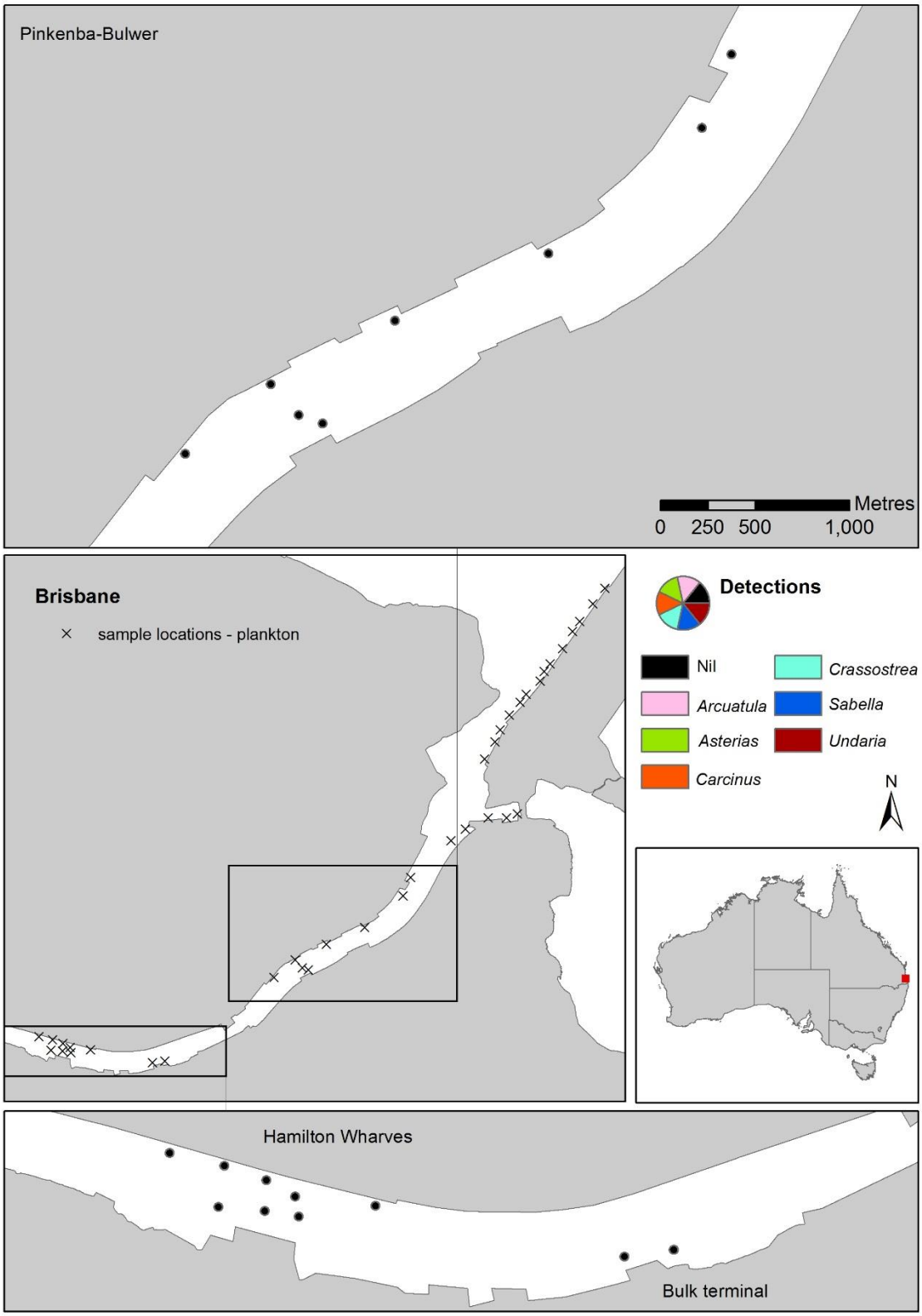

Figure 18. Map of plankton tow locations and molecular detections for Pinkenba-Bulwer, Bulk terminal and Hamilton wharves sublocations in Brisbane in autumn 2018.

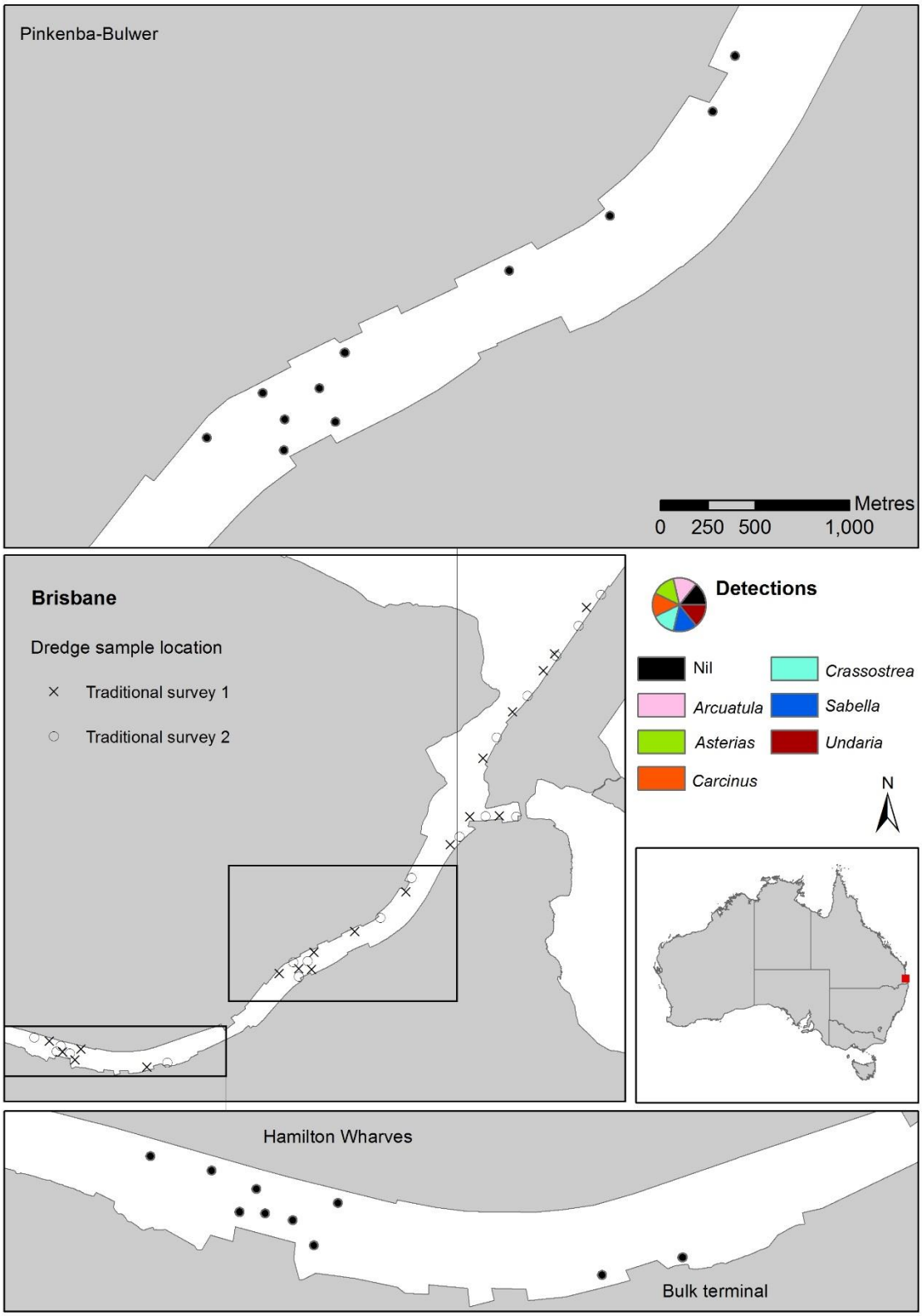

Figure 19. Map of dredge tow locations and detections for Pinkenba-Bulwer, Bulk terminal and Hamilton wharves sublocations for both traditional surveys in Brisbane.

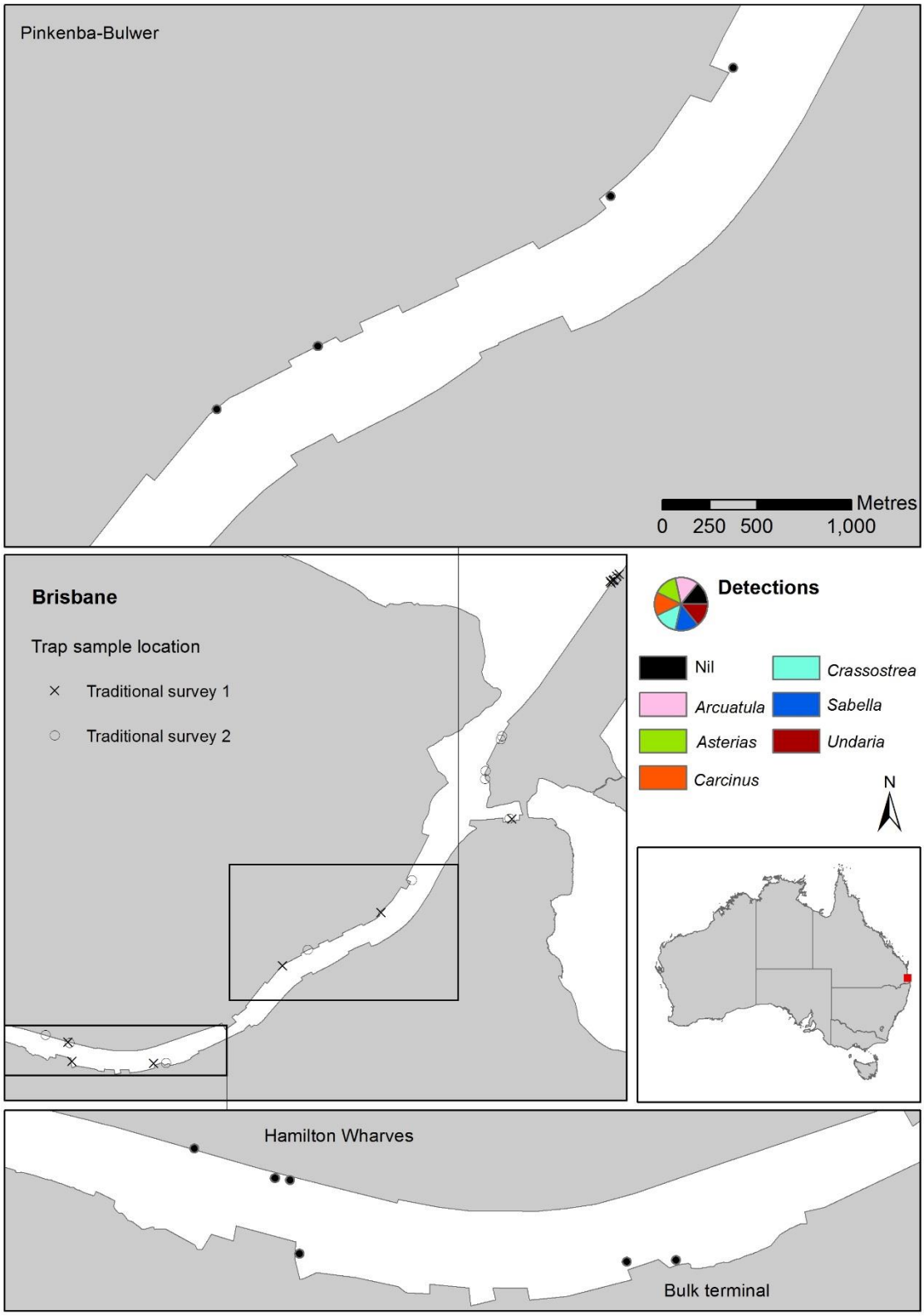

Figure 20. Map of trap locations and detections for Pinkenba-Bulwer, Bulk terminal and Hamilton wharves sublocations for both traditional surveys in Brisbane.

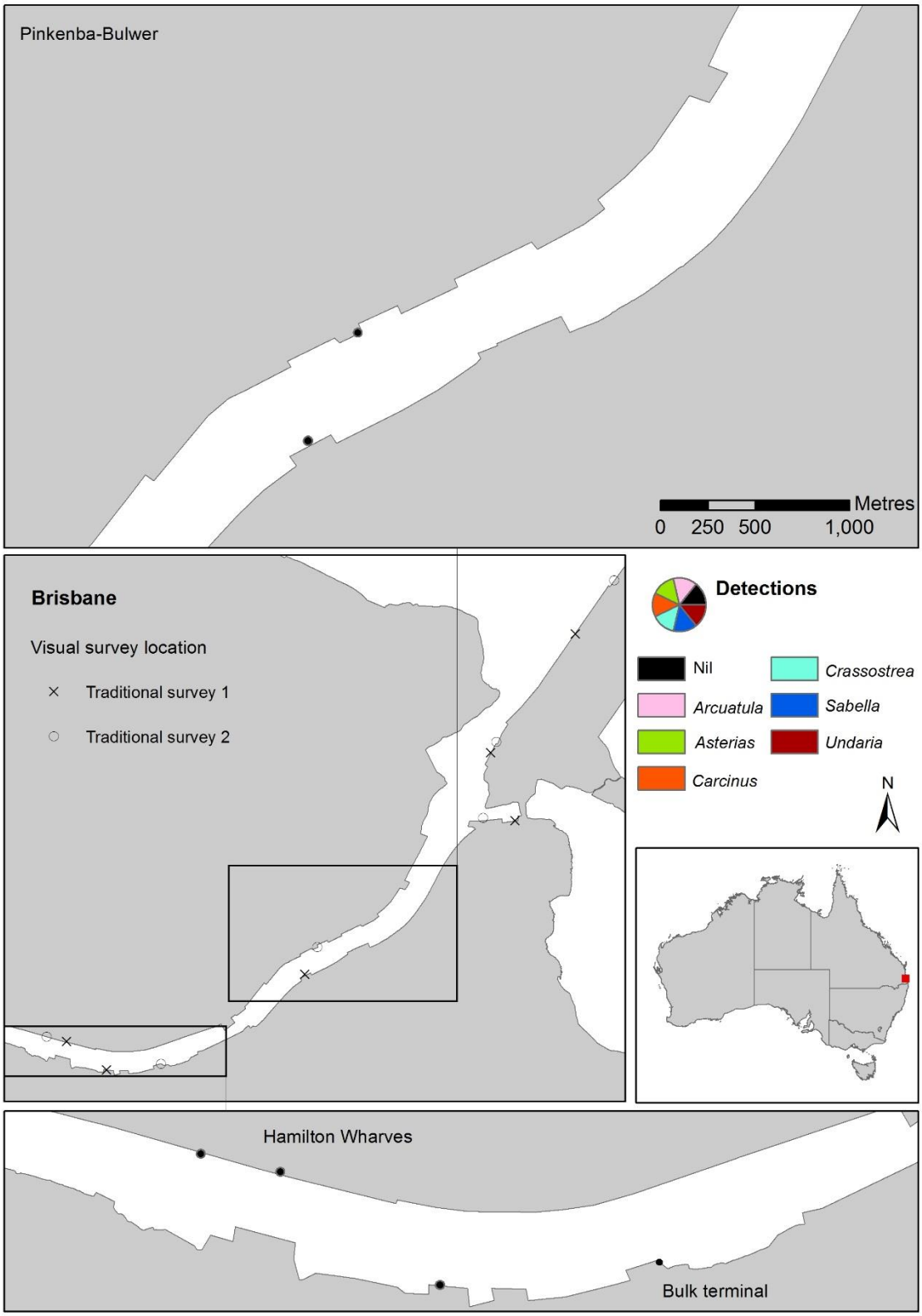

Figure 21. Map of visual survey locations and detections for Pinkenba-Bulwer, Bulk terminal and Hamilton wharves sublocations for both traditional surveys in Brisbane.

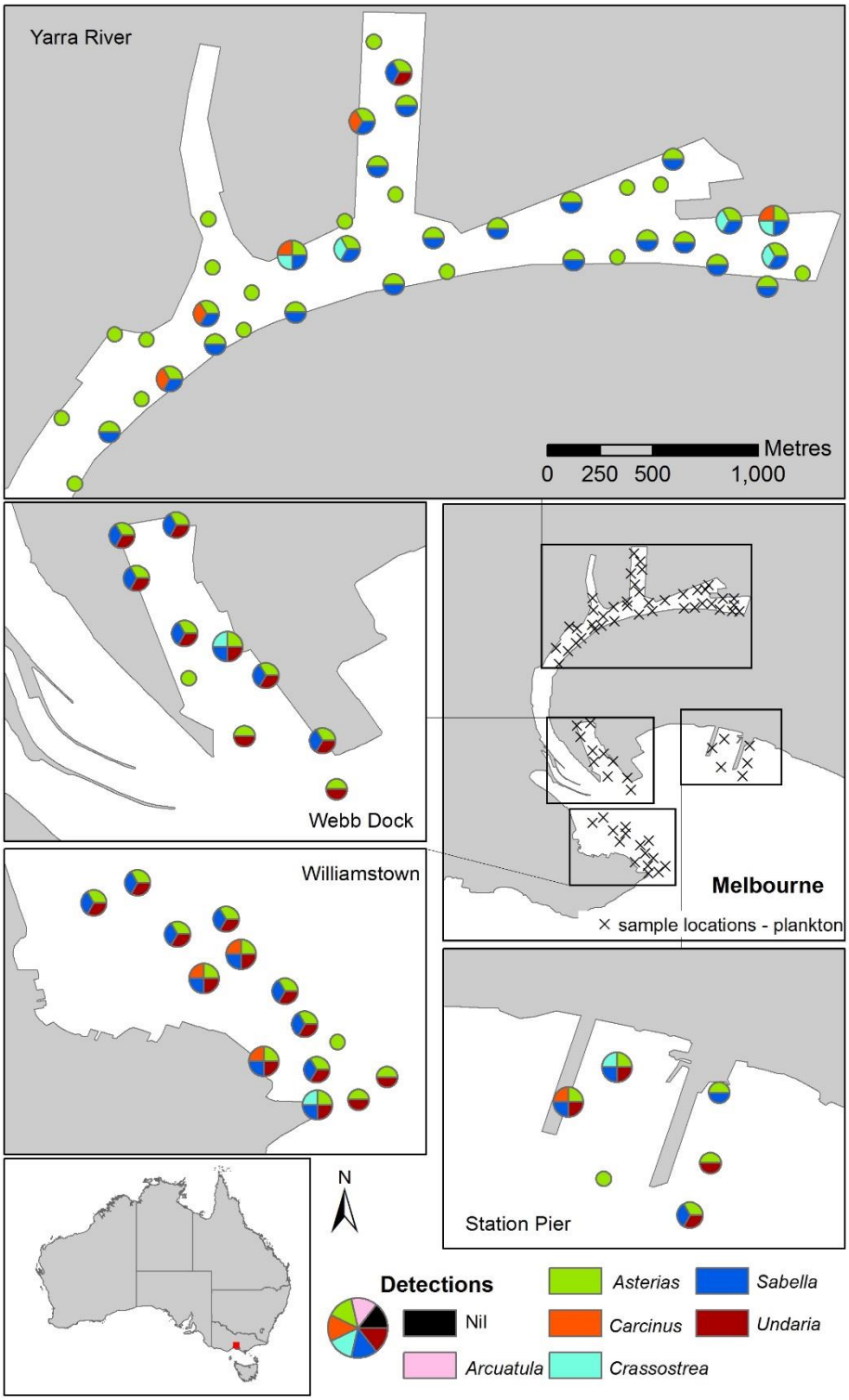

Figure 22. Map of plankton tow locations and molecular detections for Melbourne in winter 2017.

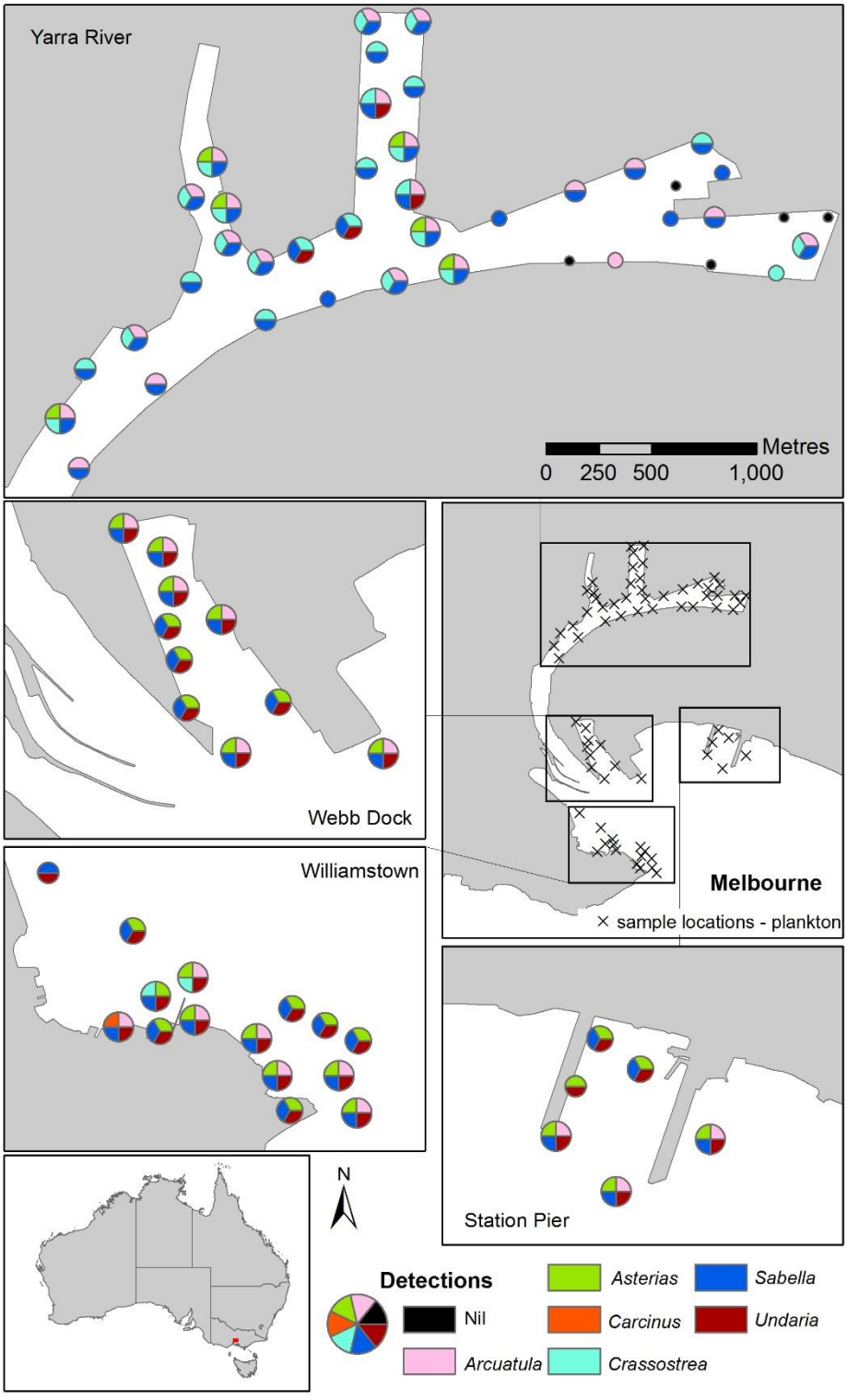

Figure 23. Map of plankton tow locations and molecular detections for Melbourne in spring 2017.

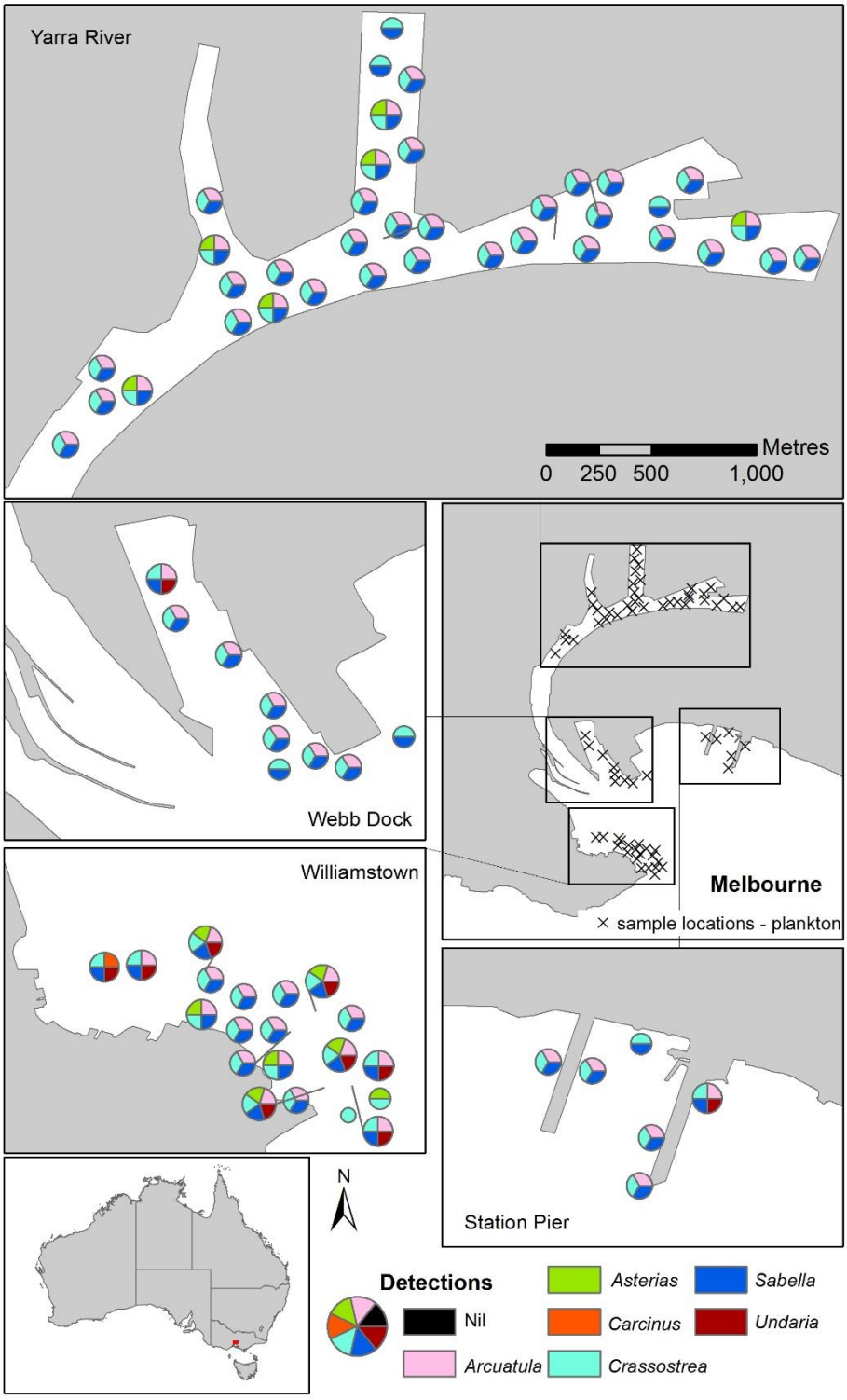

Figure 24. Map of plankton tow locations and molecular detections for Melbourne in summer 2018.

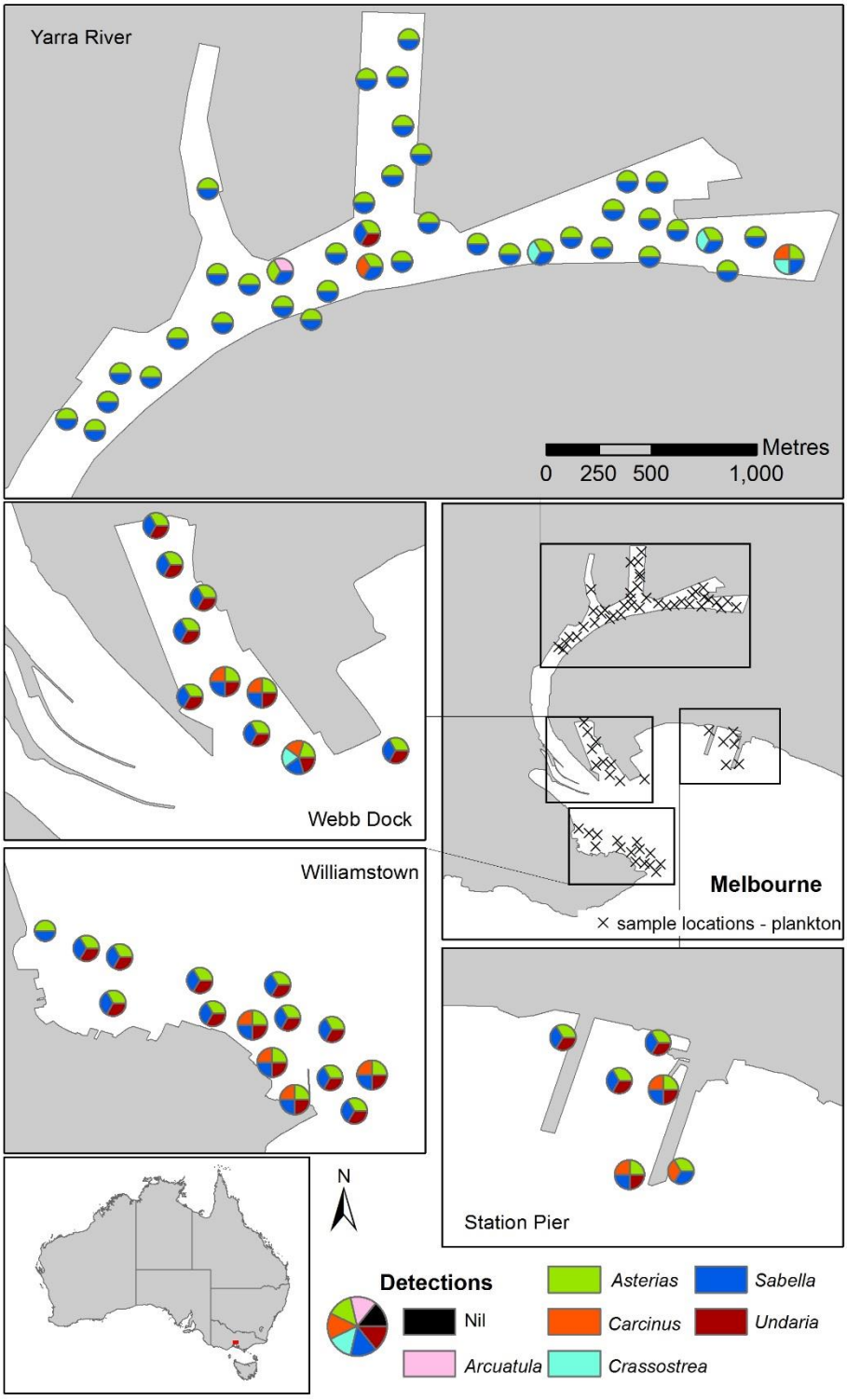

Figure 25. Map of plankton tow locations and molecular detections for Melbourne in autumn 2018.

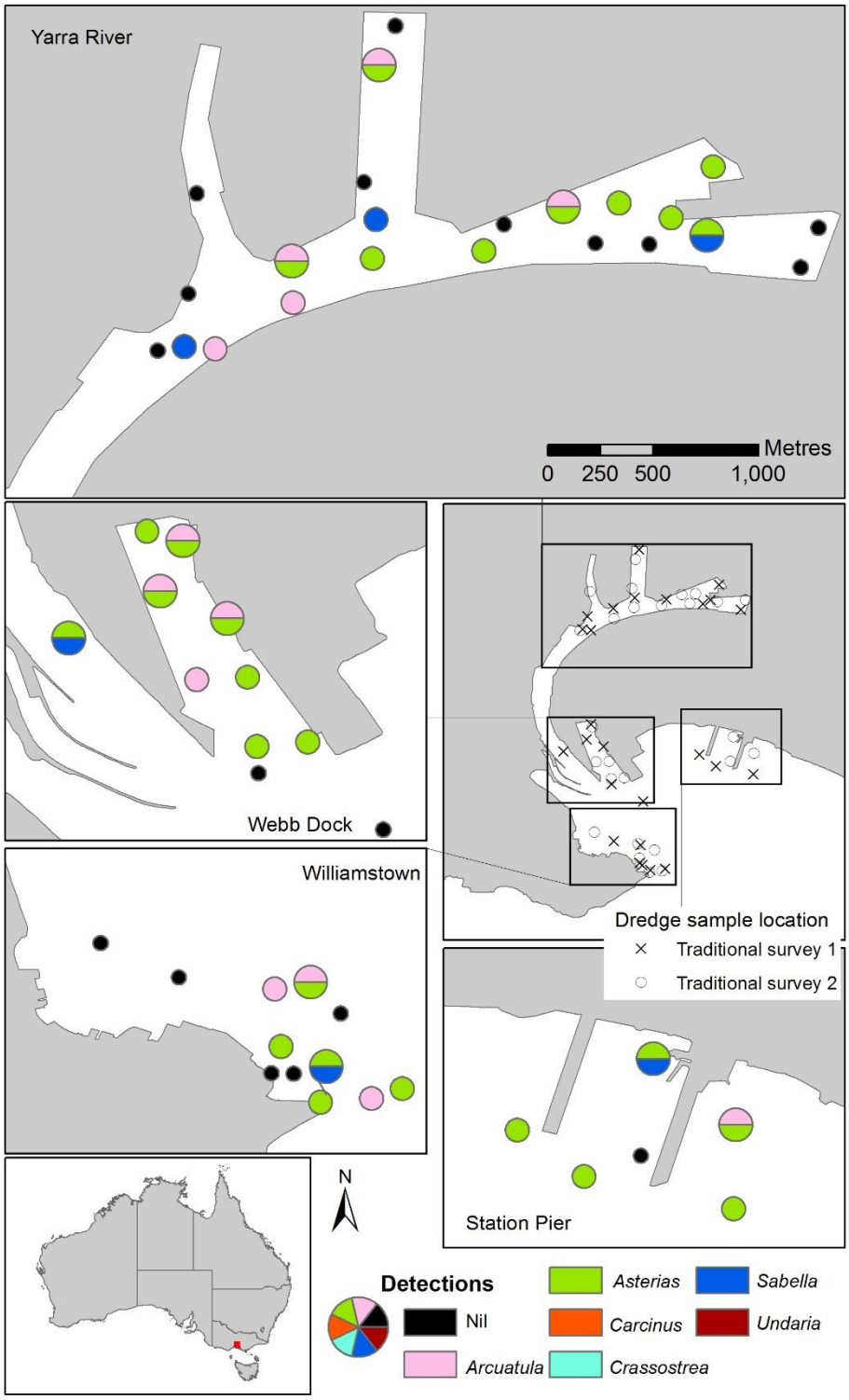

Figure 26. Map of dredge tow locations and detections for both traditional surveys in Melbourne.

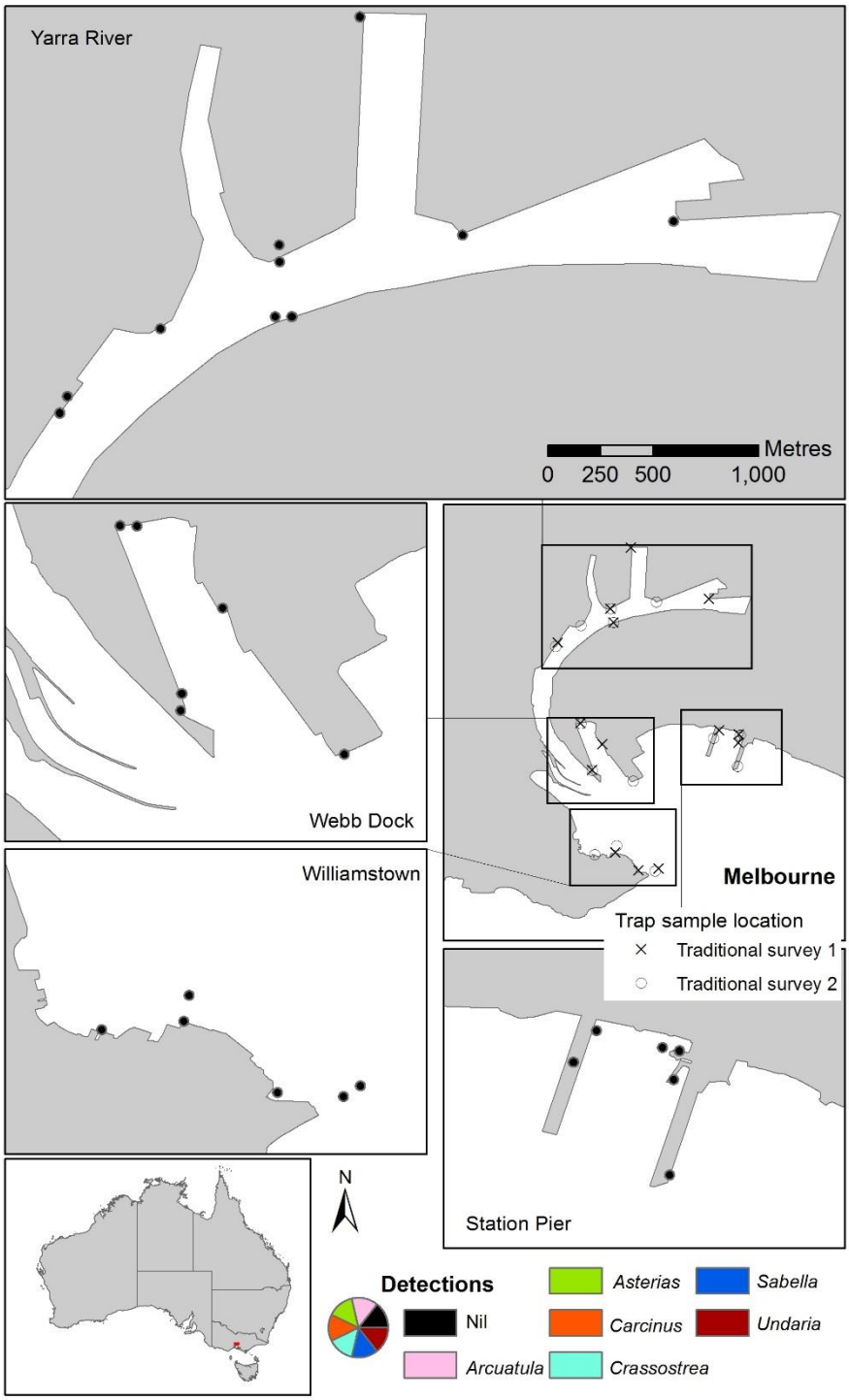

Figure 27. Map of trap locations and detections for both traditional surveys in Melbourne.

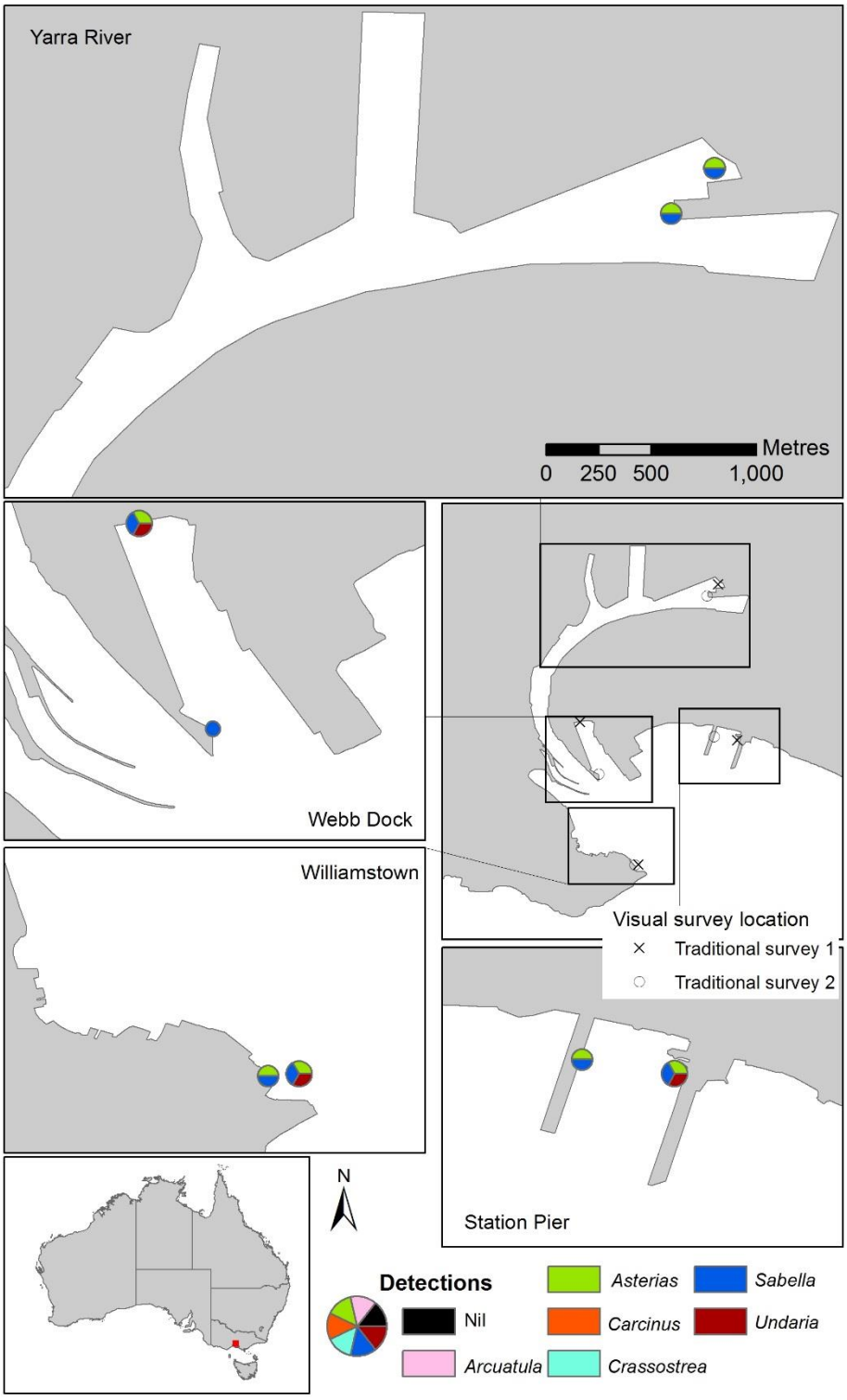

Figure 28. Map of visual survey locations and detections for both traditional surveys in Melbourne.

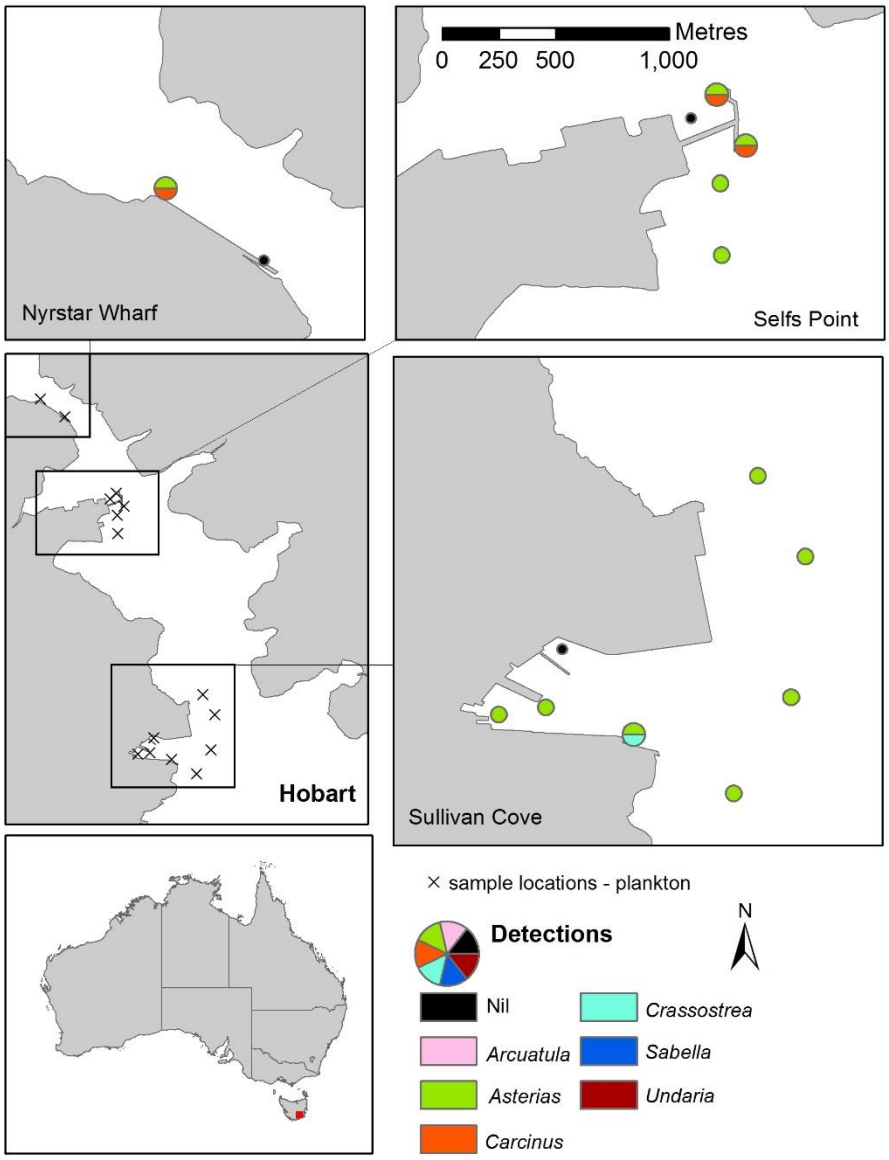

Figure 29. Map of plankton tow locations and molecular detections for Hobart in winter 2017.

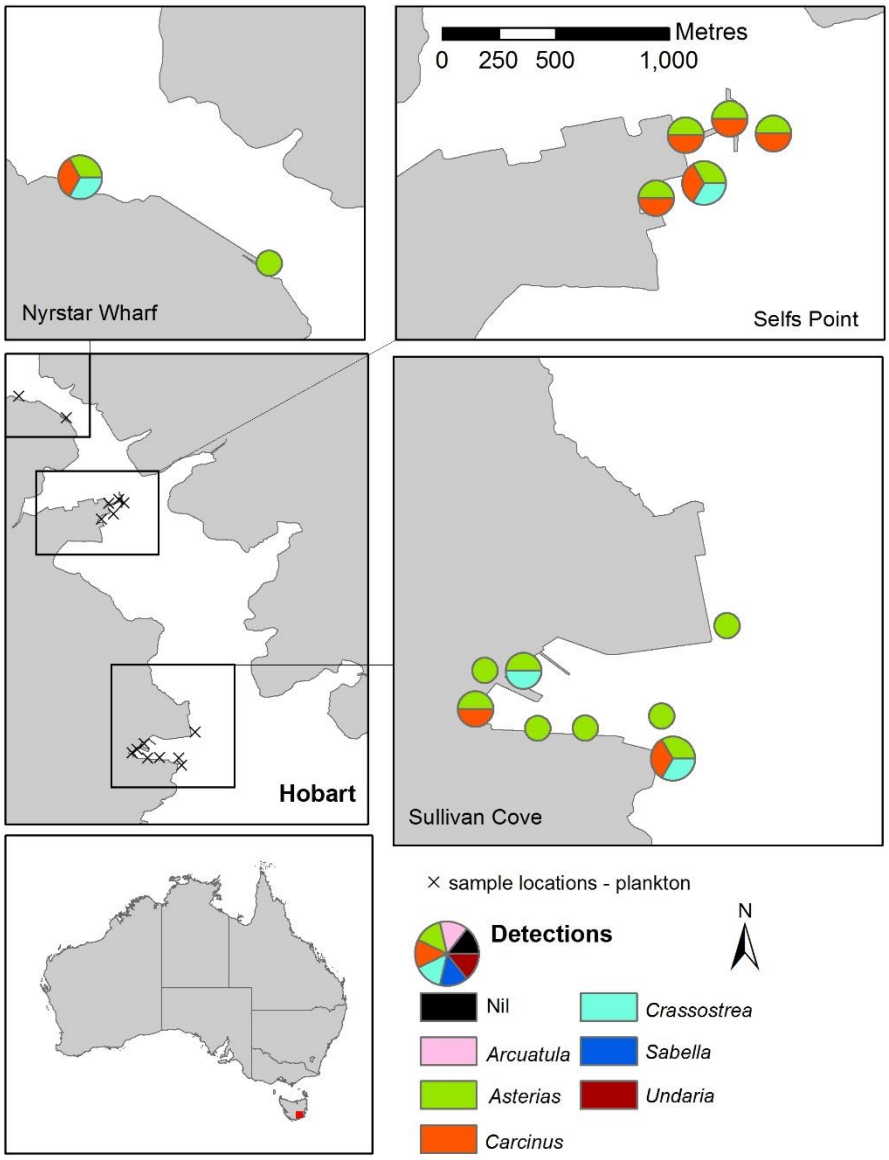

Figure 30. Map of plankton tow locations and molecular detections for Hobart in spring 2017.

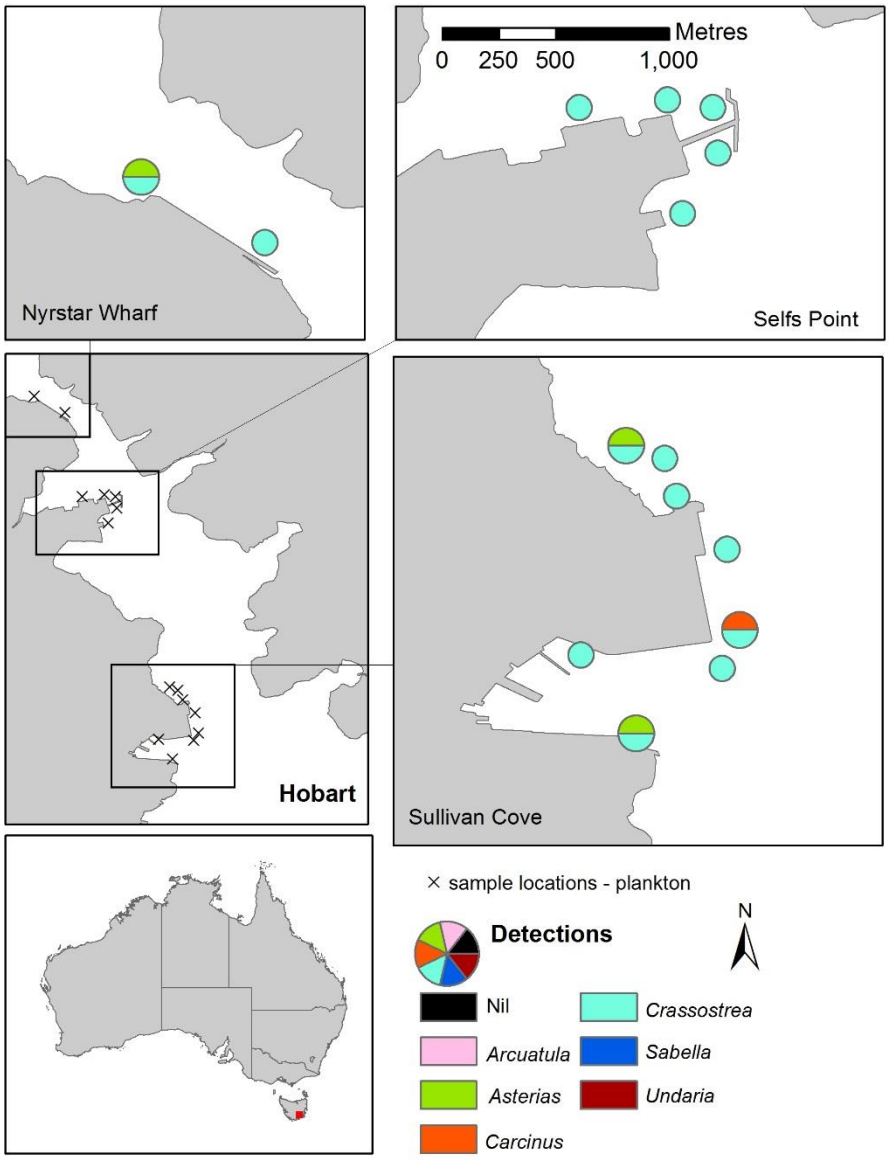

Figure 31. Map of plankton tow locations and molecular detections for Hobart in summer 2018.

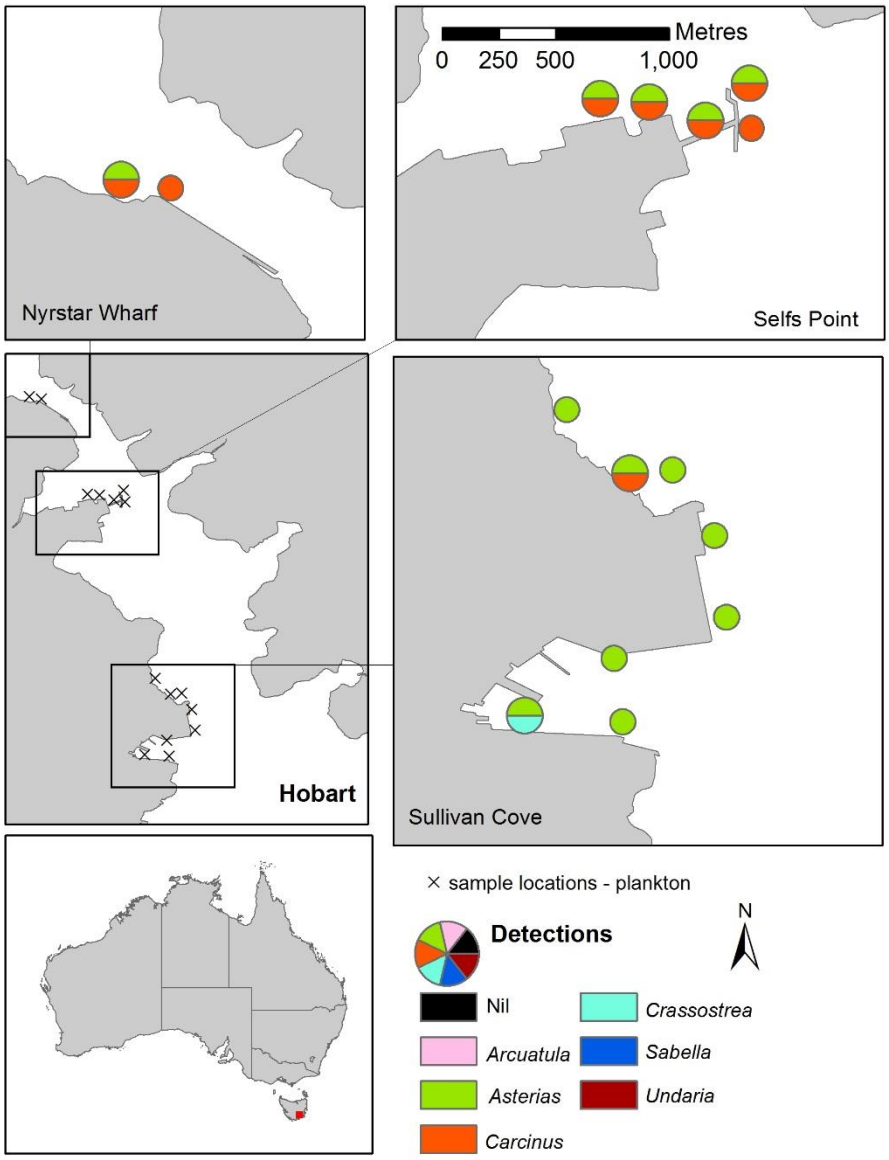

Figure 32. Map of plankton tow locations and molecular detections for Hobart in autumn 2018.

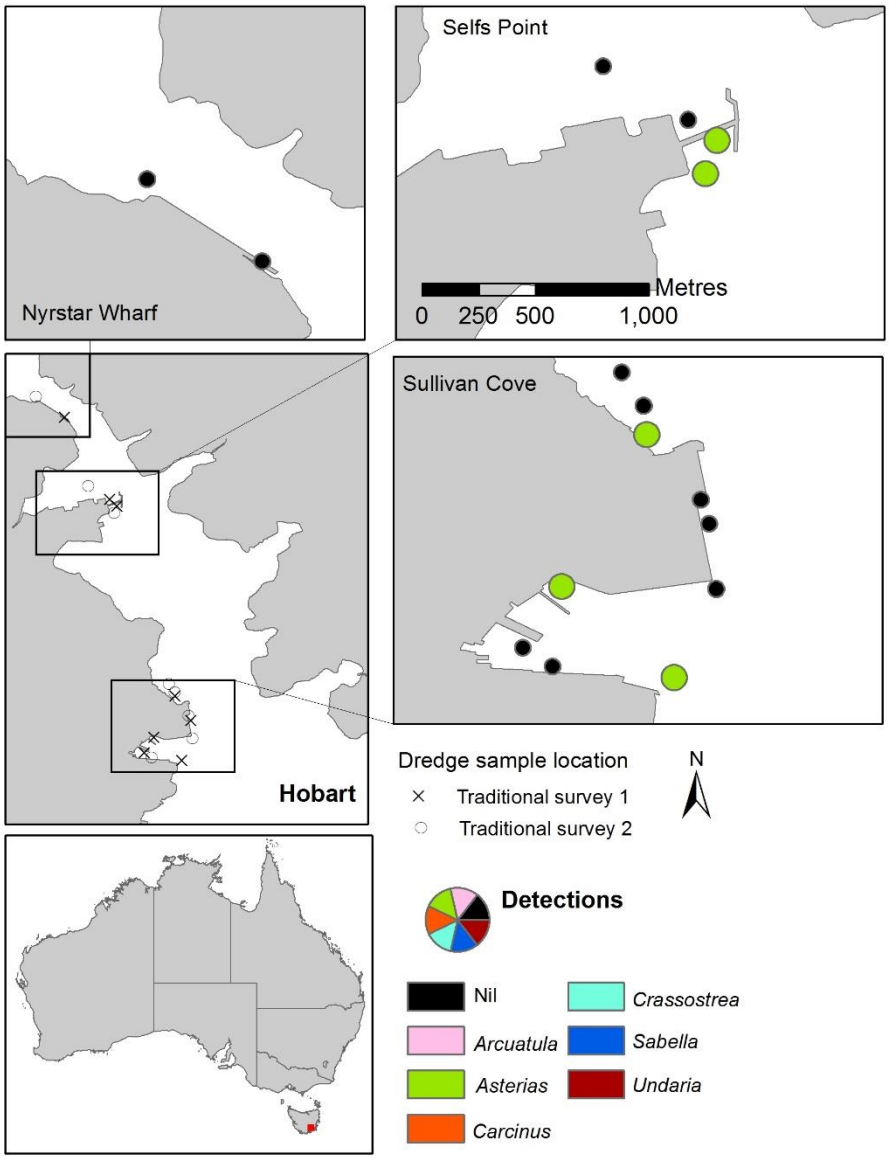

Figure 33. Map of dredge tow locations and detections for both traditional surveys in Hobart.

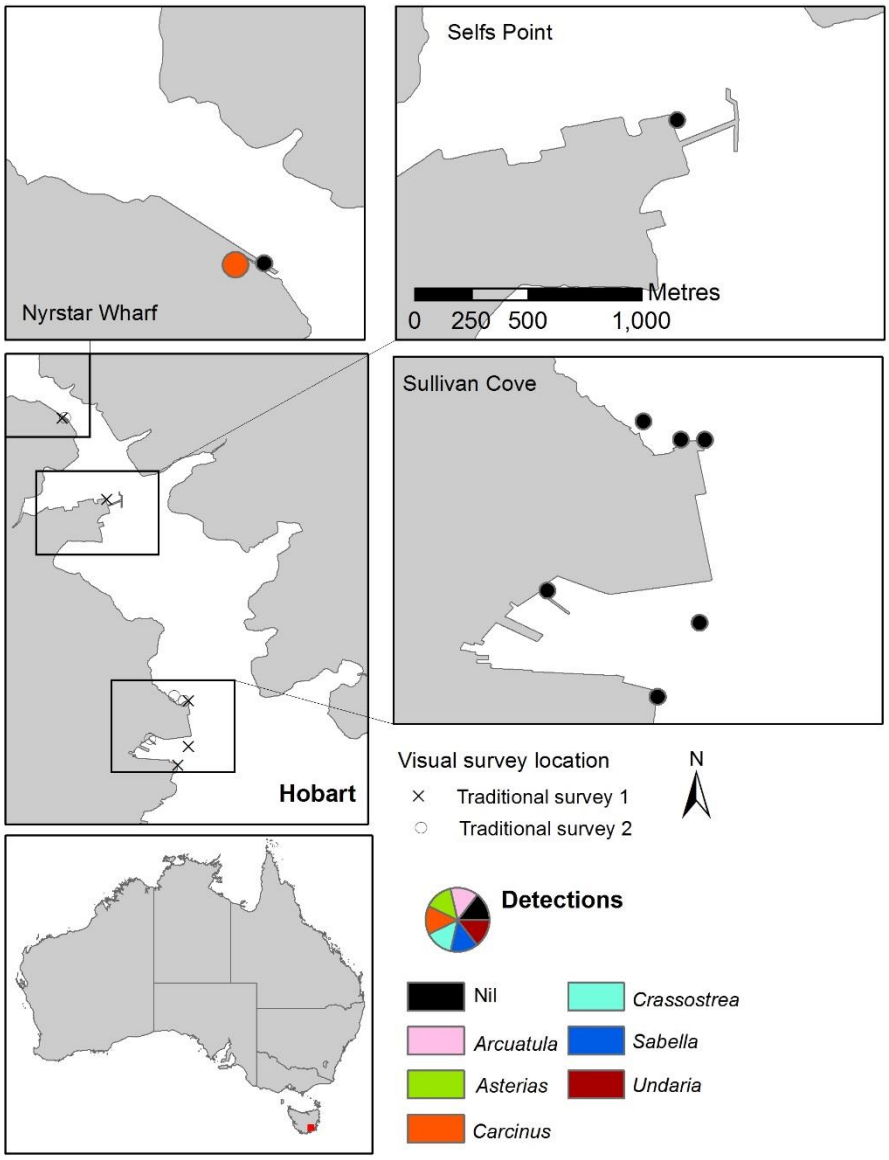

Figure 34. Map of trap locations and detections for both traditional surveys in Hobart.

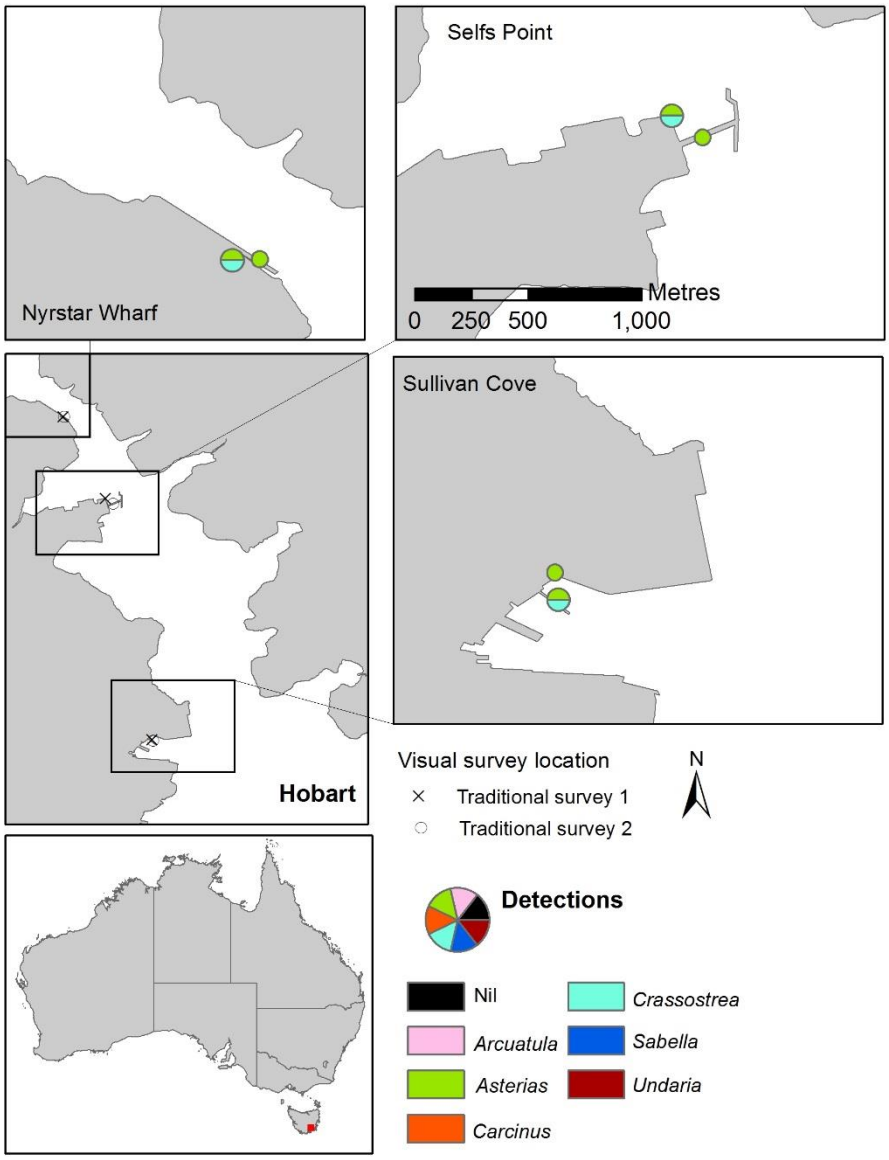

Figure 35. Map of visual survey locations and detections for both traditional surveys in Hobart.

3.3. Comparison of sampling methods

3.3.1. Method effectiveness and detection likelihood

Aside from the isolated detections in Brisbane, detections by molecular methods for all species occurred at all sublocations within each port, although not always in every sample set (see section 3.2). Where both molecular and traditional surveillance methods detected a pest, the traditional method recorded that species at all sublocations in most cases. The exceptions were *Carcinus*, which was detected in traditional samples only at the Nyrstar wharf sublocation in Hobart (Figure

34), and *Undaria,* which was not detected by traditional methods in the Yarra River in Melbourne (Figure 28). The lack of detections by traditional methods in some sublocations is unsurprising, given that DNA can be advected away from source populations, leading to detections over a wider area than the species occurrence range. The modelled relative pest concentration (Figure 36) showed little difference across sublocations in each port, except for *Carcinus* in Hobart, which had a lower relative prevalence in the Sullivan Cove (HSC) sublocation, and *Undaria* and *Asterias* in Melbourne, which both had lower relative concentration in the Yarra River (MYR), particularly *Undaria*. For *Asterias*, *Crassostrea* and *Carcinus* in both Melbourne and Hobart, modelled relative concentration was high and similar across those ports for *Asterias* and *Crassostrea*, while relative concentration of *Carcinus* was predicted to be lower in Melbourne than Hobart. Due to the uncertainty around *Arcuatula* detections in Brisbane and Gladstone, these data were not included in the probabilistic model, because these results would unduly influence the predicted performance of dredge sampling. The *Undaria* detection in Brisbane, while believed to be an isolated occurrence, was retained, with a resulting mean concentration of 0.006 predicted for the Fisherman Islands (BFI) sublocation, in comparison to 0.01 for *Undaria* in the Yarra River (MYR), which was the lowest concentration predicted for a species known to occur in a port. Note that while *Undaria* has large established populations in the port area in Melbourne, it is not clear whether there is a population in the Yarra River. The molecular detections there could be due to advected DNA from the adjacent Port Philip Bay populations. For sublocations where relatively few samples were collected such as Nyrstar Wharf in Hobart (HNW), bulk terminal, Brisbane (BBT), and Curtis East in Gladstone (GCE), the model predicts a low concentration of undetected target pests may occur, since for these sublocations the low sample numbers mean a pest occurring at low density may have been missed by chance. Given pest absence across the wider port in each case, however, it is unlikely that these pests occur. Note that these predicted densities are approximately three orders of magnitude smaller than the predicted densities of the pests that are known to occur at each site.

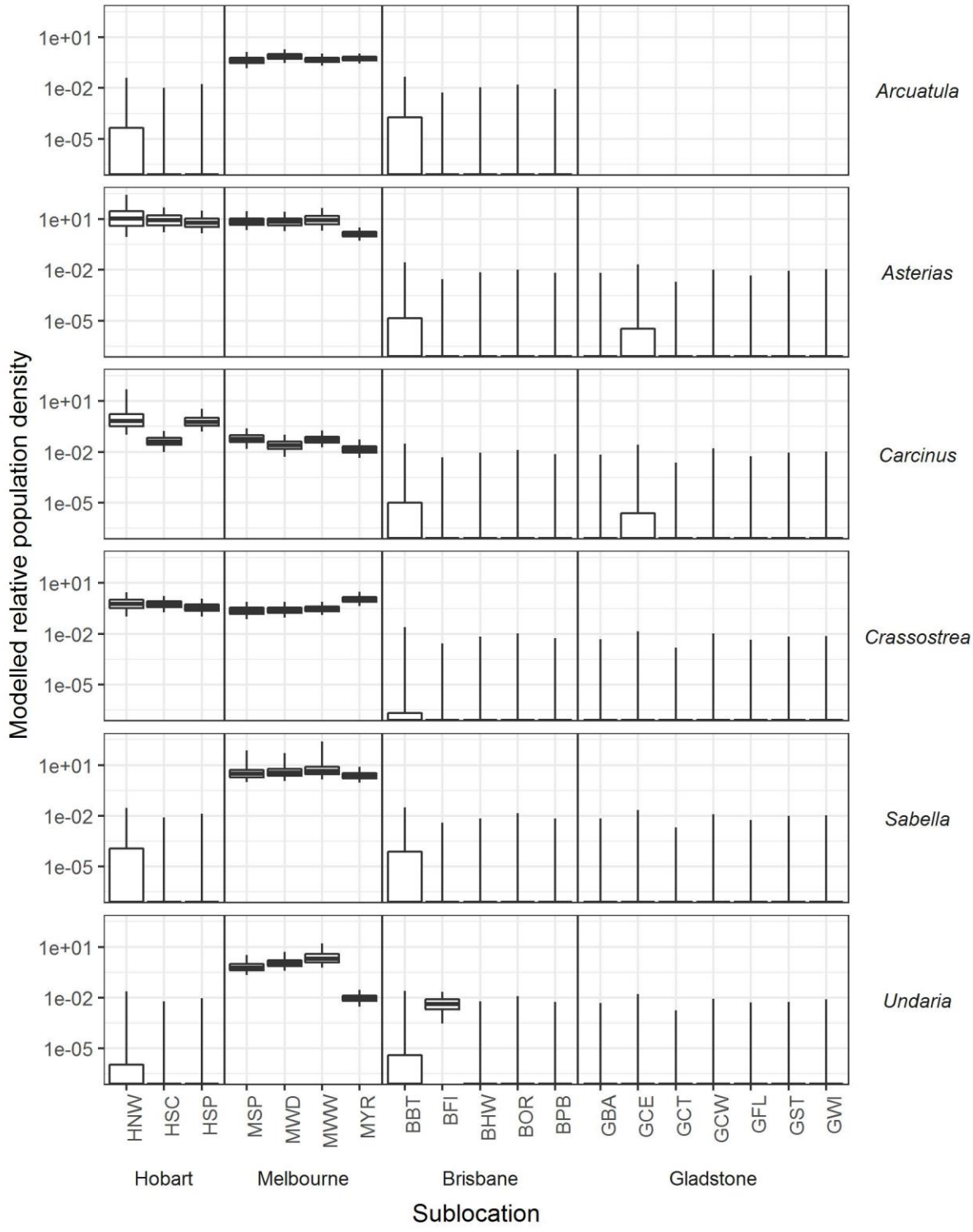

Figure 36. Predicted relative abundance of each target species by sublocation. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Note that y-axis is shown on a log10 scale.

Different traditional sampling types are applicable for the different target species. For some species there are multiple valid methods considered by the MDeT, but typically one most effective method. Traditional survey detections occurred primarily in samples collected by the methods most appropriate to each species. *Arcuatula* was detected only in dredge samples, *Carcinus* only in trap samples and *Crassostrea* and *Undaria* only in visual samples. Visual and dredge sampling are both considered valid for *Asterias*, and this species was detected by both methods, but with a higher rate of detections in visual samples (13/14 visual transects in ports where it occurred, compared with 32/68 dredge samples). *Sabella* was also detected primarily in visual samples (8/8) with additional detections in a relatively small number of dredge samples (6/52). Dredge samples are considered appropriate in the MDeT for detection of *Sabella* and also *Carcinus*, but only for occurrences in subtidal soft substrate; *Sabella*, however, is more commonly associated with hard substrate (Parry *et al.* 1996), while *Carcinus* occurs primarily in intertidal areas on either rocky or soft substrate (National System for the Prevention and Management of Marine Pest Incursions 2015). Visual surveys are considered valid by the MDeT for all species, but the current surveys did not target *Arcuatula* or *Carcinus* by this method, and no visual detections occurred of these species; traps are also considered valid by the MDeT for *Asterias*, but this species was not targeted by, and did not occur in traps. The probabilistic model showed that efficiency of the molecular method was similar to or better than the best traditional method for each species, i.e.: visual for *Asterias, Crassostrea, Sabella,* and *Undaria*; dredge for *Arcuatula*; and trap for *Carcinus* (Figure 37). Note that for all methods, this is the modelled likelihood of detection given that a pest is present in the sampling unit.

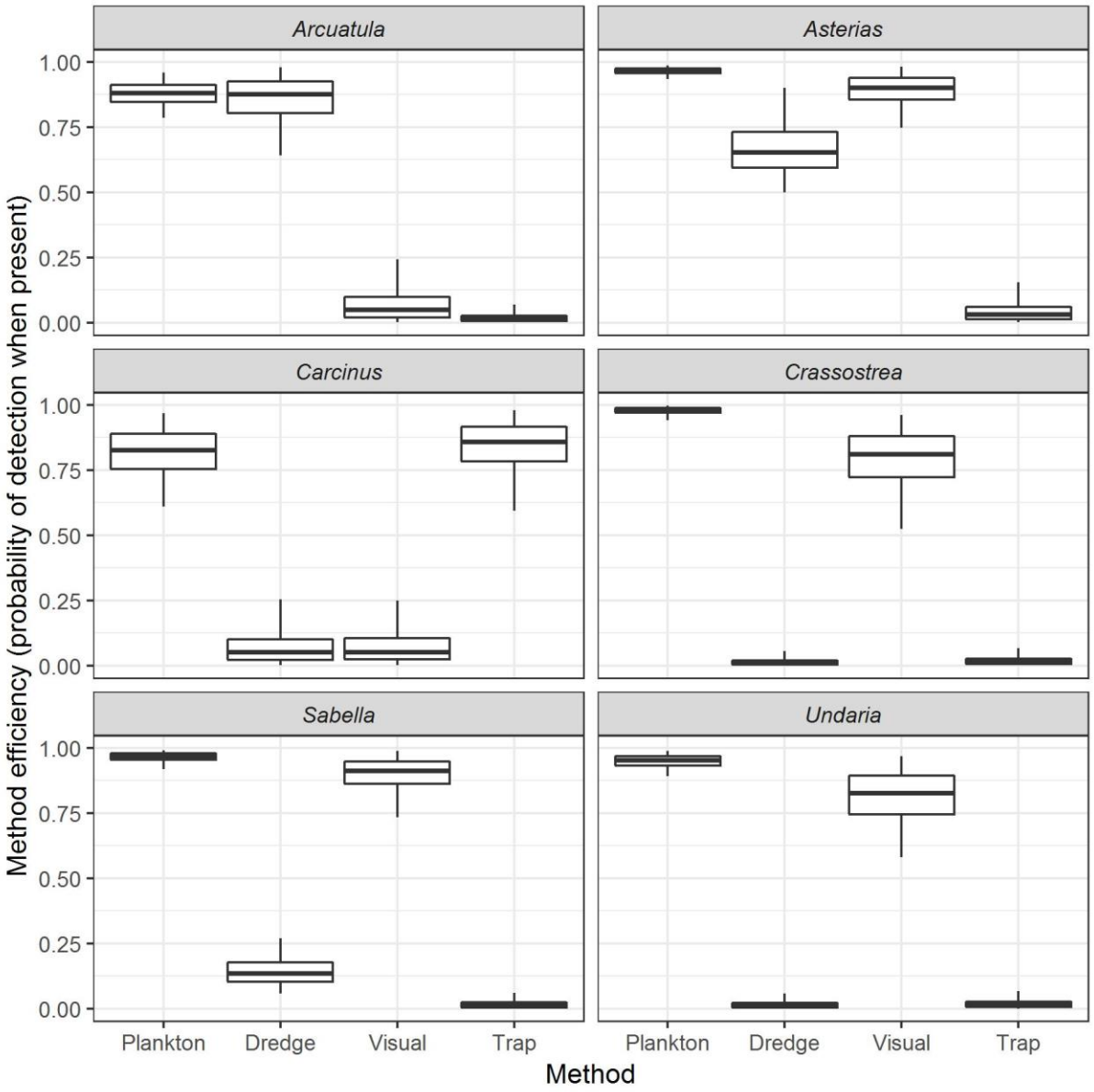

Figure 37. Posterior predictions of mean method efficiency (likelihood of detection, given pest presence in sample unit), for each target species by molecular (plankton) and traditional (dredge, visual, trap) methods. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean.

The total number of samples needed to achieve survey sensitivity (S_{SE}) (= likelihood of a detection in at least one sample) of 0.8 (the default value for the MDeT) for equivalent pest density was predicted for the most appropriate traditional method and for the molecular method across sampling sets for each species (Figure 38). The number of plankton samples required varied between sample sets but was lower than or approximately equal to the number of traditional samples required in at least one sample set. The predicted number of traditional samples required

to detect a low relative pest concentration, i.e. of 0.01, equivalent to the modelled relative concentration of *Undaria* in the Yarra River, is shown in Table 5, along with the required person hours, calculated based on the results from section 3.3.2. These numbers should be interpreted with caution due to being calculated from limited data. Detected pests in most cases appeared to be well-established and therefore are likely to be at present at a greater population size (and hence concentration) than the population size indicative of a new incursion. The prediction for a relatively low concentration therefore involves considerable extrapolation. It is clear, however, that timing of sampling will have a large influence on likelihood of molecular detections, but that when performed at an appropriate time, molecular methods are highly sensitive. For a modelled population density of 0.01, ~12–20 molecular samples are required to provide S_{SE} of 0.8, given sampling at an appropriate time of year, compared with ~90–100 visual samples for *Asterias, Crassostrea, Sabella* or *Undaria.* Modelled dredge numbers for *Arcuatula* and trap numbers for *Carcinus* to detect the equivalent density were 189 and 961 respectively (Table 5). The number of traps should be interpreted with particular caution given there was only a single trap detection during the survey, so this result is based on very limited data. The required numbers predicted should be considered to show the relative performance of the molecular and traditional methods for a low, but unmeasured, population density, not the actual numbers required for detection in a location. The actual abundance, and hence density, of pests in each sublocation is unknown, so it is unclear how the modelled population density used here relates to the population size assumed by the MDeT or whether it is an appropriate concentration to target for this type of surveillance. The number of samples required reduces rapidly with increasing population density for all pests and methods (Figure 38), and the difference in required sampling between methods becomes less, since at high population densities, pests will be present in a high proportion of sampling units, and detection likelihood will depend only on efficiency, which was high (>0.75) for all methods (Figure 37). This indicates that the main factor in better performance of the molecular method is a higher concentration of detectable targets, rather than a higher rate of detection where targets are present, although modelled efficiency of the molecular method was also generally higher than that of traditional methods. The results shown in Table 5 suggest that a combination of sampling in summer and autumn using molecular methods is likely to be most efficient, requiring fewest samples for the detection of the six target pests at a given sensitivity. The fewest samples are needed in summer for detection of *Arcuatula* and *Crassostrea,* in either summer or autumn for *Sabella*, and in autumn for *Asterias, Carcinus* and *Undaria.* Since detections of these pests occurred only in temperate ports (aside from the isolated *Undaria* detection), the seasonal pattern of detection in tropical ports is uncertain. Differences in molecular detections between

sample sets were further investigated by the zero-inflated models, which also examined relative DNA concentration. These results are presented in section 3.5.7. The probabilistic model outputs show the overall or average performance of each traditional method across the two sample sets (winter and summer) where these were applied. Some differences in detection occurred between summer and winter sample sets, but the replication of traditional surveys was inadequate for formal analysis and samples were taken at slightly different sites. Specifically, all *Undaria* detections and the single *Carcinus* detection were in winter, and a greater abundance of *Sabella* was recorded in winter than summer.

The logistic regression of detection frequency for those pests recorded in Melbourne and Hobart showed little difference in the per sample detection rate between methods (Figure 39), with 95% credible intervals of the predicted means overlapping, except in the case of the two species not detected in traditional sampling, i.e. *Carcinus* and *Crassostrea* in Melbourne. There was also generally little difference in the detection rate across sublocations except for *Carcinus* in Hobart, which had a lower rate of detection in the Sullivan Cove (HSC) sublocation, and *Undaria* in Melbourne, which was less frequently detected in the Yarra River (MYR). Based on the number of samples collected and analysed from the project, molecular surveys resulted in an overall (i.e. across all four sampling sets) S_{SE} of ≥ 99% in most cases, the exceptions being *Carcinus* with S_{SE} ranging from 89 to 100% across sublocations, and *Crassostrea* with S_{SE} between 92 and 100% (Figure 40). For traditional methods, S_{SE} varied considerably between species and sublocations, being highest for *Sabella* (95 – 96%) and *Asterias* (78 – 96%). Predicted S_{SE} was 53 – 96% for *Arcuatula*, with the variation between sublocations due to the difference in sample numbers (Table 3), and was < 60% for both *Crassostrea* (4 – 51%) and *Carcinus* (3 – 33%), with lowest values in Melbourne where these species were not detected. Results using the 0.6 and 0.8 numbers from the MDeT were very similar and are shown in Appendix 2. The total number of samples needed to achieve S_{SE} of 0.8 across sampling sets was highest for both methods for *Carcinus* in both ports (Table 6), this being the species with the lowest apparent abundance of the detected species. The sample numbers required to obtain a given S_{SE} will depend on the actual population size and hence likelihood of a sample containing the target, therefore, these sample numbers are those needed to detect the population currently present at each sublocation. Although some abundance data were collected during traditional sampling, these data are insufficient to extrapolate to population size within each area; however, it is clear from the rates of detection that most species detected are well established, as also predicted by the probabilistic model. That model also demonstrated that both methods are effective at detecting target pests where these

occur at the population densities currently found in Melbourne and Hobart. Higher sample numbers will be needed, however, to achieve equivalent S_{SE} at lower population densities, as modelled in the probabilistic model. Note that the sample numbers shown in Table 6 are annual total numbers required based on four sampling sets across seasons for the molecular method, in contrast to the numbers that maybe required when targeting an optimal season for sampling each species. Further seasonal results for the ports with detections are presented in section 3.5.7.

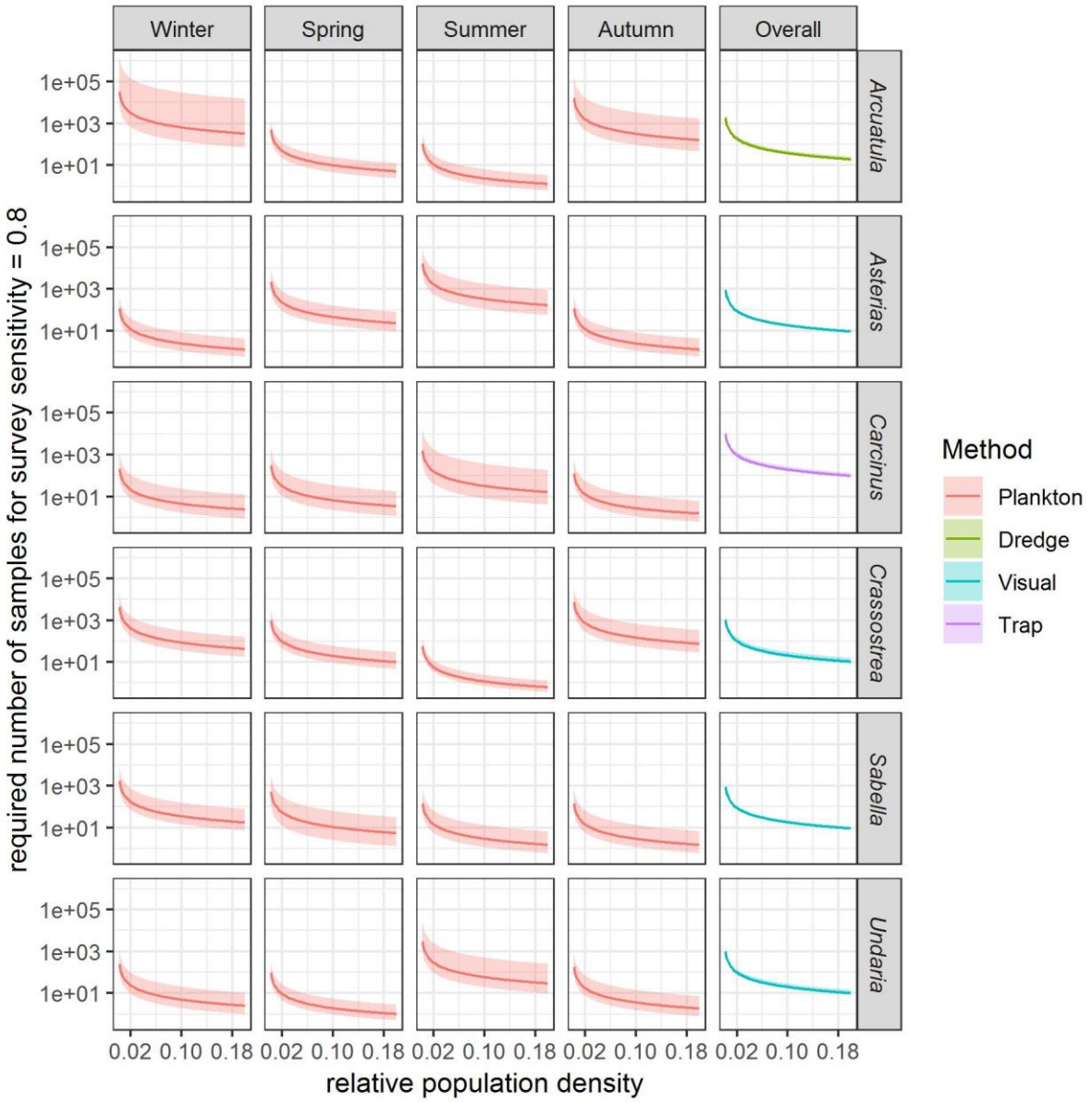

Figure 38. Posterior predictions of required sample numbers to achieve a survey sensitivity of 0.8 for a range of relative pest prevalence values. Line shows mean predicted value with shading indicating 95% credible intervals. Note that y-axis is shown on a log10 scale.

Table 5. Predicted number of samples and associated person hours required to achieve survey sensitivity (S_{SE}) of 0.6 or 0.8 for a low relative population size (modelled concentration = 0.01) of each species by molecular method by sample time and best traditional method for each species. Numbers shown are the predicted mean with 95% credible interval shown in brackets.

				Number of samples		Person-hours	
Species	**Sample type**	**Method**	**Series**	**S_{SE}=0.8**	**S_{SE}=0.6**	**S_{SE}=0.8**	**S_{SE}=0.6**
Arcuatula	Molecular	Plankton	Winter	3241 (707 - 150649)	1845 (403 - 85768)	1221	695
			Spring	50 (23 - 123)	29 (14 - 70)	19	11
			Summer	**12 (6 - 32)**	**7 (4 - 18)**	**5**	**3**
			Autumn	1592 (468 - 16914)	907 (267 - 9630)	600	342
	Traditional	Dredge		**189 (165 - 250)**	**108 (94 - 143)**	**340**	**194**
Asterias	Molecular	Plankton	**Winter**	**13 (6 - 43)**	**7 (4 - 24)**	**5**	**3**
			Spring	230 (93 - 809)	131 (53 - 461)	87	49
			Summer	1678 (564 - 9009)	955 (321 - 5129)	632	360
			Autumn	**13 (6 - 42)**	**7 (4 - 24)**	**5**	**3**
	Traditional	Visual		**91 (83 - 109)**	**52 (47 - 62)**	**514**	**294**
Carcinus	Molecular	Plankton	Winter	22 (9 - 117)	13 (5 - 67)	8	5
			Spring	32 (12 - 171)	19 (7 - 98)	12	7
			Summer	158 (41 - 1827)	90 (23 - 1040)	60	34
			Autumn	**13 (6 - 56)**	**8 (4 - 32)**	**5**	**3**
	Traditional	Trap		**961 (824 - 1341)**	**547 (470 - 764)**	**828**	471
Crassostrea	Molecular	Plankton	Winter	425 (176 - 1595)	242 (100 - 908)	160	91
			Spring	98 (46 - 298)	56 (27 - 170)	37	21
			Summer	**6 (4 - 13)**	**4 (2 - 7)**	**2**	**2**
			Autumn	733 (282 - 3354)	417 (161 - 1909)	276	157
	Traditional	Visual		**102 (84 - 150)**	**58 (48 - 86)**	**576**	**328**
Sabella	Molecular	Plankton	Winter	176 (73 - 777)	100 (42 - 443)	66	38
			Spring	54 (13 - 315)	31 (8 - 180)	20	12
			Summer	**15 (6 - 65)**	**9 (4 - 38)**	**6**	**3**
			Autumn	**15 (6 - 67)**	**9 (4 - 38)**	**6**	**3**
	Traditional	Visual		**90 (82 - 110)**	**51 (47 - 63)**	**509**	**288**
Undaria	Molecular	Plankton	Winter	25 (10 - 104)	14 (6 - 59)	9	5
			Spring	**10 (5 - 28)**	**6 (3 - 16)**	**4**	**2**
			Summer	293 (98 - 2542)	167 (56 - 1447)	110	63
			Autumn	18 (8 - 71)	11 (5 - 41)	7	4
	Traditional	Visual		**99 (84 - 139)**	**57 (48 - 80)**	**559**	**322**

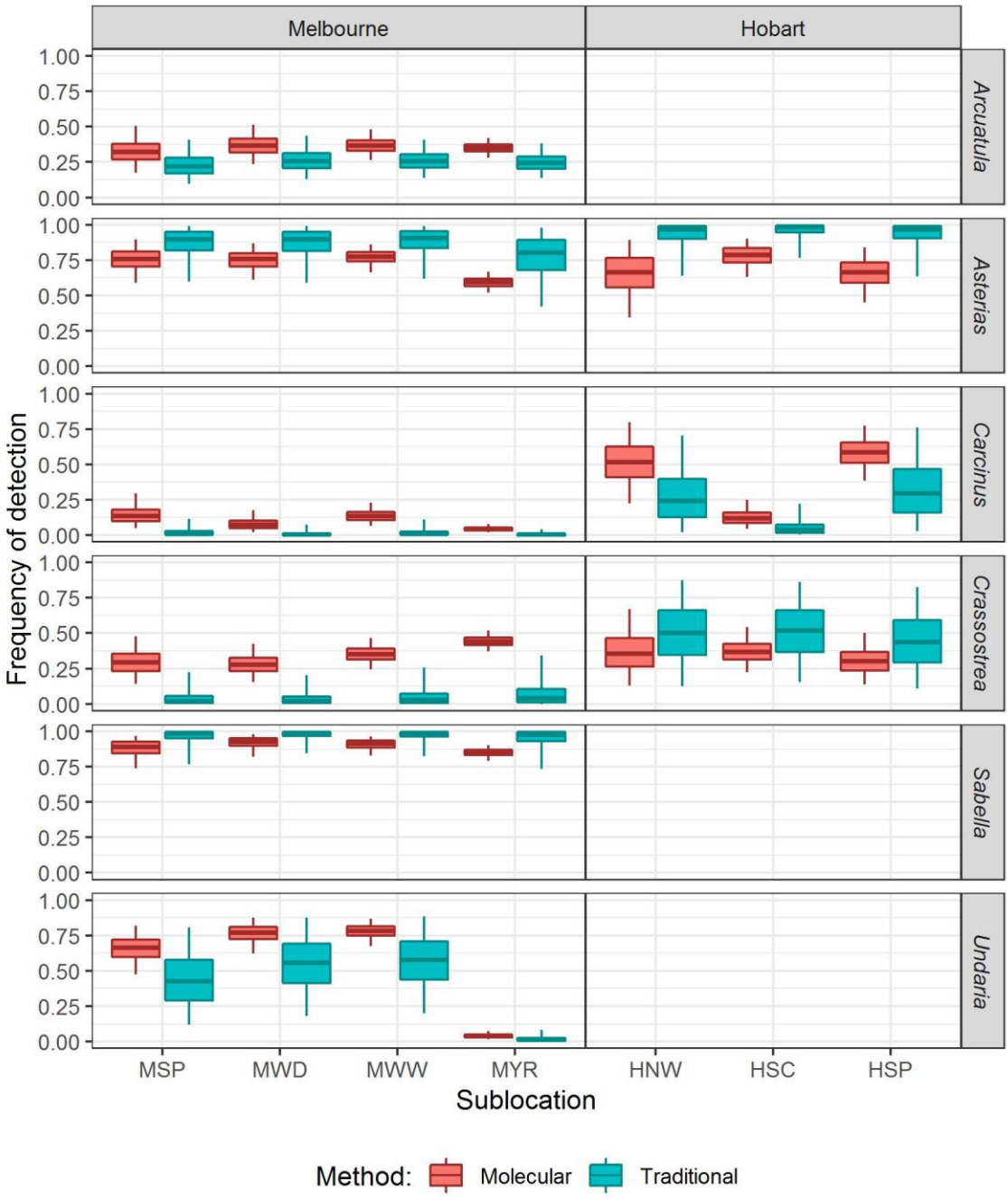

Figure 39. Posterior predictions of mean per sample likelihood of detection for the current population size of each detected target species in Melbourne and Hobart across all sample sets. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean.

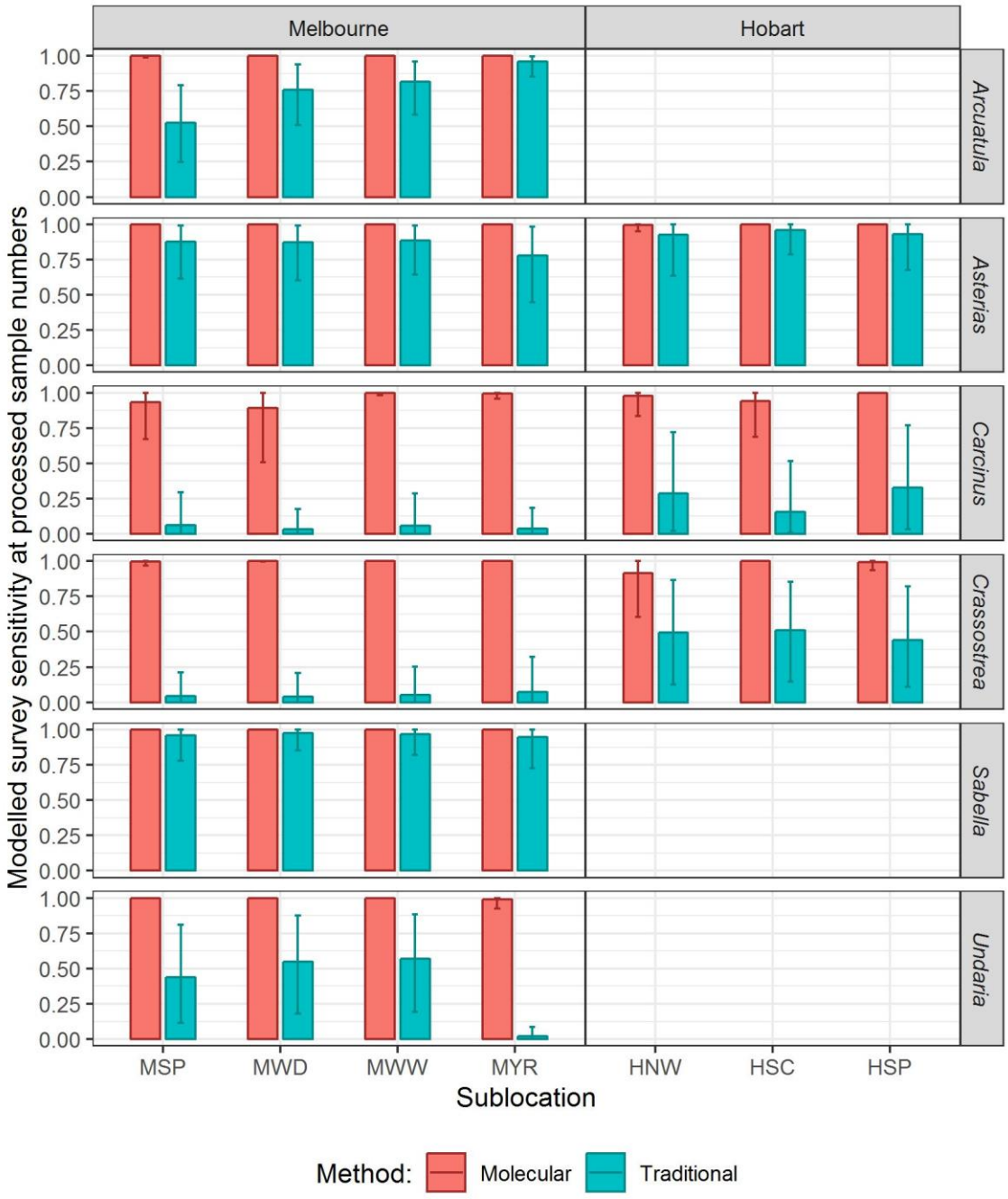

Figure 40. Predicted survey sensitivity for each detected target species in Melbourne and Hobart at the current population size present for each sublocation based on actual sample numbers collected and processed across all sample sets for each method. Bars show mean predicted sensitivity, with error bars showing 95% credible intervals.

Table 6. Predicted number of samples required to achieve survey sensitivity (S_{SE}) of 0.6 or 0.8 for each species at current population size in each port. Numbers shown are the sum of predicted numbers across sublocations with 95% credible interval shown in brackets. Numbers are for total samples in a twelve month period using four sampling sets as applied in the current project: seasonal effects are on molecular survey sensitivity for Melbourne and Hobart are considered in section 3.5. Molecular sample numbers refer to plankton tows. Methods for traditional samples are designated as D: dredge, T: trap, V: visual. The number of samples required by the MDeT for each S_{SE} is shown for comparison.

Port	**S_{SE} = 0.8**		**S_{SE} = 0.6**	
Species	**Molecular**	**Traditional**	**Molecular**	**Traditional**
Hobart				
MDeT required	94	D:15, T:8, V:3	54	D:9, T:5, V:3
Asterias	6 (3 – 9)	V: 3 (3 – 6)	3 (3 – 6)	V: 3 (3 – 3)
Carcinus	17 (10 – 48)	T: 40 (11 – 731)	11 (6 – 27)	T: 23 (6 – 416)
Crassostrea	13 (8 – 30)	V: 9 (3 – 38)	7 (5 – 18)	V: 6 (3 – 21)
Melbourne				
MDeT required	496	D:49, T:26, V:6	282	D:28, T:15, V:5
Arcuatula	17 (12 – 26)	D:25 (15 – 50)	D:11 (8 – 16)	D:15 (8 – 31)
Asterias	8 (5 – 9)	V: 5 (4 – 9)	V: 5 (4 – 6)	V: 4 (4 – 7)
Carcinus	78 (41 – 226)	T: 519 (90 – 317 797)	46 (24 – 129)	T: 297 (52 – 180 930)
Crassostrea	17 (12 – 31)	V: 125 (25 – 74 970)	11 (8 – 18)	V: 73 (16 – 42 683)
Sabella	4 (4 – 6)	V: 4 (4 – 6)	4 (4 – 4)	V: 4 (4 – 4)
Undaria	46 (24 – 112)	V: 83 (22 – 778)	26 (15 – 64)	V: 49 (14 – 444)

3.3.2. Sampling time, costs and required expertise

The average field time required per sample was calculated to be 11 minutes for plankton tows, 36 minutes for dredge tows, 52 minutes for traps (including deployment and collection) and 113 minutes for visual survey transects. The relative person hours required for the traditional component of sampling was more than double that for molecular sampling for the sample numbers collected and analysed. (Table 7).

Personnel costs for undertaking surveys will increase with the required person hours, but the different methods also require different levels of staff qualification and expertise. Traditional sampling methods require staff to have sufficient expertise to identify potential target organisms in the field (National System for the Prevention and Management of Marine Pest Incursions 2010b), and visual sampling will further require SCUBA or surface-supplied scientific or commercial diving qualifications. Collection of plankton tows, however, does not require any

specific expertise, although should still be conducted with scientific rigour to ensure surveys are comparable and that all accompanying data is accurately obtained; this is also a requirement for traditional sampling. The costs of field sampling are also likely to be considerably lower for molecular than traditional sampling due to the difference in cost for the required equipment, i.e.: plankton net for molecular, compared with dredge, SCUBA diving gear and traps for traditional surveys. Costs for vessel hire and use will also increase with required sampling time and also typically with vessel size and capability. Deployment of the plankton net is possible by hand from a small vessel, while dredge tows typically require use of a vessel with at least a davit or small crane and sufficient deck space for processing collected samples (Przeslawski *et al.* 2018). We estimate the field sampling cost of the traditional survey to be 3.4 times greater than the molecular survey in Gladstone, 2.9 times greater in Brisbane, 2.2 times greater in Melbourne and 4.5 times greater in Hobart. These cost differences are purely for operating costs, including vessel hire and relevant equipment for each method (plankton net, dredge, traps, SCUBA equipment), the costs do not include an estimate for wages, with staff costs likely to be greater for traditional sampling due to required expertise in addition to scaling with required time (Table 6). The difference in relative cost of the two methods between ports reflects differences in the relative sample numbers for the specific sample methods, which were due to port characteristics considered in the MDeT (sublocation sizes, depths and flushing rates).

Table 7. Estimated total person hours for field sampling based on collection of the same sample numbers applied in the current survey. These estimates do not include mobilisation/demobilisation times, which will be consistent between methods.

Port	Molecular (Plankton)	Traditional (Total)	Visual	Trap	Dredge
Gladstone	100.2	225.2	79.1	31.0	115.1
Brisbane	57.0	156.8	67.8	20.7	68.3
Melbourne	111.0	162.8	45.2	24.1	93.5
Hobart	23.1	71.3	33.9	8.6	28.8
TOTAL	291.3	616.1	226.0	84.4	305.7

The project was not designed to compare all aspects of the two methods, and we did not obtain estimates of laboratory time for traditional processing and identification with which to compare to molecular analysis processing. There are, however, different levels of expertise required for these components. Traditional sample sorting requires sufficient expertise to identify potential target organisms, while identification requires specific taxonomic expertise, which may be scarce for

some taxonomic groups or target species. In the current project, for example, a native oyster species *Crassostrea dactylena* was detected in Gladstone; this required examination by the Museum and Art Gallery of the Northern Territory to ascertain that it was not the target species *Crassotrea gigas* (Benthic Australia data). Taxonomic expertise is needed at the stage of assay development and testing for the molecular method, but following assay validation, molecular analysis requires only general laboratory skills and proficiency in use of the qPCR platform, although interpretation of results should always be performed with relevant biological knowledge. Formally comparing the total time required for processing and analysis for each method is difficult; for traditional methods, this is likely to be highly variable, depending on the species present and availability of relevant taxonomic expertise, and increase in proportion to the number of samples. Processing and analysis of molecular samples, however, could potentially be performed rapidly, within ~1 week post collection, with required time not dependent on the species present and increasing only marginally with increased sample numbers, since batches of samples can be processed and analysed simultaneously.

3.4. Patterns in plankton tow sampling volume and PCR inhibition

Plankton tows targeted a nominal length of 100 m, except for the Yarra River sublocation in Melbourne, where the field team used a length of 140 m for the summer 2018 sample set only. Field conditions and logistics of net deployment and retrieval can often result in an actual tow length different to that targeted, but the effective volume filtered is affected to a greater extent by net clogging, which varies within and between sampling times and locations (Deveney *et al.* 2017). For the current project, data on actual tow length was of variable accuracy and was missing for some sets of samples, so net efficiency (= effective tow distance/actual distance) could not be analysed. Flow meter readings, which give effective tow distance, were, however, recorded for the majority of samples, and varied seasonally within each port (Figure 41) being generally lowest in spring.

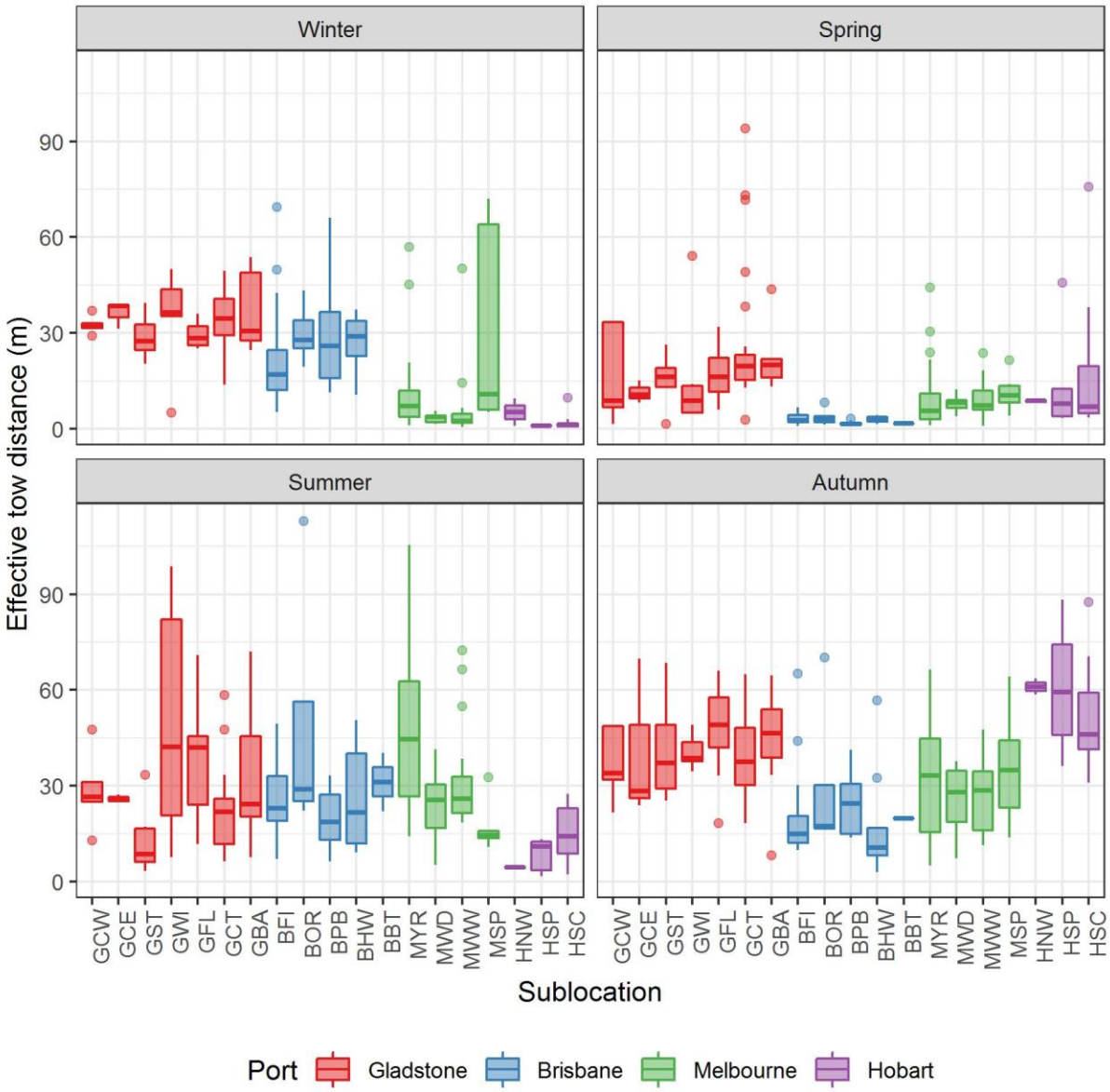

Figure 41. Effective tow distance from flow meter readings by season and sublocation.

Deveney *et al.* (2017) observed generally more net clogging and lower effective tow lengths in tropical than temperate ports, but with little difference in resulting sample mass overall. In the current project, low effective tow lengths were observed in most sublocations within the temperate ports of Melbourne and Hobart in winter, but, in general, variation in effective tow length was greater within than between locations. Sample mass was typically between 1 and 2 g filtered dry weight, and varied little between seasons or locations, with the exception of Gladstone where relatively high sample masses were obtained in all seasons except autumn (Figure 42).

PCR inhibition, as measured by a scale factor calculated using the inhibition control that was added to all samples, occurred in some samples from each port and season. Nearly all winter samples from Gladstone had moderate (> 5) to very high (some >1000) scale factors, especially at the Coal Terminal sublocation, and winter samples from the Sullivan Cove sublocation in Hobart also displayed high scale factors (Figure 43). In other seasons, only a few samples returned scale factors > 5, although some from Gladstone in spring had scale factors > 100, and there was almost no inhibition in autumn samples, except for Sullivan Cove where most samples had low to moderate scale factors and one sample had scale factor > 100.

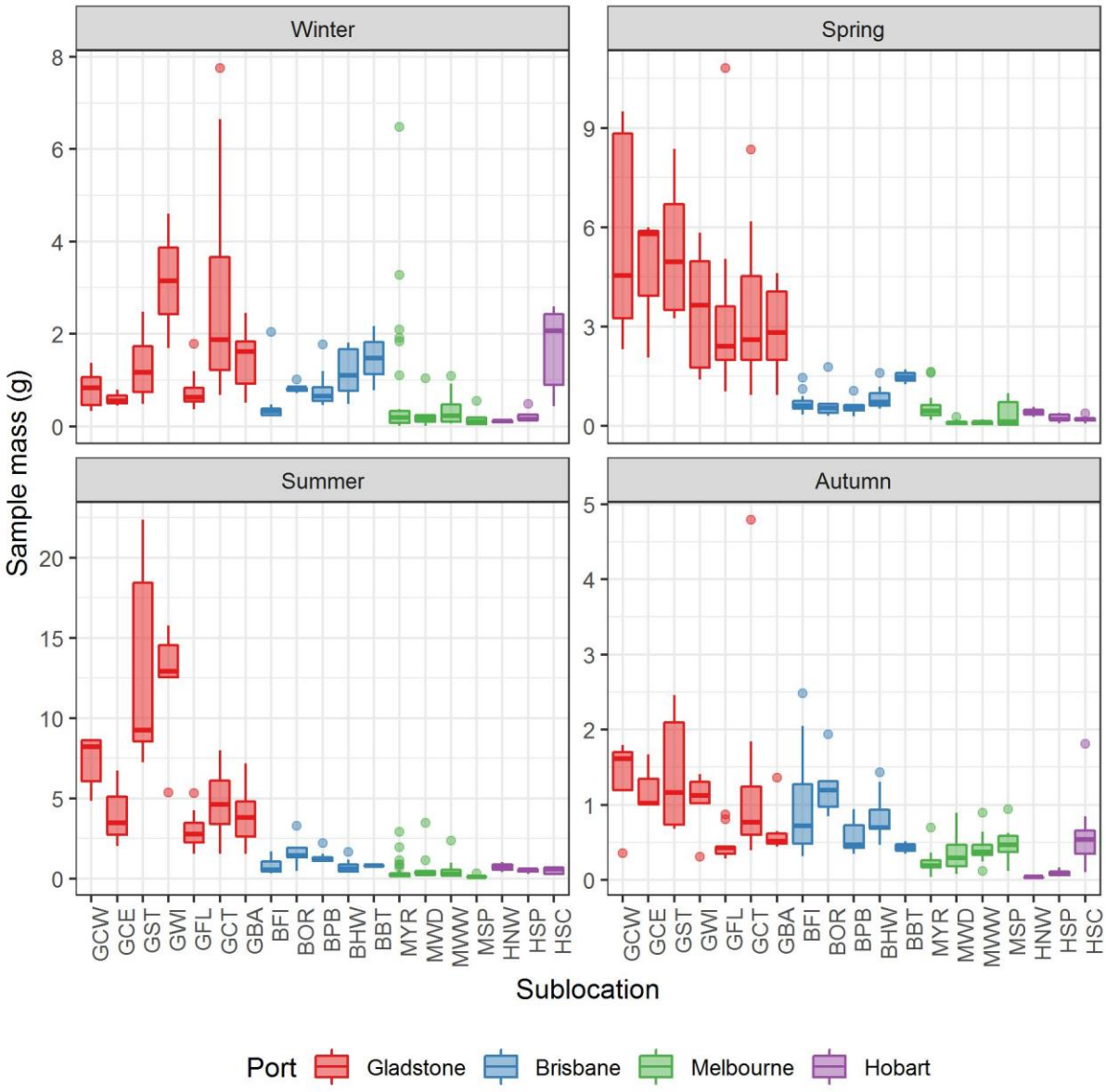

Figure 42. Sample dry weight after filtering and freeze-drying by season and sublocation.

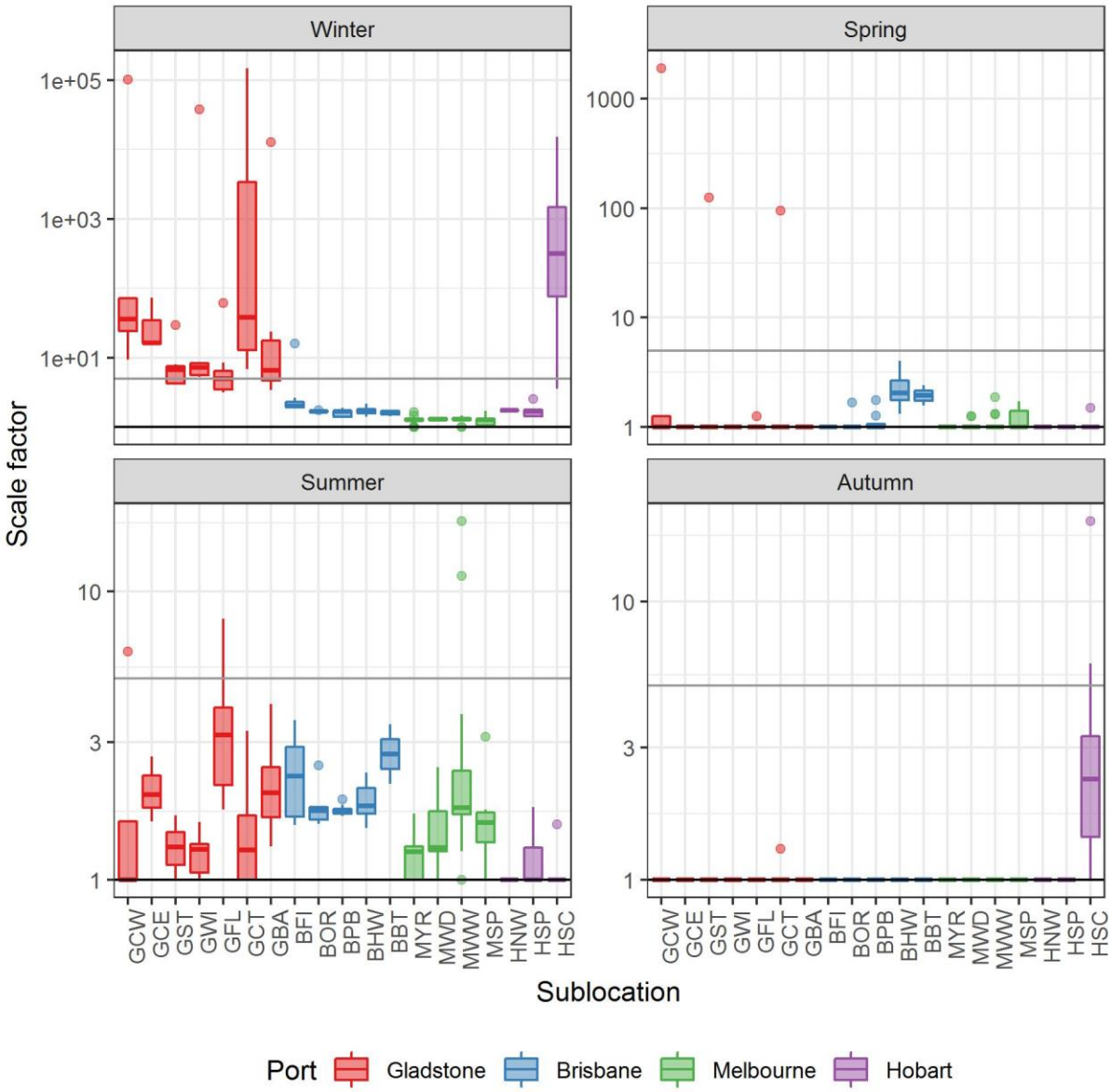

Figure 43. PCR inhibition measured as scale factor by season and sublocation. Note that y-axis is shown on log-scale.

3.5. Seasonal patterns in DNA and molecular detections

For each detected species both the relative DNA yield per sample and relative field DNA varied between sample sets, often by several orders of magnitude (Table 8) and for most species the rate of detection was also different between sample sets (detailed in sections 3.5.1 – 3.5.6). The log-normal variance for each ZALN model was between 0.68 and 1.86, indicating that the prior used for variance in the models (with a maximum possible value = 10) did not restrict the variance

estimates. The effect of the modelled log-normal variance on the predicted 95 percentile range of relative DNA values is illustrated in Table 8.

Table 8. Predicted mean and 95 percentile range of relative DNA by seasonal sampling set for each species and port with a detection. Predicted mean and 95 percentile range were calculated from the log-normal mean and variance predictions of zero altered log-normal models.

Species	Port	Season	Relative sample DNA	Relative field DNA
Arcuatula	Melbourne	Spring	2.8 (0.8 – 20)	0.5 (0.1 – 3.4)
		Summer	8.7 (1.4 – 35)	0.3 (0.0 – 1.2)
		Autumn	0.2 (0.9 – 22)	3.9E-3 (0.0 – 0.5)
Asterias	Hobart	Winter	5.5E+4 (2100 – 2.7E+5)	4.4E+4 (1400 – 2.2E+5)
		Spring	2.6E+5 (1.0E+4 – 1.3E+6)	2.8E+4 (920 – 1.4E+5)
		Summer	67 (8.3 – 1100)	7.3 (0.8 – 120)
		Autumn	6000 (250 – 3.3E+4)	110 (4.0 – 630)
	Melbourne	Winter	1.9E+6 (7.4E+4 – 9.9E+6)	3.5E+5 (1.2E+4 – 1.9E+6)
		Spring	630 (46 – 6100)	85 (5.4 – 840)
		Summer	19 (4.6 – 610)	0.5 (0.1 – 17)
		Autumn	1.4E+6 (5.7E+4 – 7.6E+6)	5.9E+4 (2000 – 3.2E+5)
Carcinus	Hobart	Winter	510 (34 – 5500)	600 (28 – 5800)
		Spring	6800 (350 – 5.6E+4)	650 (25 – 5300)
		Summer	110 (7.5 – 1200)	17 (0.6 – 130)
		Autumn	1.1E+4 (680 – 1.1E+5)	200 (9.3 – 2000)
	Melbourne	Winter	2300 (570 – 9.0E+4)	420 (77 – 1.6E+4)
		Spring	1100 (450 – 7.2E+4)	260 (58 – 1.2E+4)
		Summer	310 (180 – 2.9E+4)	7.1 (2.3 – 480)
		Autumn	2100 (350 – 5.6E+4)	91 (12 – 2500)
Crassostrea	Hobart	Winter	180 (81 – 3500)	110 (30 – 1600)
		Spring	140 (52 – 2200)	22 (6.4 – 340)
		Summer	1.3E+5 (1.2E+4 – 5.2E+5)	1.6E+4 (1300 – 6.8E+4)
		Autumn	1200 (1.2E+3 – 5.2E+4)	24 (17 – 920)
	Melbourne	Winter	43 (45 – 1900)	3.6 (3.1 – 160)
		Spring	1100 (280 – 1.2E+4)	180 (39 – 2100)
		Summer	1.1E+5 (1.0E+4 – 4.3E+5)	3300 (270 – 1.4E+4)
		Autumn	47 (77 – 3300)	2.9 (3.8 – 210)
Sabella	Melbourne	Winter	140 (10 – 1000)	25 (1.5 – 190)
		Spring	480 (28 – 2700)	82 (3.7 – 480)
		Summer	6000 (310 – 3.0E+4)	190 (7.8 – 1000)
		Autumn	2.0E+4 (1000 – 1.0E+5)	880 (36 – 4600)
Undaria	Melbourne	Winter	1900 (230 – 2.5E+4)	490 (53 – 6500)
		Spring	1900 (180 – 1.9E+4)	280 (24 – 2900)
		Summer	92 (28 – 2900)	2.9 (0.8 – 93)
		Autumn	3800 (430 – 4.5E+4)	150 (15 – 1800)

3.5.1. Arcuatula

Only data from Melbourne were analysed for *Arcuatula* seasonal detection patterns, given the uncertainty over whether Gladstone detections truly are the target species *A. senhousia.* The ZALN models for *Arcuatula* showed that likelihood of detection was greatest in summer followed by spring (Figure 44), with relative DNA per sample following the same pattern (Figure 45). Correcting for the difference in sample volume (flowmeter distance) per season, however, showed that field DNA concentration was high in both spring and summer (Figure 45). No estimate for DNA concentration in winter was possible because there were no detections, but this season had clearly the lowest (undetectable and hence effectively zero) *Arcuatula* DNA. Sampling in winter (July) 2015 in Melbourne by Deveney *et al.* (2017) also did not detect *Arcuatula*. The map of interpolated *Arcuatula* relative DNA (Figure 46), corrected for scaling and sample volume, shows a peak in spring with highest concentrations found in the Maribyrnong and lower Yarra River. The few molecular detections in autumn 2018 were all from the Yarra River sublocation. Dredge samples recorded this species in this area, but highest abundances in dredge samples occurred in the Webb dock sublocation.

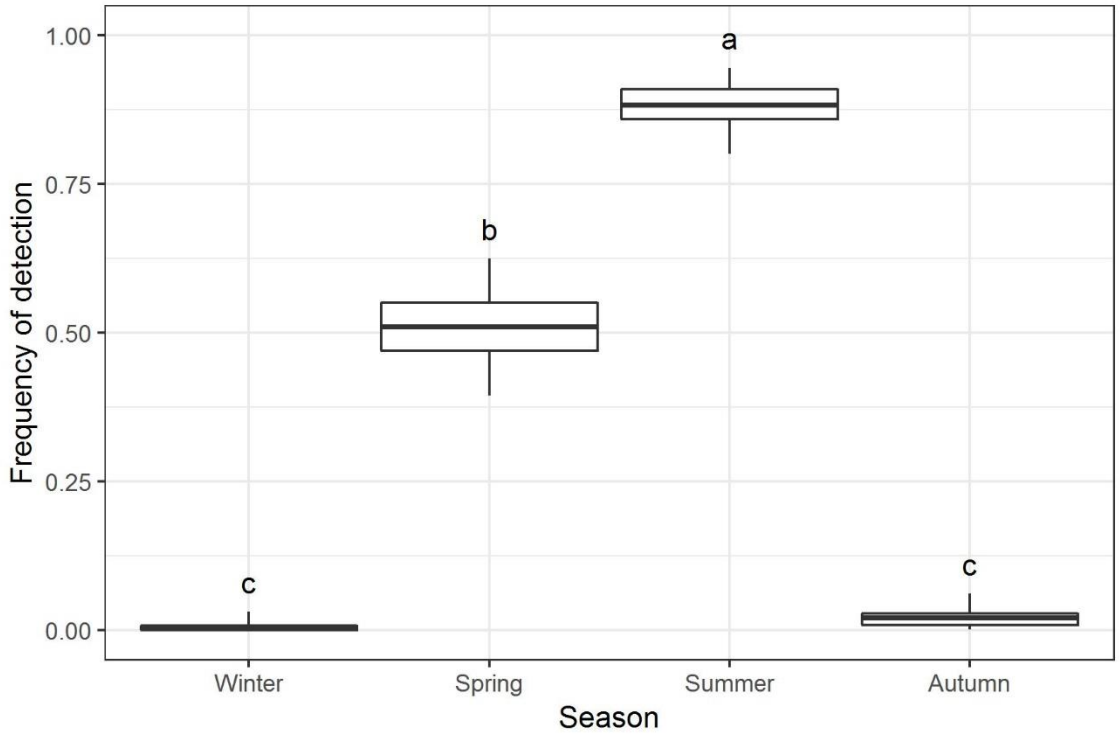

Figure 44. Posterior predictions of mean detection frequency for *Arcuatula* in Melbourne molecular samples by season. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

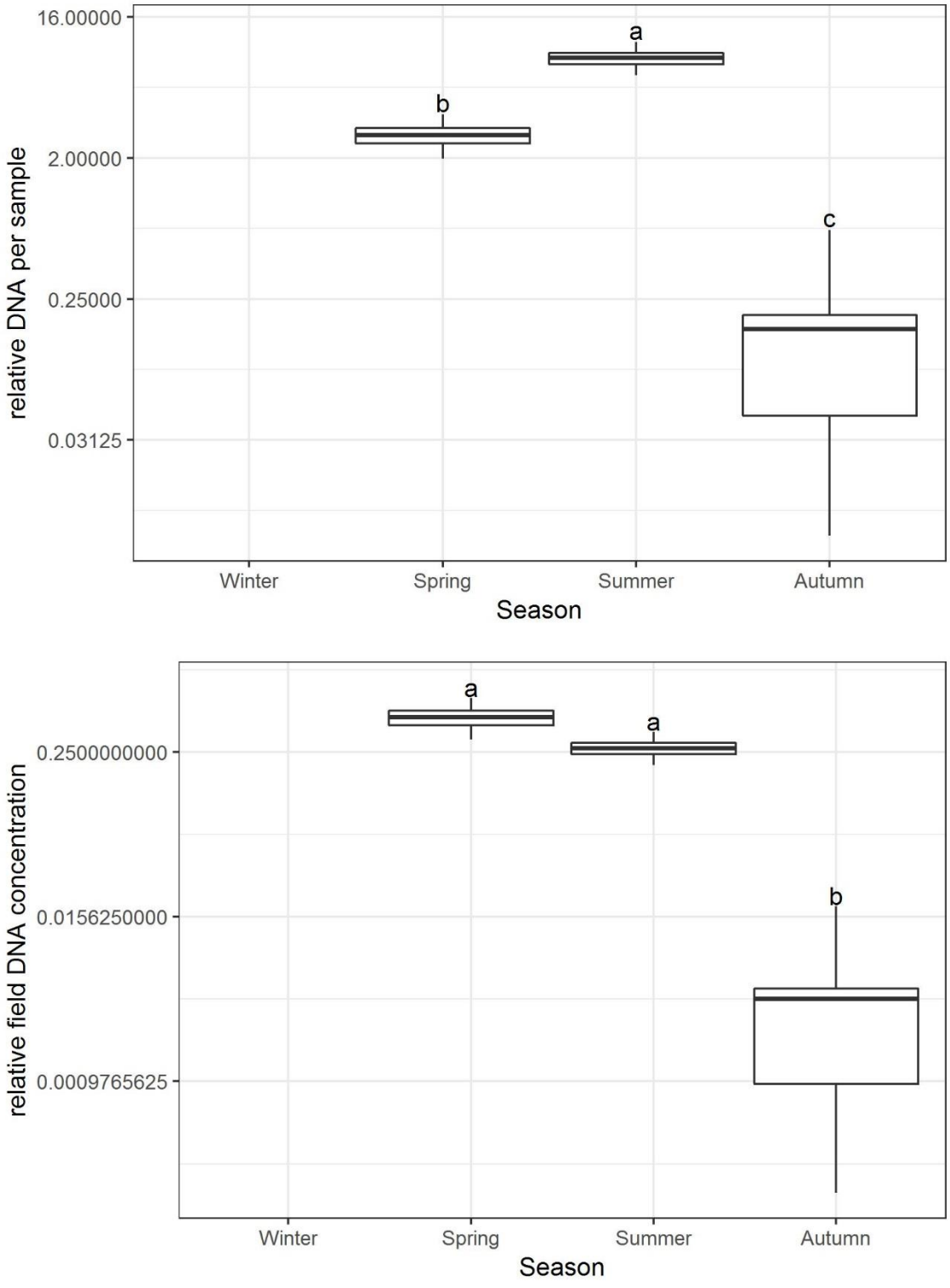

Figure 45. Posterior prediction of mean *Arcuatula* relative DNA in Melbourne molecular samples by season. Top panel shows relative DNA in samples and bottom panel shows relative field DNA accounting for sampling volume. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Note that y-axis is shown on a log scale. Different letters indicate that 95% credible intervals do not overlap.

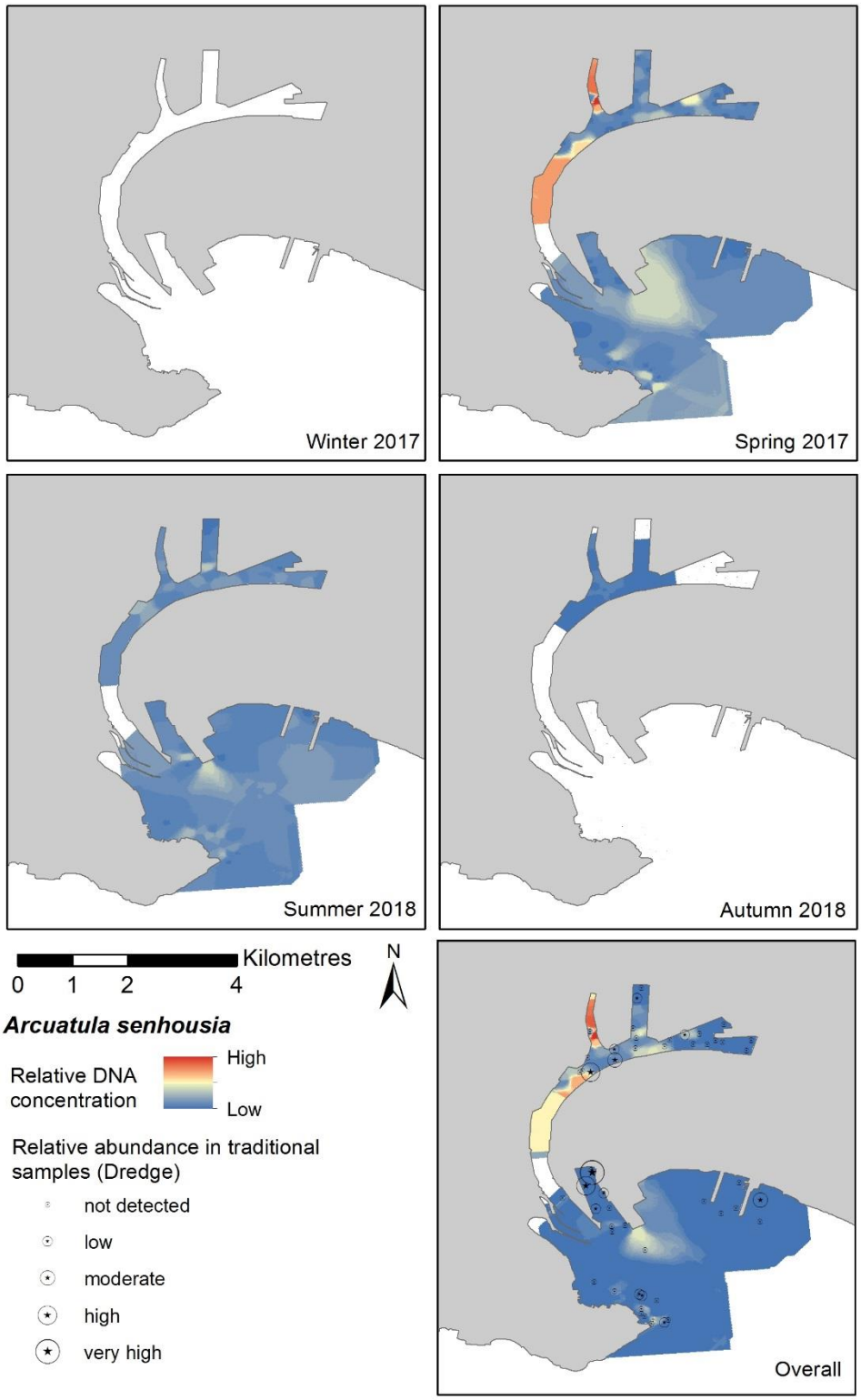

Figure 46. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Arcuatula* in Melbourne by season and overall, plus overall relative abundance in dredge samples.

In the northern hemisphere, *Arcuatula* typically spawns in summer, with larvae developing in the plankton through autumn-winter (Galil 2006; Global Invasive Species Database 2019b), although an invasive population in California was found to have year-round spawning with peaks in autumn and spring (Williams *et al.*, 2005). The reproductive pattern of *Arcuatula* in Australian populations has not been assessed (Aquenal 2008). The pattern of detections and DNA concentrations found in Melbourne suggests that spawning occurs in spring, with larvae present through summer, but with detection likelihood and DNA concentration becoming low by late autumn. Molecular detections may occur due to DNA shed from adults, not only reproductive material, but the lack of *Arcuatula* detections in autumn-winter suggests that DNA released by adults is typically insufficient to result in a detection, and so detections in spring-summer are likely to be of gametes or larvae of this species.

3.5.2. Asterias

The ZALN model for *Asterias* showed that detection frequency was highest for autumn and winter in Melbourne, declining in spring and summer (Figure 47). In Hobart, detection frequency was high from autumn through winter and spring but low in summer (Figure 47). The relative DNA in samples was highest in autumn-winter in Melbourne and spring in Hobart (Figure 48), but after accounting for differing sample volumes between seasons, field concentrations were predicted to be highest in winter for Melbourne, though still high in autumn, and highest in winter-spring for Hobart (Figure 48). This suggests a similar seasonal pattern between ports but with a slight lag, i.e. an earlier peak in DNA, and also in detection rates, in Melbourne than Hobart. Overall *Asterias* DNA concentrations were higher in Melbourne than Hobart. Maps of interpolated *Asterias* DNA show highest concentrations in winter for both Melbourne (Figure 49) and Hobart (Figure 50). In Melbourne, highest relative DNA was found in the Swanston dock area of the Yarra River, while highest abundances detected by traditional sampling occurred near Station Pier. In Hobart, DNA concentration was greatest around Sullivan Cove, and traditional sampling recorded high abundances in this location and at Selfs Point.

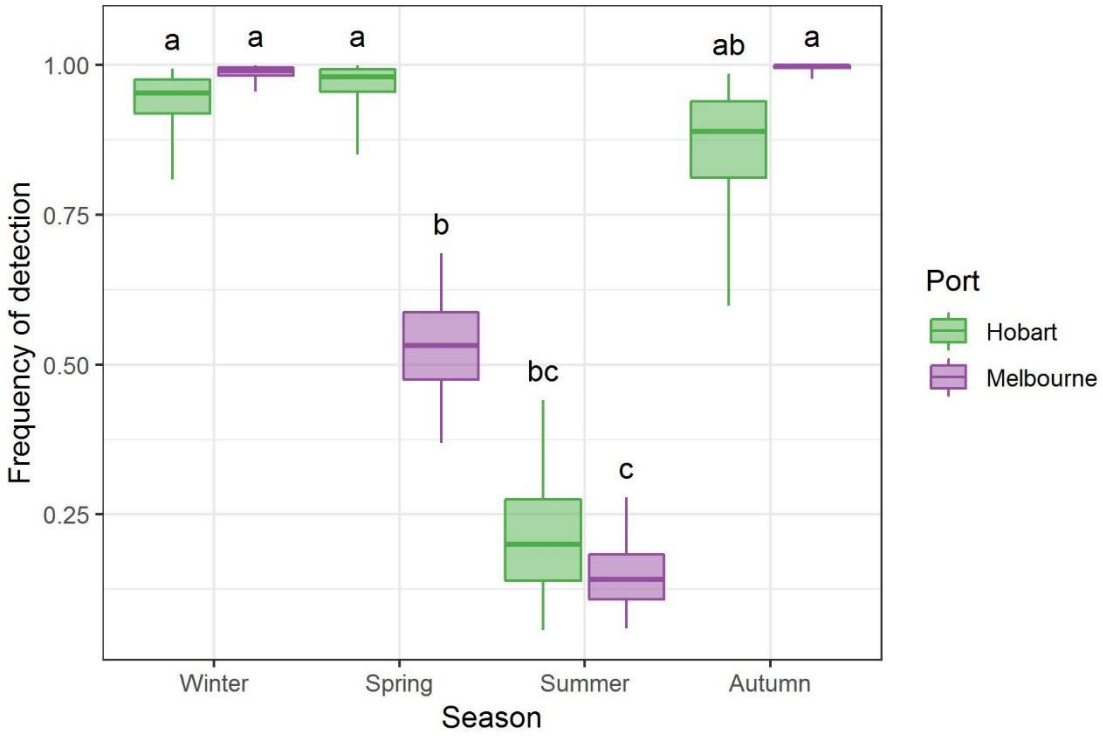

Figure 47. Posterior predictions of mean detection frequency for *Asterias* in Melbourne and Hobart molecular samples by season. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

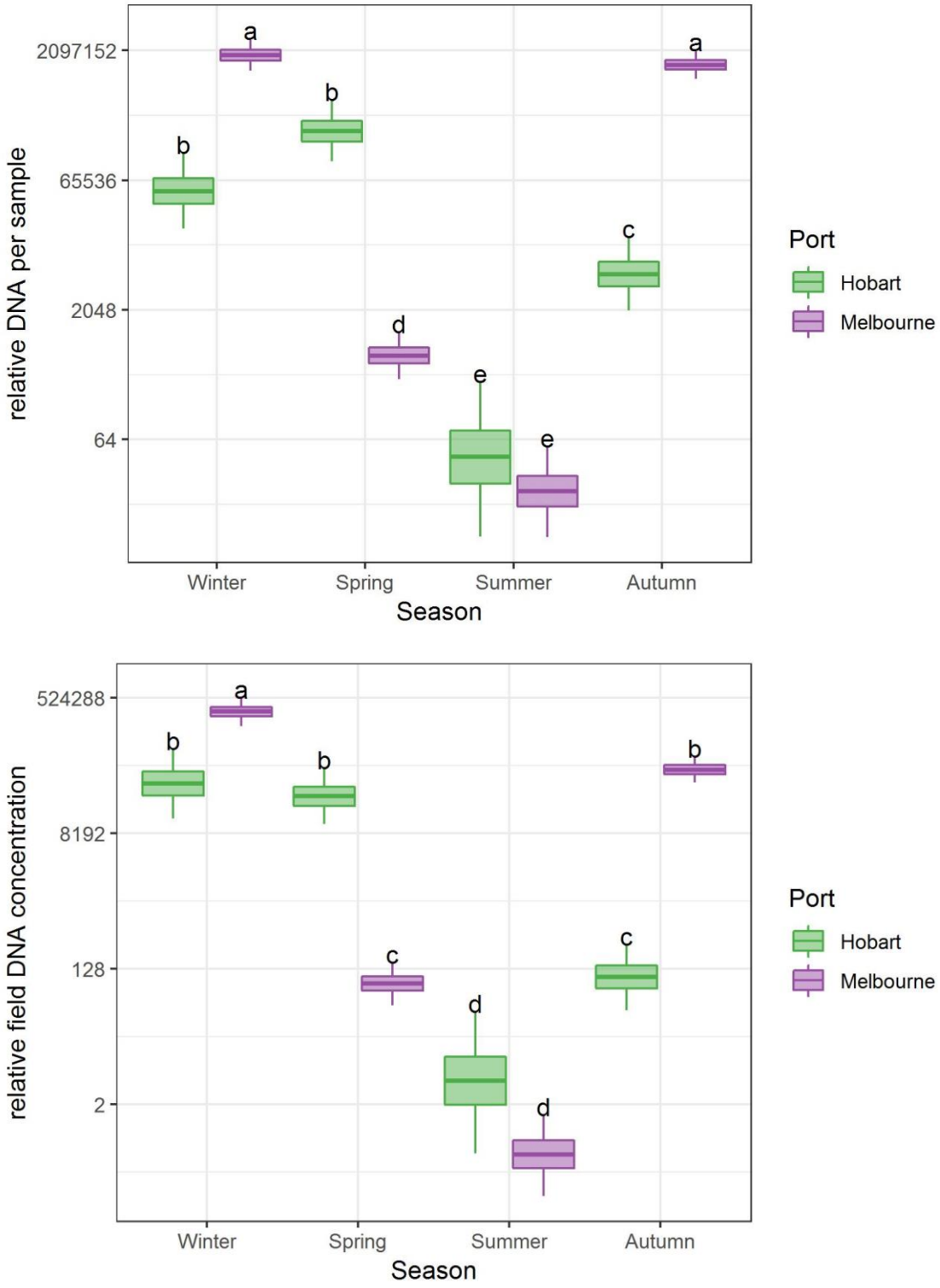

Figure 48. Posterior prediction of mean *Asterias* relative DNA in Melbourne and Hobart molecular samples by season. Top panel shows relative DNA in samples and bottom panel shows relative field DNA accounting for sampling volume. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Note that y-axis is shown on a log scale. Different letters indicate that 95% credible intervals do not overlap.

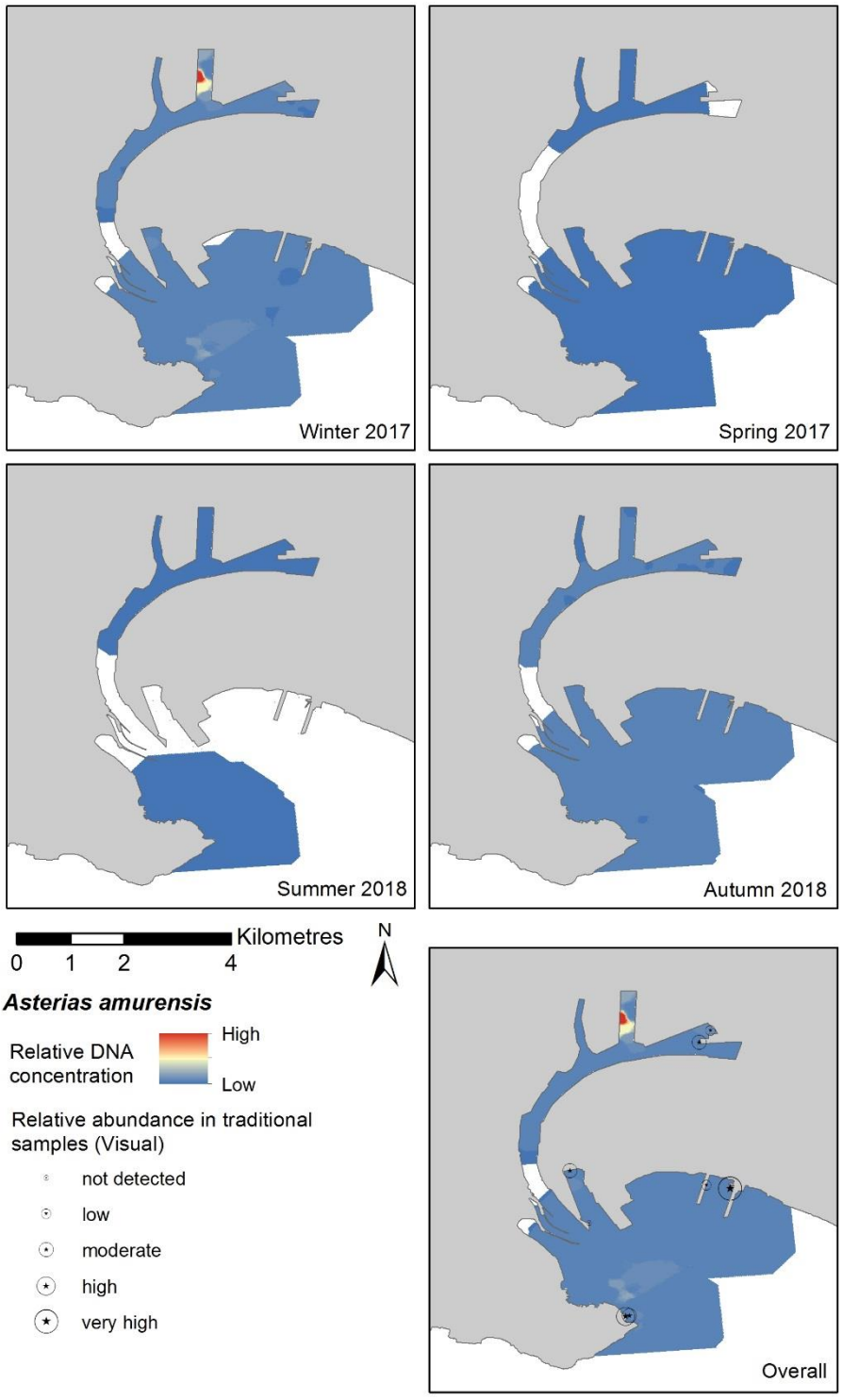

Figure 49. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Asterias* in Melbourne by season and overall, plus overall relative abundance in dredge samples.

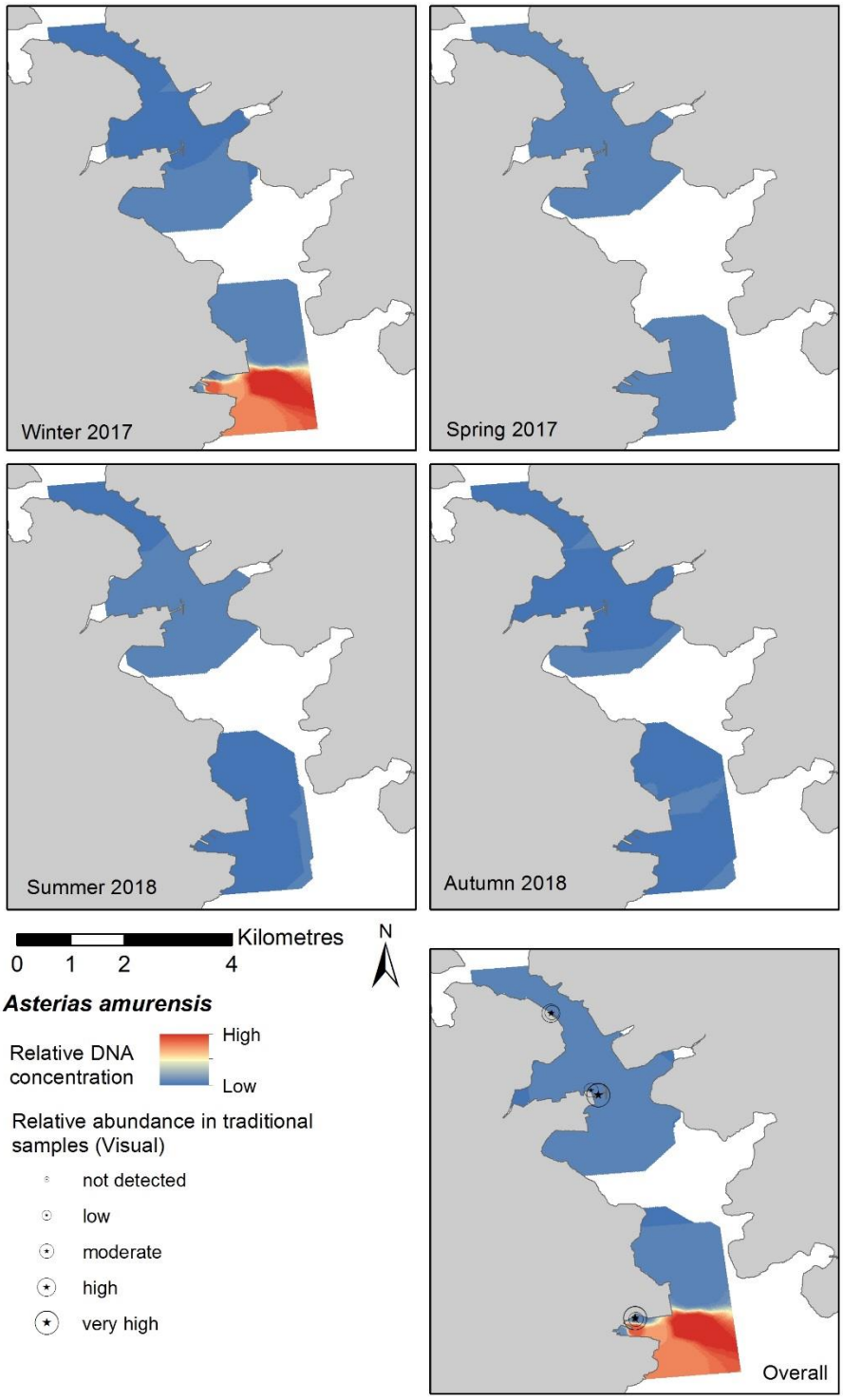

Figure 50. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Asterias* in Hobart by season and overall, plus overall relative abundance in dredge samples.

In many parts of its range *Asterias* spawns predominantly in winter-spring, but *Asterias* larvae have been recorded in Melbourne and Hobart from mid-late autumn through to early summer, with a longer spawning and larval period in Hobart than Melbourne (Dommisse and Hough 2004). The observed patterns of detections and DNA concentration from the current project coincide with this period, especially since autumn sampling for the current project took place at the end of autumn, and our data also indicate longer spawning period or larval duration in Hobart. Dommisse and Hough (2004), however, noted a variation in the presence of larvae between years, with earlier occurrence linked to cooler water temperatures, so there may be inter-annual variation in this pattern. Winter is likely to be the best overall season for detection of *Asterias,* but with high likelihood of detection also from late autumn and in spring, especially in cooler years or locations.

3.5.3. Carcinus

The ZALN for *Carcinus* showed that detection frequency fluctuated but was similar across seasons within each port (Figure 51). Detection frequency was generally lower in Melbourne than Hobart, especially in spring. DNA concentration in samples also fluctuated, but without a clear seasonal pattern (Figure 52). Field concentration, correcting for sample volume, was also similar across seasons, but appeared to be consistently higher through autumn, winter and spring, declining in summer (Figure 52). Reduced detection rates and sample DNA in winter may therefore have been due to the low effective sampling volume for both Melbourne and Hobart in this season. The maps show DNA concentrations of *Carcinus* were highest in winter in Melbourne, especially in the Williamstown wharves sublocation (Figure 53), and in spring in Hobart, with greatest concentration in the Selfs point sublocation (Figure 54). Only a single trap sample captured *Carcinus*, with four individuals caught, this was at the Nyrstar Wharf sublocation.

In cooler waters, *Carcinus* typically spawns in summer, but in warmer temperate areas multiple spawning events may occur each year (National System for the Prevention and Management of Marine Pest Incursions 2015). In South Australia, ovigerous female *Carcinus* were only observed in winter and spring trap samples, suggesting this may be the predominant reproductive season, but few females were caught in other seasons so a rigorous comparison could not be made (Dittmann *et al.* 2017). *Carcinus* detections may also be of DNA shed by post-settlement crabs, possibly released during moulting. Our data do not demonstrate a defined best season for detection of *Carcinus,* but across the two ports where this species occurred, autumn appears to be marginally better than other seasons.

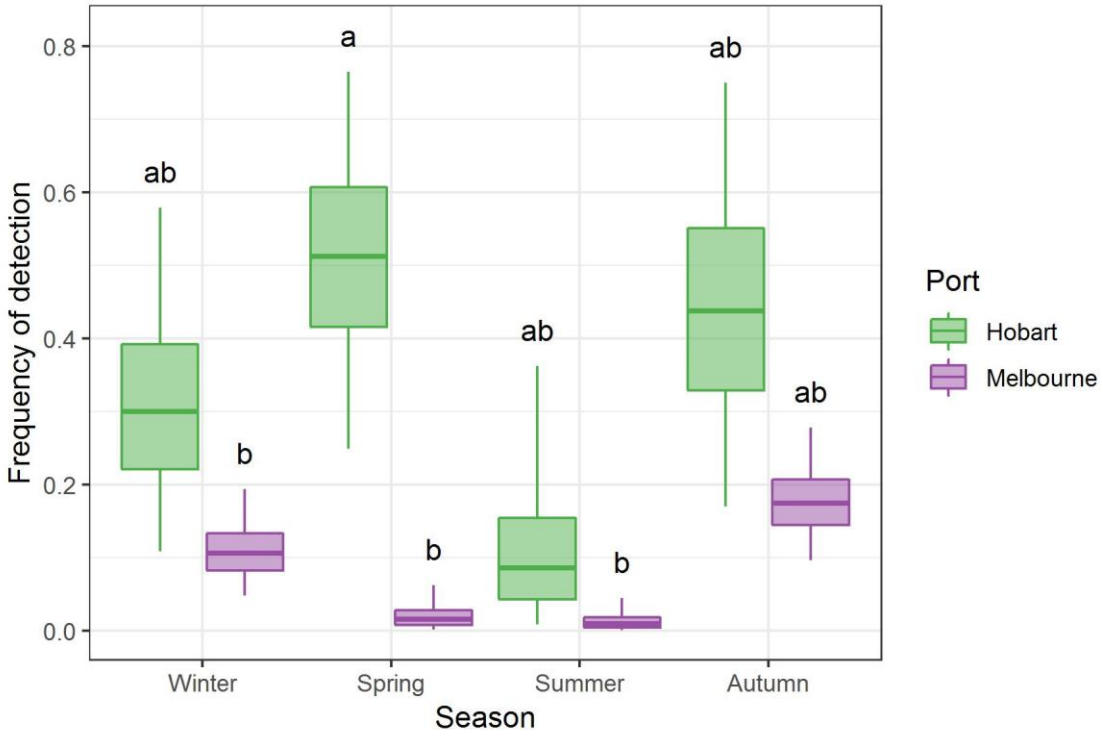

Figure 51. Posterior predictions of mean detection frequency for *Carcinus* in Melbourne and Hobart molecular samples by season. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

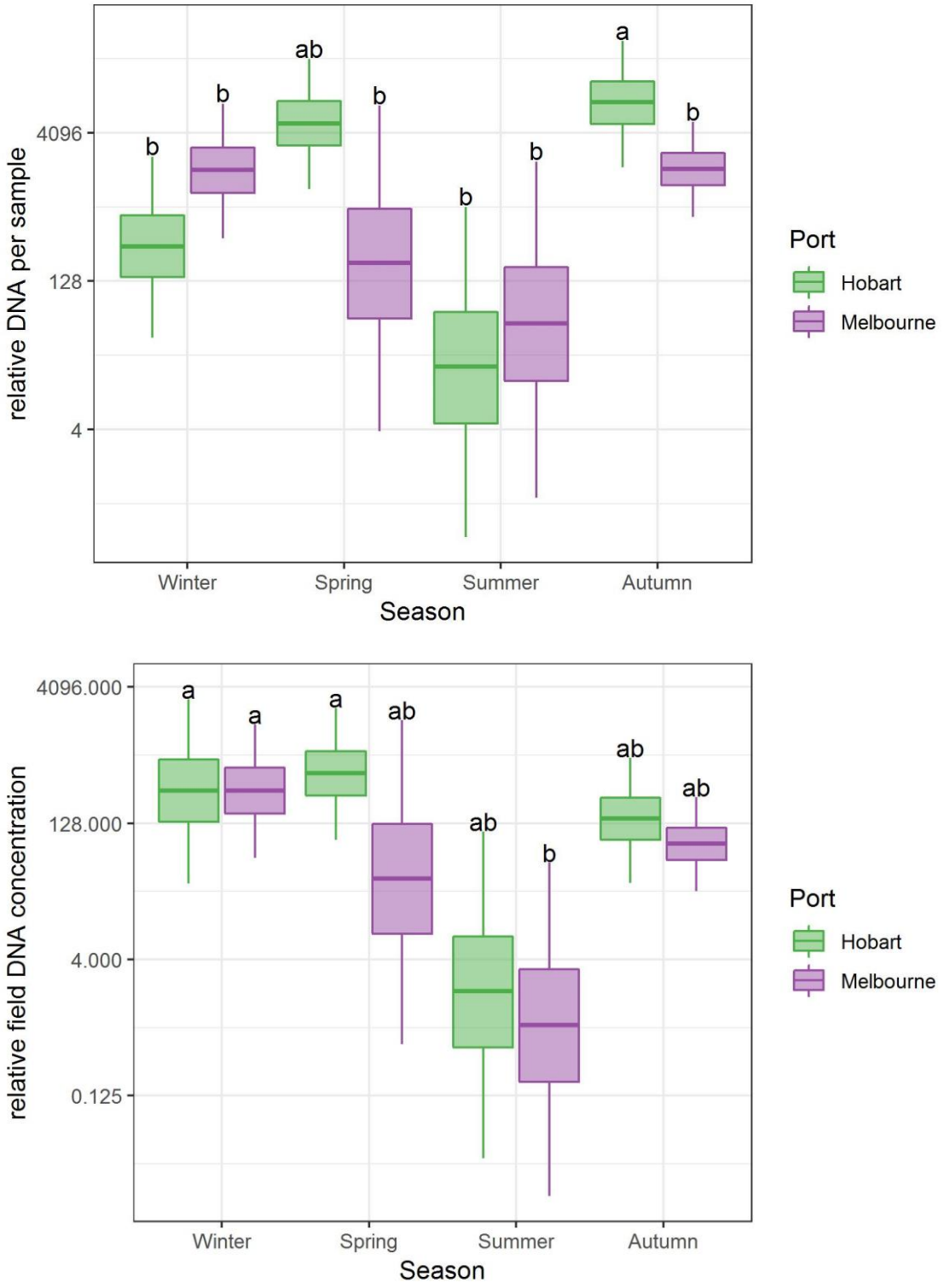

Figure 52. Posterior prediction of mean *Carcinus* relative DNA in Melbourne and Hobart molecular samples by season. Top panel shows relative DNA in samples and bottom panel shows relative field DNA accounting for sampling volume. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Note that y-axis is shown on a log scale. Different letters indicate that 95% credible intervals do not overlap.

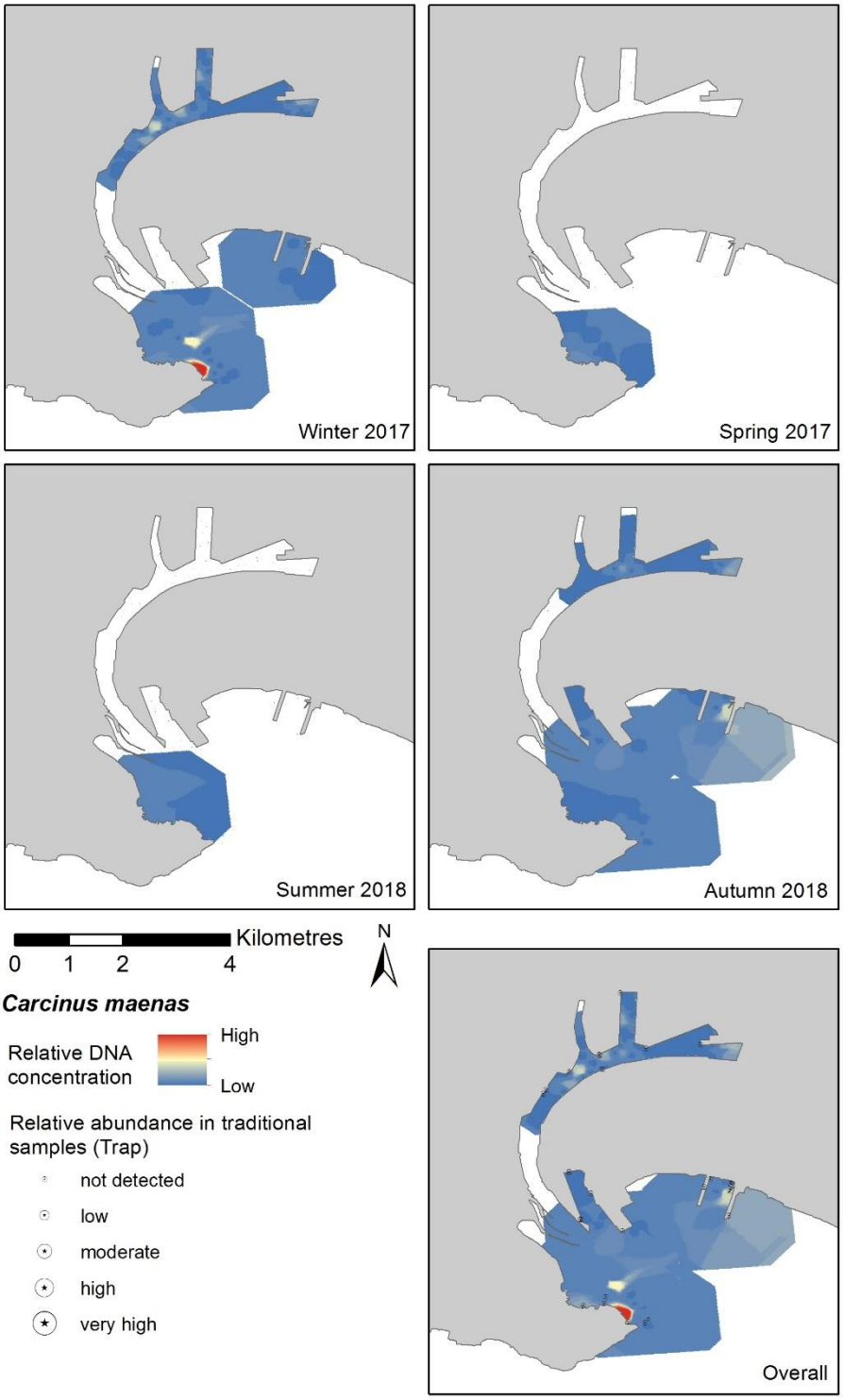

Figure 53. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Carcinus* in Melbourne by season and overall, plus overall relative abundance in dredge samples.

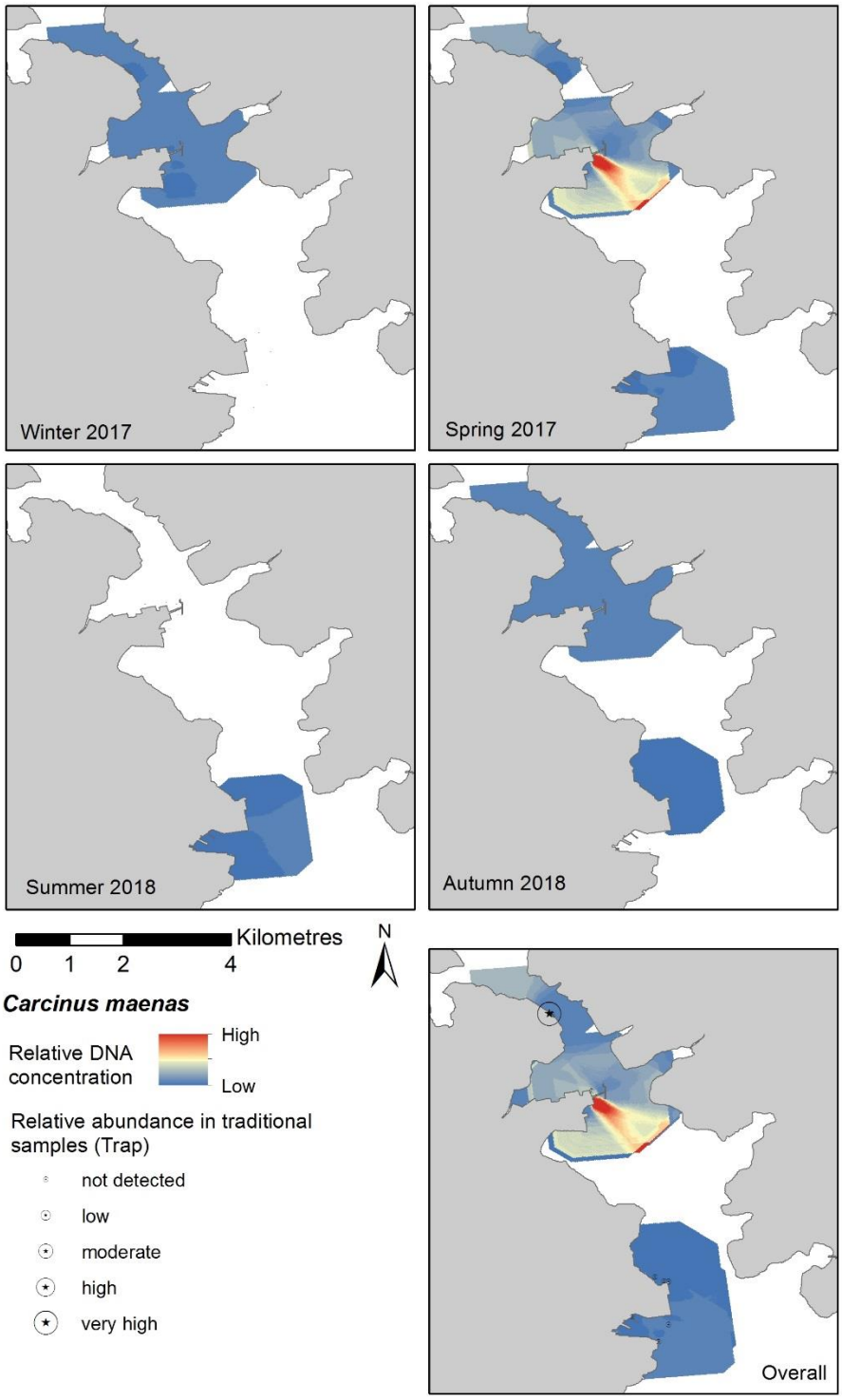

Figure 54. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Carcinus* in Hobart by season and overall, plus overall relative abundance in dredge samples.

3.5.4. Crassostrea

The ZALN showed that both Melbourne and Hobart had very similar seasonal patterns of detection likelihood and DNA of *Crassostrea* (Figure 55). The detection frequency of *Crassostrea* was much higher in summer than other seasons, with winter and autumn having few detections. DNA concentration in samples and the field followed a similar seasonal pattern (Figure 56). The maps also show that highest *Crassostrea* DNA concentrations occurred in summer in both Melbourne and Hobart. In Melbourne, greatest concentrations occurred in the Station Pier sublocation, but there were also samples with high concentration from the Yarra River near the Swanston Dock and Maribyrnong, and around the Williamstown wharves (Figure 57). In Hobart, highest concentrations were found around Selfs Point and Sullivan Cove (Figure 58). The traditional survey collected most specimens of this species in Selfs Point, but no abundance counts were recorded during visual surveys so it is unclear whether this site had higher abundance than other Hobart sublocations. *Crassostrea* was recorded at the other sublocations in Hobart, but not observed in Melbourne. The visual survey locations in Melbourne, however, did not occur in the same areas as high DNA, except around Station Pier.

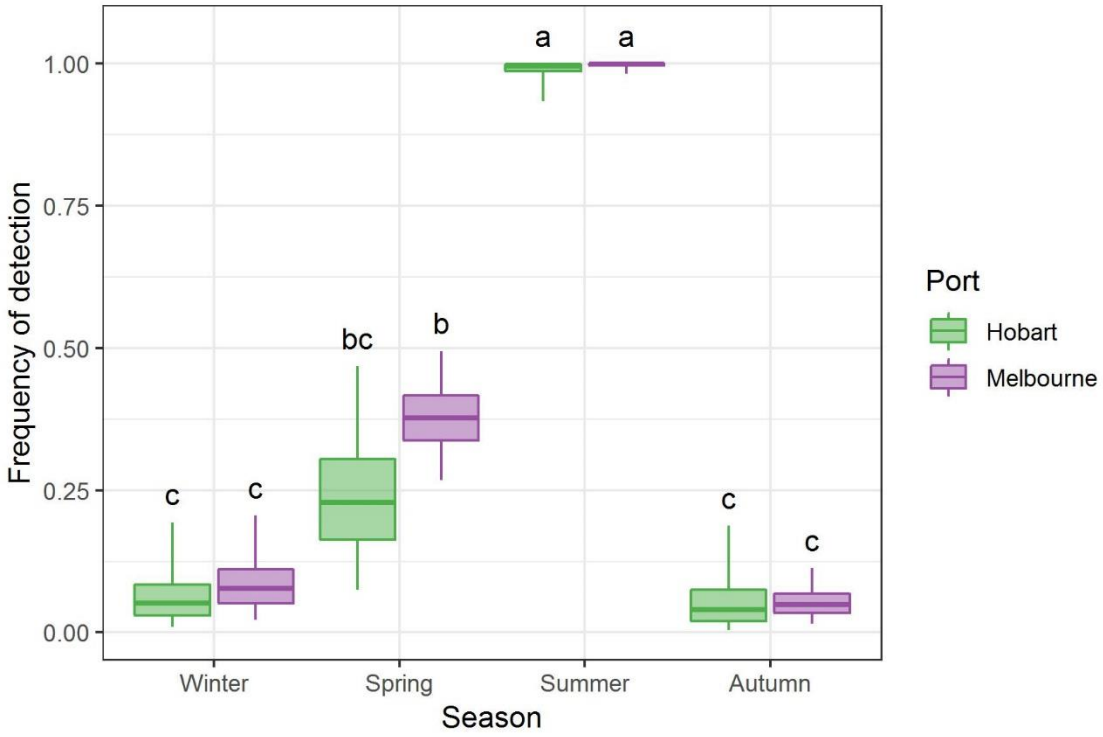

Figure 55. Posterior predictions of mean detection frequency for *Crassostrea* in Melbourne and Hobart molecular samples by season. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

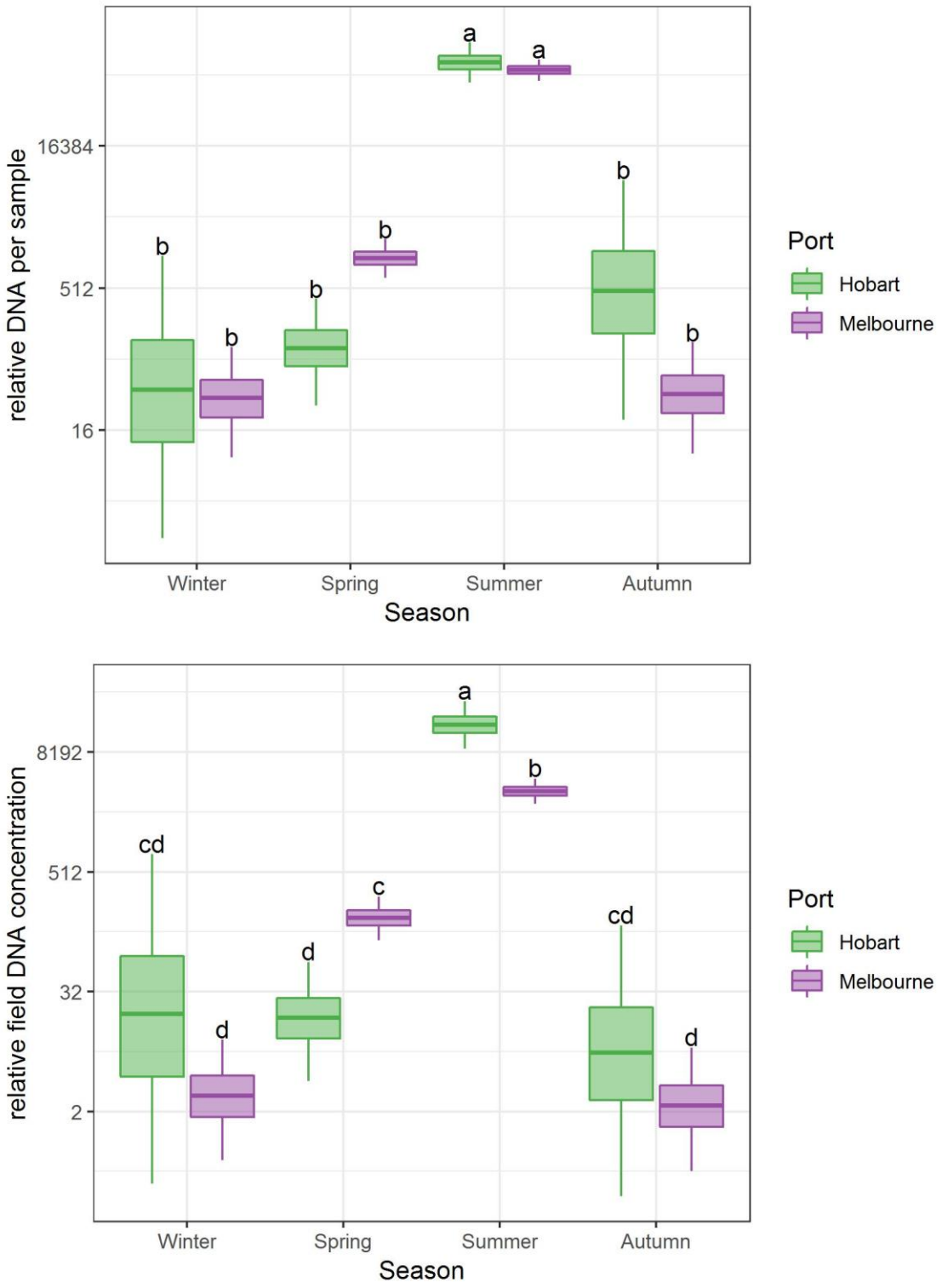

Figure 56. Posterior prediction of mean *Crassostrea* relative DNA in Melbourne and Hobart molecular samples by season. Top panel shows relative DNA in samples and bottom panel shows relative field DNA accounting for sampling volume. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Note that y-axis is shown on a log scale. Different letters indicate that 95% credible intervals do not overlap.

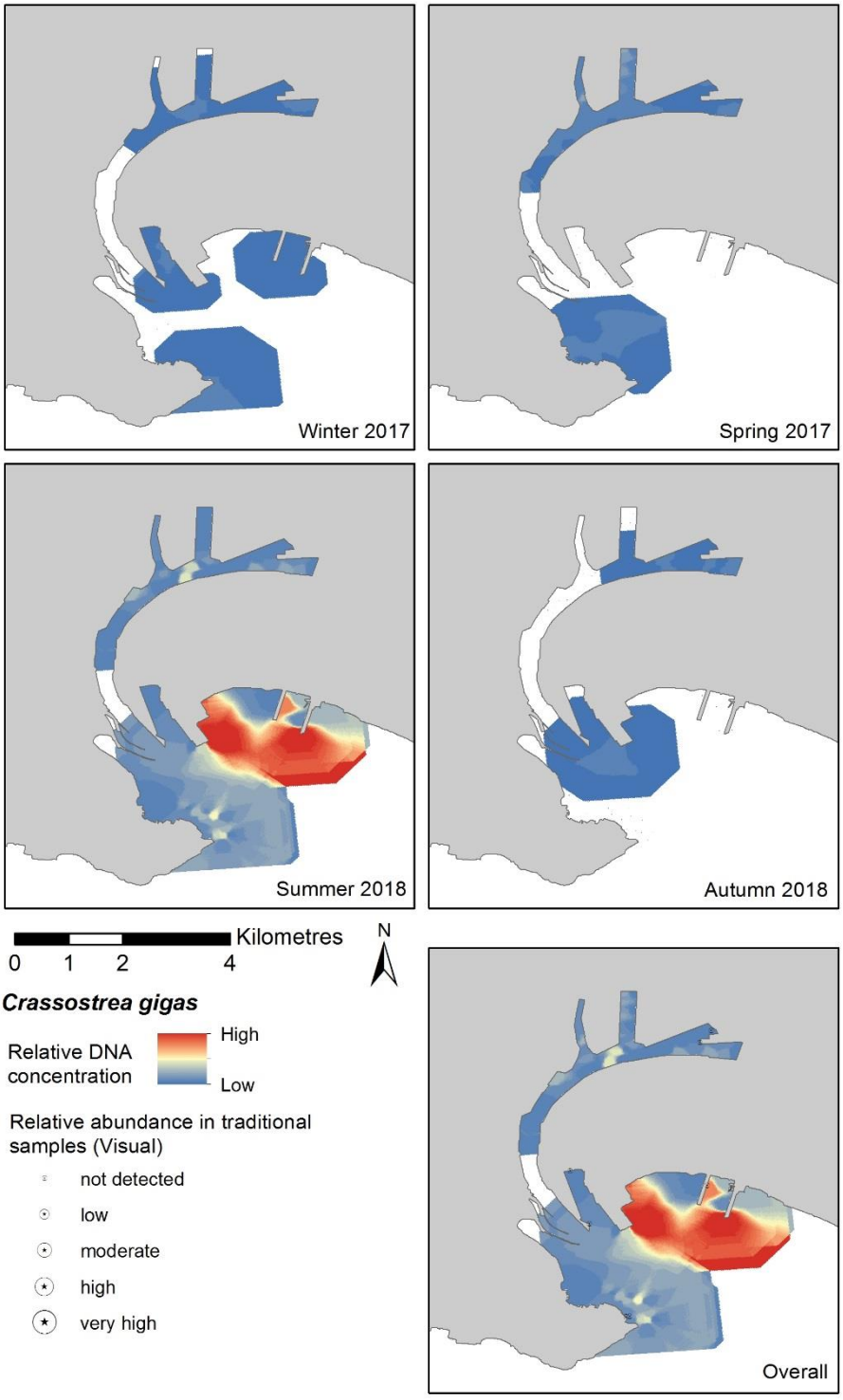

Figure 57. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Crassostrea* in Melbourne by season and overall, plus overall relative abundance in visual samples.

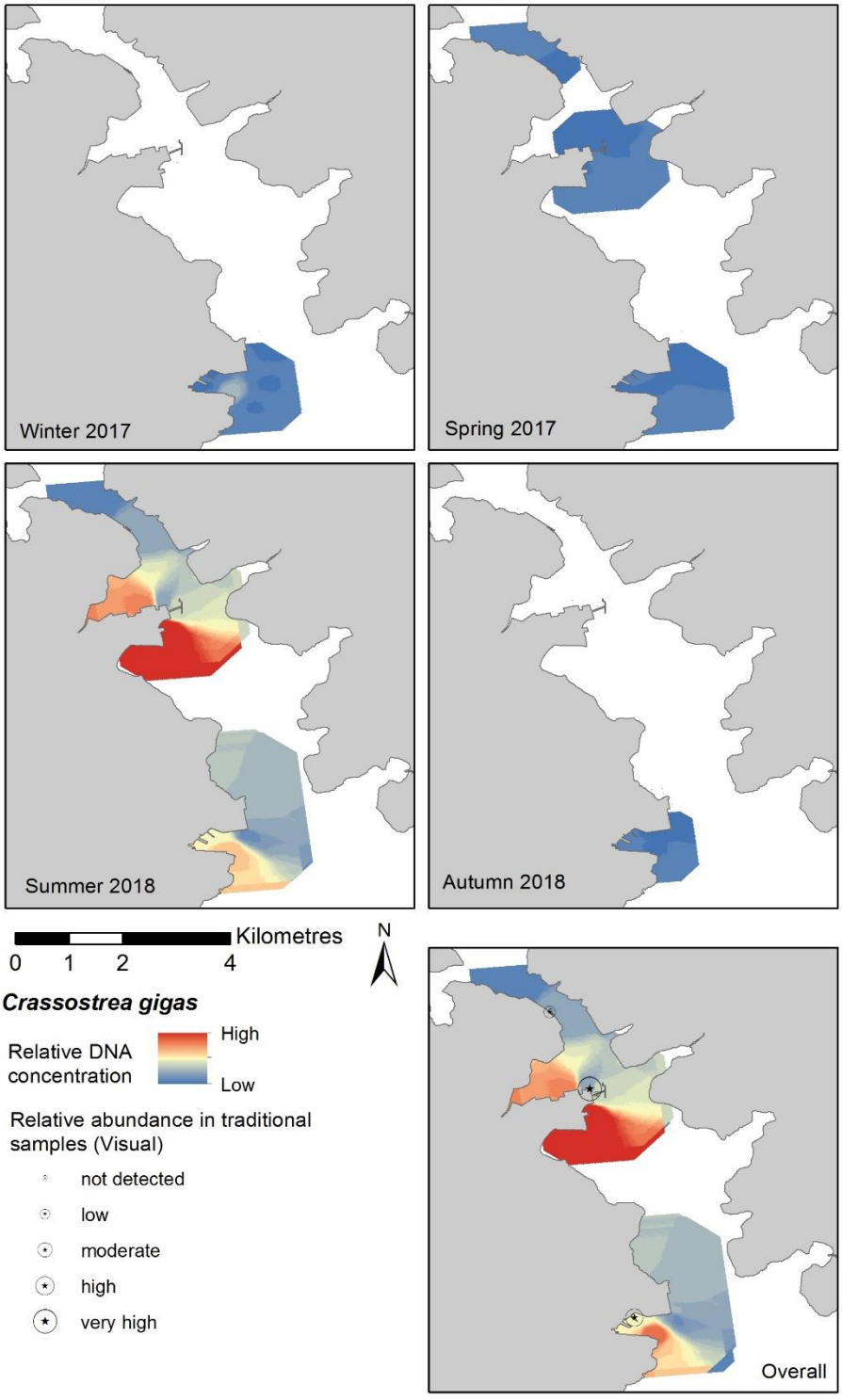

Figure 58. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Crassostrea* in Hobart by season and overall, plus overall relative abundance in visual samples.

Crassostrea reproduction is temperature dependent with a clear seasonal cycle in natural populations; in temperate locations spawning occurs in late spring through summer (Fabioux *et al.* 2005), which corresponds to the seasonal pattern observed in this project. A slightly higher rate of detections and DNA concentration in Melbourne than Hobart in spring suggests spawning may commence earlier in Melbourne where water temperatures are slightly higher. Summer is likely to be the best season for detecting *Crassostrea.*

3.5.5. Sabella

The likelihood of detection for *Sabella* from the ZALN was highest in summer-autumn and lowest in winter (Figure 60). DNA concentration in both samples and the field increased from winter through spring-summer to peak in autumn (Figure 61). The maps show highest concentrations of *Sabella* DNA occurred in autumn, especially in the Swanston Dock and adjacent Yarra River, and near the entrance to the Webb Dock (Figure 61). The traditional survey recorded highest abundance of this species in the Williamstown wharf sublocation, however, visual surveys in the Yarra River and Webb Dock did not coincide with the specific areas having high DNA in plankton samples.

The reproductive season for *Sabella* appears to be variable. Autumn-winter was recorded as the main reproductive period in the Mediterranean (Giangrande *et al.* 2000; Giangrande *et al.* 2010) and in Port Philip Bay (Currie *et al.* 2000), but with a shift towards gamete maturation earlier in Autumn in more recent Mediterranean studies (Giangrande *et al.* 2010). In South Australian populations, no consistent reproductive period was observed but gamete abundance in adult worms was generally lowest in summer, suggesting gamete release in spring (Lee 2013; Lee *et al.* 2018). These studies were based on histology, which, in the case of *Asterias*, was not always well correlated with natural occurrence of larvae (Dommisse and Hough 2004). Our results suggest that summer-autumn is the best season for detection of *Sabella.*

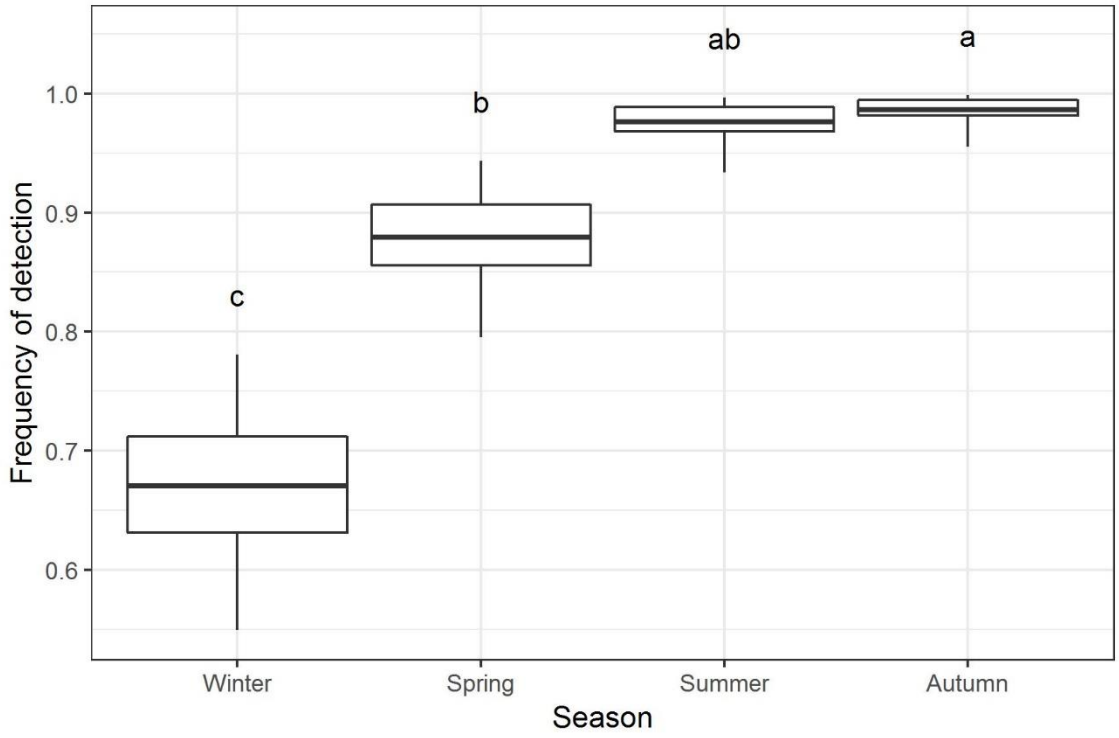

Figure 59. Posterior predictions of mean detection frequency for *Sabella* in Melbourne molecular samples by season. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

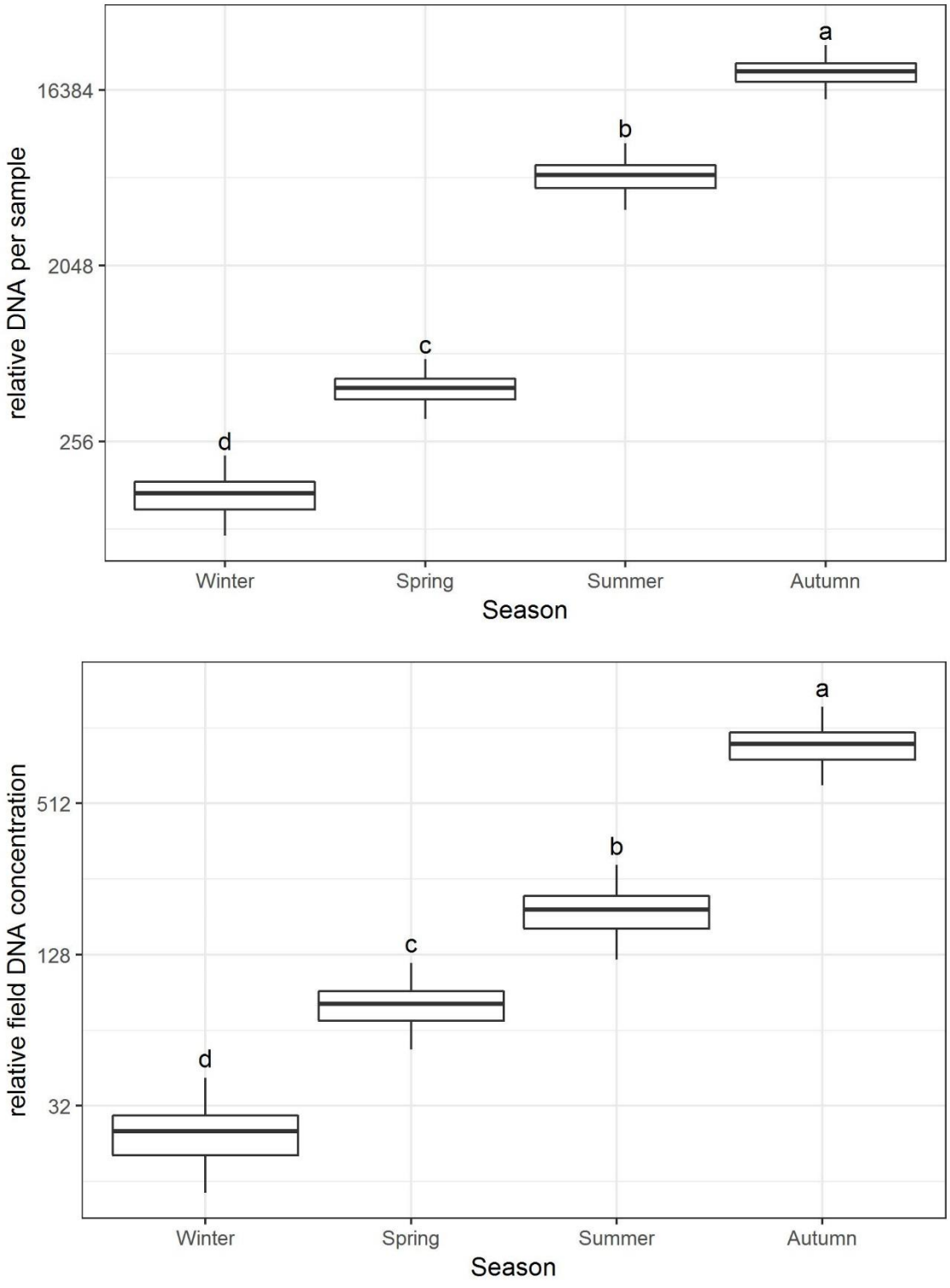

Figure 60. Posterior prediction of mean *Sabella* relative DNA in Melbourne molecular samples by season. Top panel shows relative DNA in samples and bottom panel shows relative field DNA accounting for sampling volume. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Note that y-axis is shown on a log scale. Different letters indicate that 95% credible intervals do not overlap.

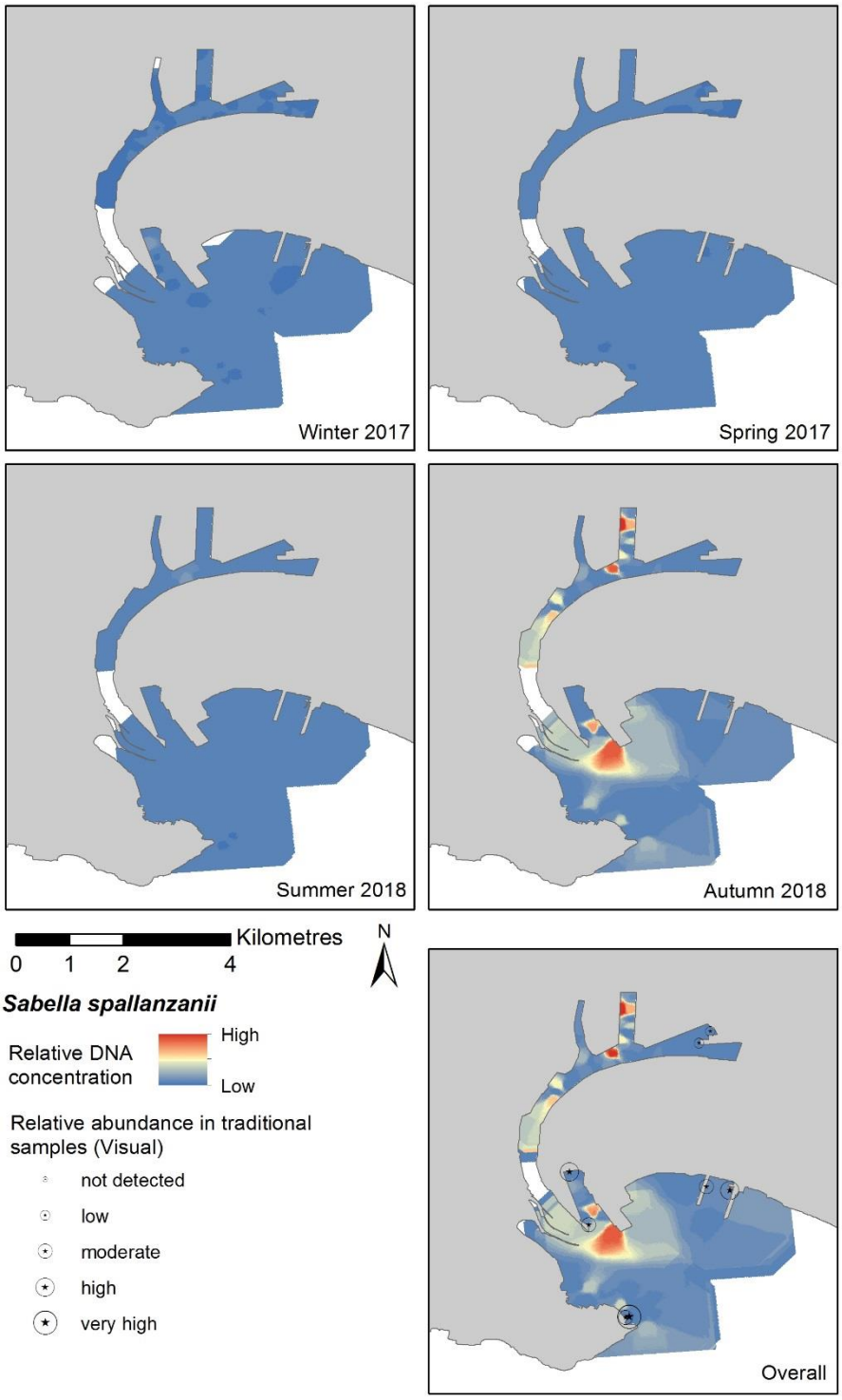

Figure 61. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Sabella* in Melbourne by season and overall, plus overall relative abundance in visual samples.

3.5.6. Undaria

The ZALN for *Undaria* showed that likelihood of detection was lowest in summer but similar for all other seasons (Figure 62), and DNA concentration in both samples and the field showed a similar pattern (Figure 63). The maps of interpolated DNA show *Undaria* DNA concentrations were high in winter and spring, especially around the Williamstown Wharves sublocation (Figure 64). This is also the area where highest abundance was observed in the traditional survey.

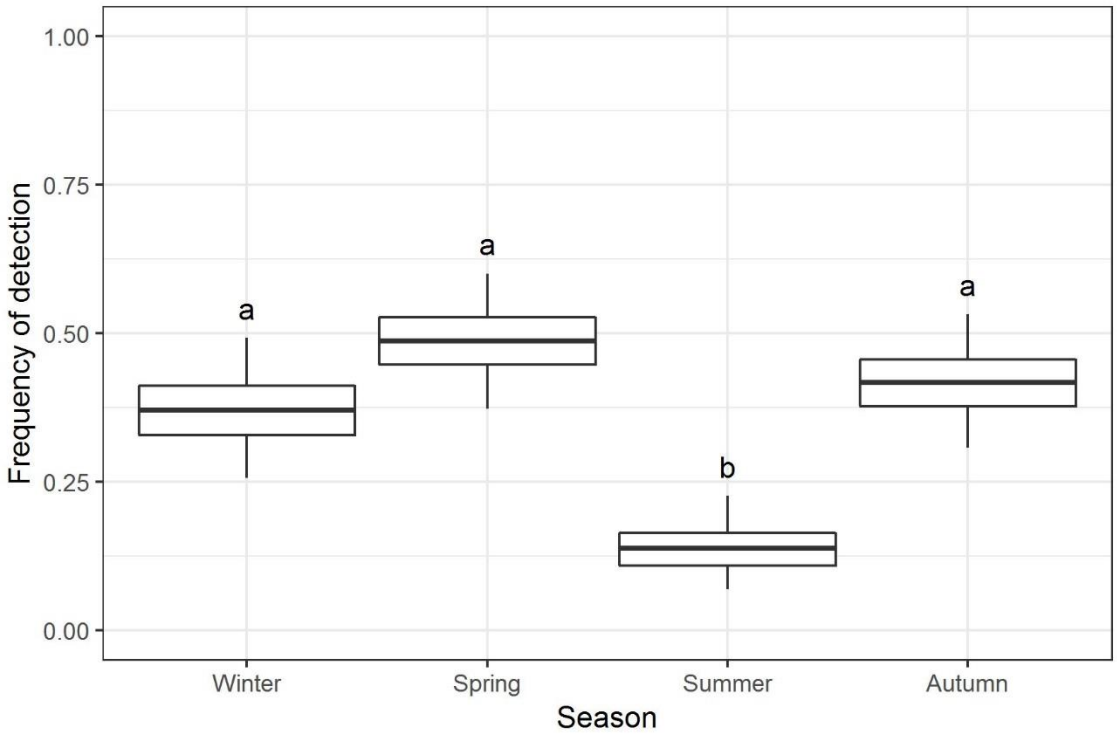

Figure 62. Posterior predictions of mean detection frequency for *Undaria* in Melbourne molecular samples by season. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Different letters indicate that 95% credible intervals do not overlap.

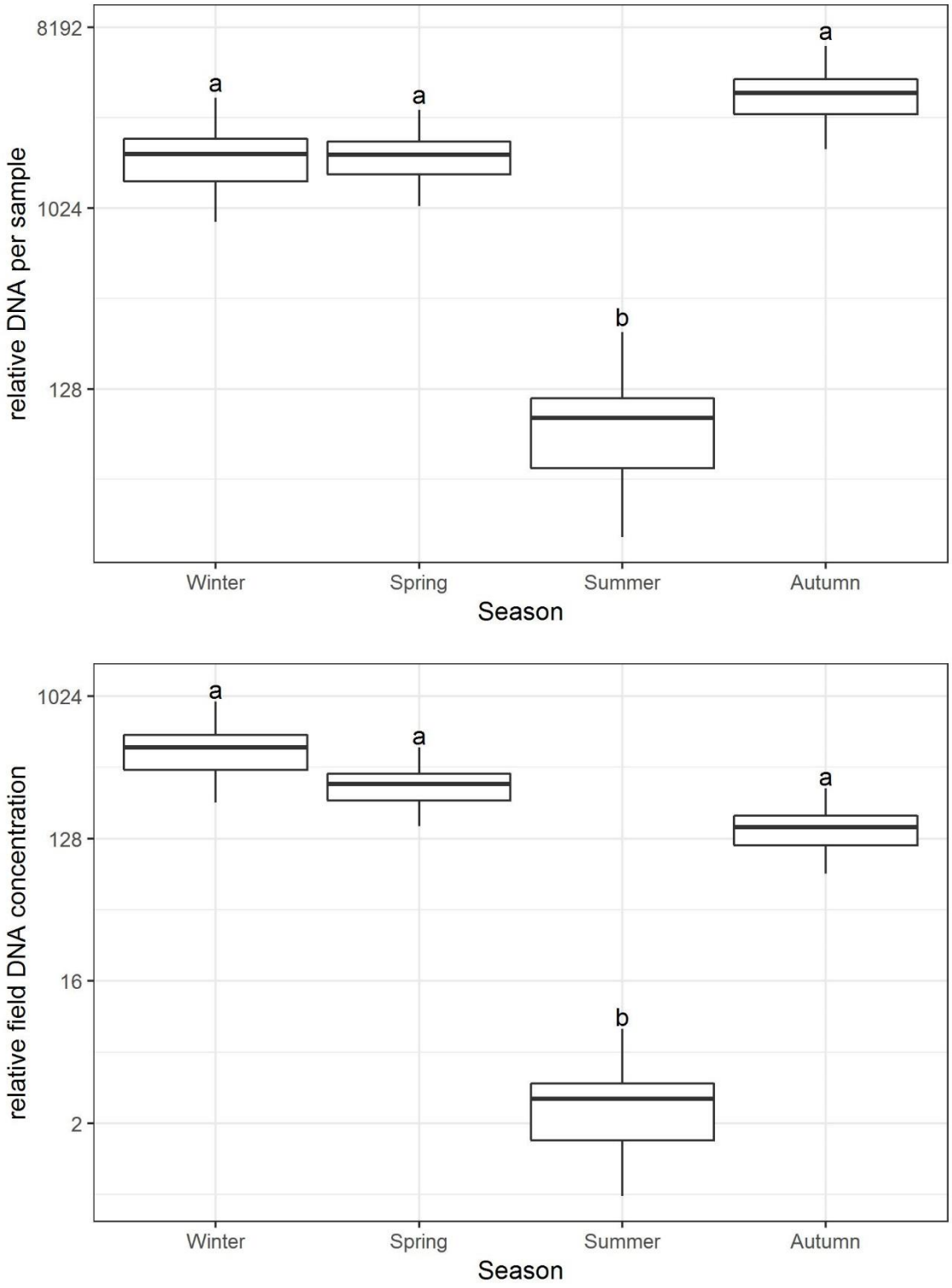

Figure 63. Posterior prediction of mean *Undaria* relative DNA in Melbourne molecular samples by season. Top panel shows relative DNA in samples and bottom panel shows relative field DNA accounting for sampling volume. Box plots boxes show mean and interquartile range, with whiskers showing 95% credible intervals of the predicted mean. Note that y-axis is shown on a log scale. Different letters indicate that 95% credible intervals do not overlap.

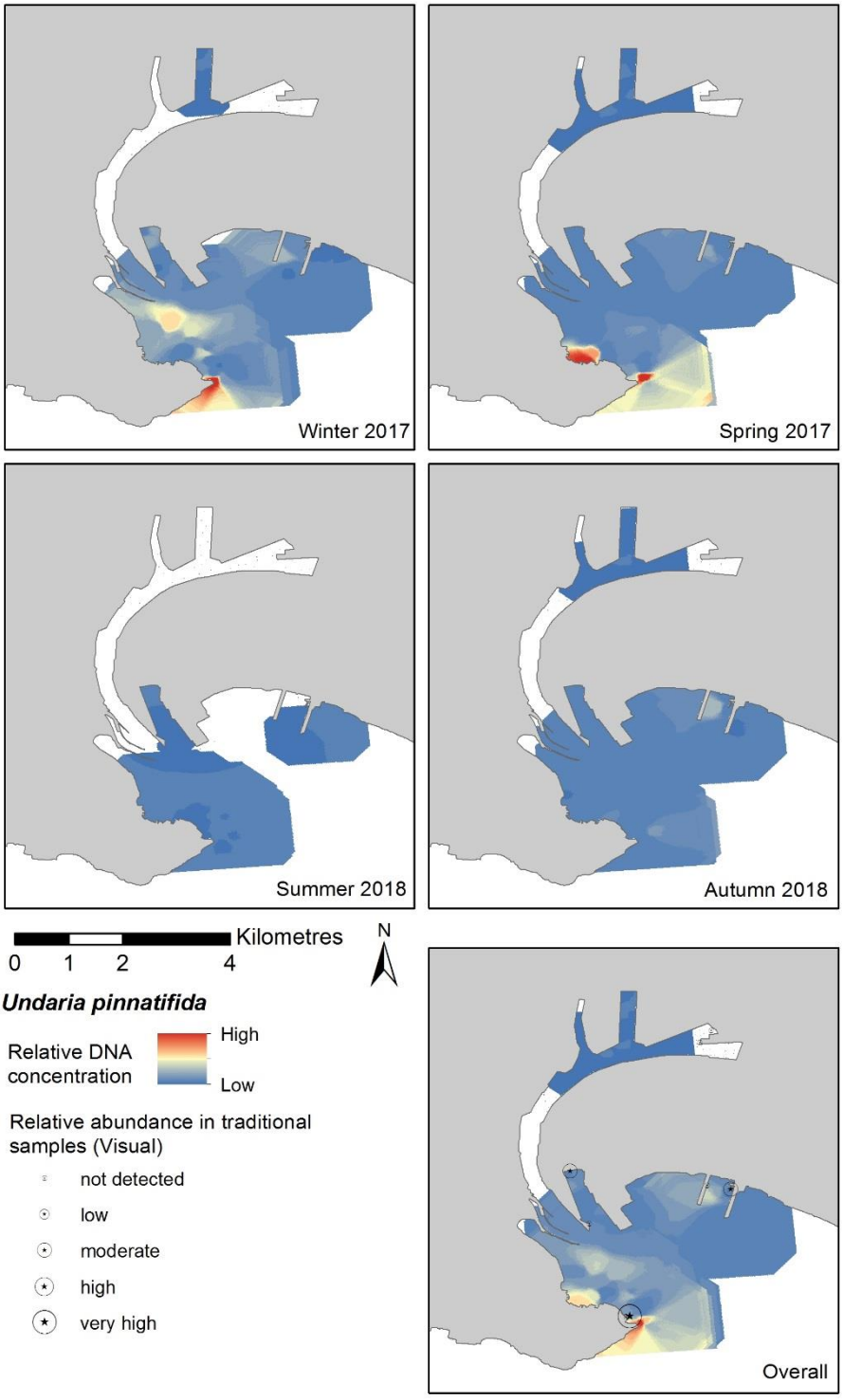

Figure 64. Map of interpolated relative DNA concentration (corrected for scaling and sample volume) for *Undaria* in Melbourne by season and overall, plus overall relative abundance in visual samples.

Undaria exhibits a seasonal and bi-phasic growth cycle with macroscopic sporophyte plants present from winter through spring to early summer in Port Philip Bay; spore release occurs primarily in late winter-spring, and mature plants decompose and senesce after reproducing, with the species remaining present as microscopic gametophytes through summer-autumn (Primo *et al.* 2010). Detections in Melbourne occurred in most seasons but were lowest in the summer senescence period. It is therefore possible that plant fragments generated by decomposition, water movement or herbivory, and not only spores, are responsible for detections. *Undaria* populations do not occur in the immediate Hobart area, so a lack of detection of this species in Hobart is not surprising. In Tasmanian populations, spore release occurs later (late spring-early summer) than in Melbourne and macroscopic plants may not appear until winter (Schaffelke *et al.* 2005) suggesting that the optimum time for detecting *Undaria* may vary with location.

3.5.7. Survey sensitivity by season

The total number of samples needed to achieve S_{SE} of 80% (the default value for the MDeT) varied greatly between seasons for those species where detection likelihood showed a distinct seasonal pattern (Table 9). Numbers were highest for the apparently scarcest species (*Carcinus*), but, even then, S_{SE} of 0.8 could be achieved for the larger port of Melbourne by collecting fewer than 10 samples in the optimal season (autumn) for this species. Note that the results presented here show sample numbers required specifically to detect the current established population size of those pests that occur in each location, while the numbers shown in Table 5 are those extrapolated for each species to a relatively low (but unmeasured) population density.

For sampling across both ports of Melbourne and Hobart, spring would be the season requiring the least samples to be collected overall to achieve S_{SE} of at least 0.8 across all six species at their current population sizes: a total of 85 samples, comprising 6 from Hobart (based on *Crassostrea*) and 79 in Melbourne (based on *Carcinus*). For Melbourne, where all six target species were detected, there is little difference between spring and autumn as the best season to sample, with spring requiring a minimum of 79 samples (for *Carcinus*) and autumn a minimum of 78 (for *Arcuatula*). These numbers are based on conducting one single survey in a year. If surveys were conducted in at least two seasons, the total number required could be reduced. At the current population sizes of the detected species, summer surveillance is highly effective for detection of *Arcuatula, Crassostrea* and *Sabella* (1 sample needed for S_{SE} = 0.8), while autumn surveillance is effective for *Asterias, Carcinus, Sabella* and *Undaria* (maximum 9 samples needed). For comparison, the total number of samples analysed in the current project was 288 in Melbourne (72 per sample set) and 60 in Hobart (15 per set). As noted in section 3.3.1, the

population sizes within these ports are likely to be representative of well-established pests and a larger number of samples will be needed to detect lower population densities.

The seasonal patterns in required sample numbers for the molecular method predicted by the probabilistic model vary slightly from the ZALN outputs. The probabilistic model determined the overall effect of sample time across all sublocations rather than individually by port. Detections occurred only in Melbourne and Hobart, therefore predicted results from the probabilistic model model effectively show the overall temporal pattern of detections across these two ports. The ZALN models show the differences between the ports in likelihood of detection and in relative DNA concentration. Differences between the probabilistic model (Section 3.3.1) and the ZALN are also due to the ZALN showing required numbers for the current pest populations, while predictions from the probabilistic model (Table 5) were extracted for a modelled low population size. For example, the probabilistic model suggests that a relatively large number of samples (131–230 for S_{SE} of 0.6 or 0.8 respectively) may be needed to detect *Asterias* in spring where this species occurs at low density, but since this species has large established populations in both Melbourne and Hobart, it can be detected with relatively few samples regardless of sampling time. Across both sets of results, however, the combination of summer and autumn sampling is predicted to be most efficient, requiring the fewest samples to achieve a given S_{SE} for a given population density.

All detections occurred at temperate ports, therefore seasonal patterns at tropical locations are uncertain, but few pests are recorded as established in tropical ports in Australia.

Table 9. Predicted required sampling numbers for the molecular method to achieve survey sensitivity (S_{SE}) of 0.8 and 0.6 for each species by port and season. Numbers are the predicted posterior mean with 95% credible intervals shown in brackets. The season requiring the lowest mean number of samples, is in bold for each port species combination. Note some 95% credible intervals overlap, so the optimal season is not always clearly better than other seasons. See Table 2 for the sample numbers applied at each port.

			Required sample numbers	
Species	**Port**	**Season**	**S_{SE} = 0.8**	**S_{SE} = 0.6**
Arcuatula	Melbourne	Winter	278 (51 – 520 036)	158 (29 – 296 069)
		Spring	3 (2 – 4)	2 (1 – 2)
		Summer	1 (1 – 1)	1 (1 – 1)
		Autumn	78 (26 – 950)	45 (15 – 541)
Asterias	Hobart	**Winter**	1 (1 – 1)	1 (1 – 1)
		Spring	1 (1 – 1)	1 (1 – 1)
		Summer	7 (3 – 28)	4 (2 – 16)
		Autumn	1 (1 – 2)	1 (1 – 2)
	Melbourne	**Winter**	1 (1 – 1)	1 (1 – 1)
		Spring	3 (2 – 4)	2 (1 – 2)
		Summer	10 (5 – 27)	6 (3 – 15)
		Autumn	1 (1 – 1)	1 (1 – 1)
Carcinus	Hobart	Winter	5 (2 – 15)	3 (2 – 8)
		Spring	3 (2 – 6)	2 (1 – 4)
		Summer	14 (4 – 185)	8 (3 – 106)
		Autumn	3 (2 – 9)	2 (1 – 5)
	Melbourne	Winter	14 (8 – 33)	8 (5 – 19)
		Spring	79 (25 – 1001)	45 (15 – 570)
		Summer	121 (35 – 2012)	69 (20 – 1146)
		Autumn	9 (5 – 16)	5 (3 – 10)
Crassostrea	Hobart	Winter	25 (8 – 167)	14 (5 – 95)
		Spring	6 (3 – 21)	4 (2 – 12)
		Summer	1 (1 – 1)	1 (1 – 1)
		Autumn	29 (8 – 455)	17 (5 – 260)
	Melbourne	Winter	18 (8 – 75)	11 (4 – 43)
		Spring	4 (3 – 6)	2 (2 – 3)
		Summer	1 (1 – 1)	1 (1 – 1)
		Autumn	30 (14 – 104)	17 (8 – 60)
Sabella	Melbourne	Winter	2 (2 – 3)	1 (1 – 2)
		Spring	1 (1 – 2)	1 (1 – 1)
		Summer	1 (1 – 1)	1 (1 – 1)
		Autumn	1 (1 – 1)	1 (1 – 1)
Undaria	Melbourne	Winter	4 (3 – 6)	2 (2 – 4)
		Spring	3 (2 – 4)	2 (1 – 2)
		Summer	11 (7 – 23)	7 (4 – 13)
		Autumn	3 (3 – 5)	2 (2 – 3)

Table 10. Posterior mean coefficient estimates for the effects of sample mass (dry weight), effective volume (flow meter distance) and PCR inhibition on likelihood of detection for each species from zero-altered log-normal Bayesian models.

Parameter	Species	Posterior mean (95% C.I.)
Mass	*Arcuatula*	0.71 (-0.26 – 1.75)
	Asterias	0.18 (-0.64 – 1.00)
	Carcinus	0.27 (-0.44 – 0.92)
	Crassostrea	1.51 (0.70 – 2.40)
	Sabella	0.13 (-0.55 – 0.95)
	Undaria	-0.29 (-1.01 – 0.31)
Volume	*Arcuatula*	0.52 (-0.63 – 1.78)
	Asterias	0.37 (-0.55 – 1.27)
	Carcinus	0.38 (-0.55 – 1.29)
	Crassostrea	-0.08 (-1.37 – 1.12)
	Sabella	0.48 (-0.77 – 1.92)
	Undaria	-0.31 (-1.09 – 0.42)
Inhibition	*Arcuatula*	-0.09 (-5.95 – 5.78)
	Asterias	-2.43 (-7.17 – -0.30)
	Carcinus	-3.22 (-9.15 – -0.12)
	Crassostrea	-1.15 (-4.00 – 0.40)
	Sabella	-0.11 (-6.05 – 5.90)
	Undaria	0.00 (-5.98 – 5.91)

3.6. Effects of inhibition, sample volume and mass

The ZALN models showed that there were no consistent effects of sample mass, effective volume or PCR inhibition on the likelihood of detection (Table 10). Sample mass had a generally positive effect on likelihood of detection, but 95% credible intervals of the posterior estimate contained zero except in the case of *Crassostrea.* Sample volume effects on detection likelihood were mixed across species, and 95% credible intervals of the parameter estimates contained zero in all cases. The effect of PCR inhibition on likelihood of detection was generally negative but the magnitude of this effect varied between species. The 95% credible interval contained zero except in the case of *Asterias* and *Carcinus.* Although some effect of inhibition was observed, detections still occurred in some samples with high to very high scaling, with *Crassostrea* detections occurring in samples with scale factors of up to 1497 from Hobart. The maximum scale factor for detection of other species ranged from 1.8 to 19.5, however, as several species were only detected in

Melbourne where few samples had high inhibition, we cannot determine how very high inhibition might impact detections of these species from current data.

The effect of sample mass on DNA yield of samples was positive for *Asterias* and *Sabella* (Table 11). These were the two species with the lowest C_t values for detection (i.e. high relative DNA), which may have allowed detection of this pattern. The effect of sample mass was, however, minor in comparison to seasonal differences.

Table 11. Posterior mean coefficient estimates for the effects of sample mass (dry weight), and estimated scale factor multiplier on relative sample and field DNA concentration for each species from zero-altered log-normal Bayesian models.

Model	Parameter	Species	Posterior mean (95% C.I.)
Sample DNA	Scale Factor multiplier	*Arcuatula*	0.64 (0.19 – 1.10)
		Asterias	0.17 (-0.40 – 0.74)
		Carcinus	1.04 (0.08 – 2.00)
		Crassostrea	0.66 (0.30 – 1.02)
		Sabella	0.83 (0.16 – 1.52)
		Undaria	0.13 (-0.75 – 1.02)
	Mass	*Arcuatula*	-0.23 (-0.51 – 0.06)
		Asterias	1.03 (0.64 – 1.43)
		Carcinus	0.31 (-0.88 – 1.52)
		Crassostrea	0.05 (-0.30 – 0.39)
		Sabella	1.01 (0.58 – 1.43)
		Undaria	-0.68 (-1.78 – 0.42)
Field DNA	Scale Factor multiplier	*Arcuatula*	0.43 (-0.08 – 0.94)
		Asterias	0.03 (-0.55 – 0.62)
		Carcinus	0.98 (0.02 – 1.95)
		Crassostrea	0.53 (0.11 – 0.93)
		Sabella	0.63 (-0.09 – 1.35)
		Undaria	0.11 (-0.78 – 1.00)
	Mass	*Arcuatula*	-0.21 (-0.55 – 0.13)
		Asterias	0.83 (0.41 – 1.25)
		Carcinus	0.17 (-1.17 – 1.51)
		Crassostrea	0.08 (-0.31 – 0.47)
		Sabella	0.99 (0.51 – 1.48)
		Undaria	-0.47 (-1.65 – 0.72)

4. CONCLUSIONS

4.1. Fitness for purpose of the molecular surveillance method

These results demonstrate that the molecular surveillance method is fit for purpose and can achieve equivalent or greater survey sensitivity (S_{SE}) than traditional methods with lesser field time and lower cost for sampling. We detected all target pests established in the surveyed areas using molecular methods, while traditional methods did not detect *Carcinus* or *Crassostrea* in Melbourne. Pets detected in the current project are, in most cases, likely to be well established and abundant. Molecular methods are likely to require far fewer samples than traditional survey methods to detect pests at low density.

The National System surveys were designed to detect 'established populations' (National System for the Prevention and Management of Marine Pest Incursions 2010b) and MDeT calculations use a set population size, and therefore a minimum number of individuals rather than a target density. The target population sizes were chosen arbitrarily by a working group, and it remains unclear if other values would be more appropriate, or how an established population could be defined in terms of density, particularly for mobile species. A consequence of the use of population size rather than density, however, is that the required sample numbers increase proportionally to the sublocation size for all methods. To make the sampling design feasible for implementation, sublocations for this project were defined to include only the areas immediately around wharves and adjacent suitable substrate (Wiltshire and Deveney 2017). This is the area in which presence of pests presents the greatest risk for ballast water uptake and therefore spread, but pests that occur outside the immediate wharf area can still contribute propagules (larvae or spores) to the ballast uptake zone. Pest populations outside the defined sublocations will not be detected by traditional methods, but propagules and other sources of DNA can be detected by the molecular method, contributing to discrepancies in detection from the different approaches.

Arthur *et al.* (2015) suggest that the density of propagules in the ballast water uptake zone is likely to be a better indicator of ballast water risk than the pest population size considered by the MDeT. The molecular method is clearly better suited than the traditional techniques to assess this risk, but relating qPCR results and DNA concentration estimates to the biomass or density of a pest in the plankton is problematic. The DNA content of larval marine organisms has not been quantified and changes with growth. We chose assay targets that have multiple copies per cell, and copy numbers can further vary with growth and physiological status of the organism. The molecular method also does not distinguish between viable propagules and other sources of DNA, although

the highest observed DNA concentrations and likelihood of detection found in this project often corresponded to known reproductive periods of the target species. Discrepancies may be due to variation in spawning period between locations or years, or due to differences in invasive and native reproductive biology (Munguia and Shuster 2013) for species where reproductive period has not been assessed in Australian populations such as *Arcuatula*. Where several detections and relatively high DNA concentrations occur, however, it is likely that an established population of that species exists. For these detections, it is likely that viable propagules are being detected, or, where detections may be due to other sources of pest DNA, that propagules are likely to occur in the same area during reproductive periods. Molecular results could therefore demonstrate relative risk from pest propagules in ballast water uptake areas, even if the actual propagule density cannot be quantified.

This project demonstrated that plankton collection and processing methods based on Deveney *et al.* (2017) are logistically feasible and suitable for obtaining high S_{SE} for the target pests. A standard plankton tow length of 100 m was applied as this was shown by Deveney *et al.* (2017) to be suitable for obtaining detections, with longer tows providing little if any benefit due to net clogging. Effective tow distances in the current study were typically much less than 100 m, indicating that substantial net clogging occurred. Pest detections were still reliably obtained, however, and flow meter distance had little to no effect on likelihood of detection. MDeT assumes that effective tow distance is equal to actual tow distance. This should be reconsidered in future design approaches. It is important that flow meter readings are obtained with future sampling, as these facilitate interpretation of molecular results with respect to field DNA concentration. Further flow meter data from other ports and sampling times will also be useful to determine whether applying a shorter tow length is adequate, although performing a shorter tow is unlikely to represent a significant time saving, given that the time to wash down the net and collect the sample is not trivial and will not change with altered tow length. Sample mass was also found to have at most a minor effect on detection likelihood or DNA concentration; it is likely that inorganic matter comprises a highly variable fraction of the sample, and so mass may not relate closely to pest DNA yield.

Assessing PCR inhibition is important for all environmental DNA detection systems (Goldberg *et al.* 2016), and effects of PCR inhibition should be investigated continually as molecular surveillance is implemented. PCR inhibition occurs when products are co-extracted or inadequately removed from the DNA matrix used for PCR. Inhibitors include products that interact with DNA or interfere with polymerases and their cofactors that drive PCR reactions. These

include a wide variety of organic substances including structural proteins, enzymes, alcohols, complex polysaccharides and humates, and inorganic substances such as ionic detergents, calcium, urea, chlorides and detergents (Bessetti 2007). Inhibition occurred in some samples from the current project but did not have a large effect on our results, with detections occurring even in some samples with high scale factors. Very high inhibition will reduce diagnostic sensitivity (the likelihood of detection when a target is present), but aside from the winter sampling in Gladstone, we rarely encountered high inhibition. Determining at what level inhibition becomes problematic for the pest assays is difficult from the current project data given that few samples with high inhibition were from locations where target pests occur. Further data on inhibition from environmental samples will assist in determining whether inhibition is more common in some areas than others and whether seasonal patterns of inhibition are consistent. Where inhibition occurs, it will also compromise calculation of pest DNA yield, given that it is not certain that the pest assays respond equivalently to inhibition as the assay for the internal control. Inhibition is also unlikely to have a linear effect, further complicating the correction of DNA yield in samples, particularly with higher scale factors. Current data did not permit full assessment of the response. This project does not provide data on the diagnostic sensitivity or specificity of the molecular assays, but these are being investigated in a parallel project. Understanding diagnostic sensitivity, and how this may be affected by inhibition, is important for developing improved survey design tools. The MDeT assumes perfect diagnostic sensitivity and specificity, but the effects of imperfect detection should be considered in future design approaches.

4.2. Pests status of the surveyed ports

Data obtained by this project provide updated information on the status of six priority pests for the surveyed ports. All six of the target pests were detected by molecular methods within the port of Melbourne, but *Carcinus* and *Crassostrea* were not detected by the traditional survey. In the case of *Carcinus*, it is likely that this species is currently present at low population size, below the detection limit for the traditional survey, especially given that it was detected in a lower proportion of molecular samples than other pests. *Carcinus* populations are known to fluctuate in size (Thresher *et al.* 2003); in Adelaide, this crab was commonly found in the early 2000s, but was not detected by traps in pest surveys conducted in 2007-8 or 2010-11 (Rowling 2009; Wiltshire *et al.* 2010; Wiltshire and Deveney 2011), before becoming relatively abundant over recent years (Dittmann *et al.* 2016; Dittmann *et al.* 2017). *Crassostrea* has been recorded in a number of locations in Victoria, including in Port Philip Bay (ALA 2019), and has been noted to occur in the Maribyrnong River (unpublished data), although upstream of the port area included in surveys for

this project. Molecular results from this project suggest that *Crassostrea* is widespread in the Melbourne area, as detections occurred within all sublocations even in the poorest season for detection (winter). Overall the highest DNA concentrations were in the Station Pier sublocation, indicating a higher abundance is likely in this area. The adult population may, however, be outside the immediate wharf area that was surveyed. *Asterias, Carcinus* and *Crassostrea* were detected in Hobart, where these pests are known to be established. *Carcinus* appears to be currently present at a low population size in Hobart given detection in a single traditional sample and relatively few molecular samples. Brisbane appears free of the target pests with no detections in traditional sampling and the low-level individual molecular detections likely to be due to hull fouling or ballast release, or possibly a cross-reaction in the case of *Arcuatula*. The status of *Arcuatula* in Gladstone remains unclear, but will be informed by further investigation of collected bivalves and sequencing of PCR product from samples with molecular detections. There was no evidence for occurrence of the other target pests in Gladstone.

The assay for *Arcuatula* provided several positives with normal amplification curves and a range of C_t values from Gladstone, and one high C_t value detection from Brisbane, two locations where *A. senhousia* is not known to occur. These detections are under further investigation. The assay is known to detect the target species *A. senhousia*, but it is possible that it also detects non-target organisms, possibly related mytilid mussels. The C_t values from Melbourne are consistently higher than those from Gladstone, despite *A. senhousia* being common in the traditional survey samples from Melbourne. This suggests that the species detected by the *Arcuatula* assay in Gladstone is abundant, but occurs outside the berth pocket areas that were targeted by traditional sampling in the current project. It is also possible that the *Arcuatula* assay more efficiently detects a non-target, probably closely related species, leading to lower C_t values than occur with true *A. senhousia* detections. If the assay detects non-target organisms, it could still be used, but a confirmatory test would need to be developed. This assay has the lowest efficiency of our currently used assays (Bott and Giblot-Ducray 2011b), however, and is likely to be re-designed along with a confirmatory test that provides longer sequence reads than the qPCR primers.

The Brisbane detection for *Undaria* re-tested positive, had no sign of cross contamination from the internal controls, and may have been due to material in a ballast discharge or hull fouling. Given that there were no other molecular detections and the habitat is unlikely to be suitable for *Undaria,* it is likely this detection was an isolated occurrence and does not indicate an established population. This raises the question of how to respond to new molecular detections of pests. An approach with escalating investigation is outlined in section 4.4.

4.3. Implications for survey design

We used the MDeT for survey design because it is the tool that was used to design National System surveys and it facilitated design of traditional and molecular surveys with theoretically equivalent S_{SE} for robust comparison between methods. The sampling that was ultimately implemented for traditional methods provided a design S_{SE} = 0.8, while the molecular survey provided a design S_{SE} = 0.6 since only a subset of samples were analysed. The molecular method, however, achieved equivalent or greater effective S_{SE} than traditional sampling for the surveyed ports where pests were detected, and modelling indicated that, for a given pest density, relatively few plankton samples would be needed for detection to equivalent S_{SE} in comparison to the best traditional method for each species. These results show that the MDeT greatly underestimated survey sensitivity of the molecular method, particularly relative to traditional surveillance techniques. The sensitivity calculations used by the MDeT assume that pests are evenly distributed through each sublocation, while most pests, particularly during the early phases of colonisation, have aggregated distributions. Even established pests will not occur evenly on all available substrate. *Crassostrea* occurs primarily in the intertidal to shallow subtidal (Global Invasive Species Database 2019a), *Undaria* grows most densely at depth of 1−3 m (Global Invasive Species Database 2019c), while *Sabella* occurs more densely in deeper water and sheltered microhabitats (Parry *et al.* 1996). Aggregated distributions lead to underestimation of sensitivity if sampling is conducted randomly over the available habitat (Arthur *et al.* 2015). The National System monitoring manual protocols that accompany the MDeT call for sampling to be conducted on a regular spatial grid rather than randomly, and to target likely areas of aggregation (National System for the Prevention and Management of Marine Pest Incursions 2010b), which may mitigate the difficulties of detecting pests with a patchy distribution. Proposed locations for sampling were spatially distributed as widely as possible (Appendix 1), but the final sampling locations at some sites for traditional methods, particularly visual surveys, were not well distributed. This may have further reduced effective S_{SE} of the traditional methods relative to plankton sampling. It is further likely that the MDeT overestimates sensitivity of the visual survey method, with the number of required samples provided by the MDeT being low, even for large sublocations such as the Yarra River, given the target pests for this method (*Crassostrea*, *Sabella*, *Undaria*) are likely to be highly aggregated. The distribution of DNA in water also shows aggregation (Furlan *et al.* 2016), and we observed patchiness in DNA concentration in our results, but with a suitable spatial distribution of sampling, detections were reliably obtained. The locations for applying traditional sampling methods around ports are often restricted due to access constraints and logistical considerations. Obtaining a suitable spread of plankton samples is more

likely to be feasible than for other sample types, particularly given that direct access to port infrastructure is not required, and samples can be collected rapidly, providing flexibility to work around shipping movements. A 100 m plankton tow also provides better spatial coverage than many traditional methods, is not affected by visibility or target pest catchability, and can be used over substrates that are unsuitable for dredge sampling, further emphasising the differences between the approaches.

The use of a defined reproductive output for a population size is another reason for the relative under-estimation of molecular survey sensitivity by the MDeT. MDeT assumes 50 detectable units (larvae or other propagules) are present in the plankton per adult pest. The target pests, however, have high fecundity: *Sabella* typically releases ~50 000 eggs per female (Currie *et al.* 2000), *Crassostrea* over 50 million (FAO 2004), and *Undaria* can release several million spores per cm of fertile tissue per hour (Primo *et al.* 2010). The other pests are also highly fecund (Thresher *et al.* 2003; Dommisse and Hough 2004; Global Invasive Species Database 2019a). More than a single propagule may need to be present in a plankton sample for detection to occur, e.g. Deveney *et al.* (2017) found that plankton samples that had ~10 D-stage *Crassostrea* larvae added did not always provide a positive qPCR detection, while detections occurred reliably in samples where ~100 larvae were added. Even allowing for this, however, it appears certain that the number of detectable units per adult pest is considerably more than 50, especially given that detections by molecular methods could additionally occur due to other environmental DNA for at least some of the target species, not just larvae or spores. Results from Deveney *et al.* (2017) and this project show that target species are detected outside known spawning seasons, although targeting the spawning season often maximises detection likelihood. Seasonality of detections appears to be much greater for some species, e.g. *Arcuatula, Crassostrea*, than others, e.g. *Carcinus, Sabella, Undaria.* This may be due to more DNA being shed from adults of the latter class of species outside the main spawning period, or to multiple spawning events occurring each year. Targeting reproductive periods will increase sensitivity of the molecular method, but the MDeT assumes sampling is conducted randomly over time, which may be a further reason for it underestimating sensitivity of the molecular method. This study helps to inform the best seasons to sample, within which fewer samples are needed to obtain the target S_{SE}. Extrapolating the data to obtain the number of samples required to achieve a given S_{SE} in other locations is, however, difficult. It is likely that most of the pests detected in Melbourne and Hobart are well established, and hence at a larger population size or density than the minimum population that may be indicative of a new establishment or of concern for ballast water management. *Carcinus* is a possible exception,

given that it is present at a population size below the detection limit of the traditional survey in Melbourne, and at the detection limit in Hobart. Even for *Carcinus*, the number of samples required to achieve S_{SE} of 0.8 by applying the molecular method in a suitable season was low (<10). The probabilistic model was used to predict required sample numbers for given S_{SE} where each pest occurs at low density, but this required considerable extrapolation, and is best taken as a guide to relative performance of molecular and traditional methods than as an indication of actual sample numbers needed. This model suggested that better performance of the molecular method in comparison to traditional sampling methods is due to a higher concentration of detectable targets being present at certain times of year, giving further evidence that the population size multiplier used by the MDeT is inappropriate, and to the importance of considering sample timing.

Modifications to the design approach are required, particularly to prevent costs of future surveys being unduly high. A new survey design method would use a more realistic measure of population size or density than applied in the MDeT, and account for seasonal patterns, providing a design with a required number of samples in different seasonal sampling periods, and facilitating choice of the best season or combination of seasons for detecting all target species. The seasonal patterns observed in this project were similar between Melbourne and Hobart for the species detected at both ports, but additional data would be needed to confirm if these patterns are valid in other ports, across years, and at different times within each season.

Across the current target pests, there is no clear single season that is ideal for sampling, but if seasonal patterns are broadly consistent, at least within temperate areas, then sampling over summer and autumn would be sufficient to obtain high survey sensitivity for all species with the lowest required sample numbers in these areas. The winter survey had overall lowest sensitivity across the six species, while spring was suitable for detection of the target species in Melbourne and Hobart, where population sizes are likely to be large, but may be less suitable to target low density populations, particularly of *Asterias or Crassostrea*. We note that the current study is inadequate to fully describe seasonal patterns, as sampling was conducted over a single year and only once per season. Sampling in the current study was conducted late within each season, in particular the ‘summer’ sampling extended into early autumn, and ‘autumn’ sampling extended into early winter. Seasonal effects are likely to follow a continuum throughout the year, so, for example, early summer sampling may yield results more similar to those found by the current survey ‘spring’ sampling than to those found in the ‘summer’ sampling. Future surveillance data will assist in determining whether seasonal patterns are consistent across locations and years.

Few tropical pests have been identified in Australia and the current priority pest species for which qPCR assays are available are not known to be established at any tropical Australian port. Data for seasonality in tropical areas are therefore lacking, and optimising seasonal sampling for these areas is unlikely to be informed by results from temperate ports, even for species that may occur in both temperate and tropical areas. Assays for other pests of concern, including additional tropical species, could be implemented in future, following development and validation to ensure fitness for purpose. Our results suggest that knowledge of spawning seasonality will help to inform the best times to sample for additional species, particularly for those with a distinct reproductive period, but any new assay for an established pest should be assessed over a range of seasons to confirm seasonal patterns of detection. For surveillance to detect pests that are currently exotic to Australia, best available data on reproductive periods should be used to inform sampling season, or where data is lacking, sampling conducted at least biannually in two opposite seasons.

4.4. Interpretation of molecular detections

The high sensitivity of the molecular surveillance method means that detections may sometimes occur of transient material, e.g. from ballast water release or hull fouling, such as the detection of *Undaria* in Brisbane. These detections do not necessarily indicate a new incursion or pest range expansion. Where multiple detections occur, however, especially with relatively high DNA yield (low C_t values), it is likely that a pest population is present. A policy framework for response to new molecular detections will need to be decided between State, Territory and Commonwealth governments, but responses within this framework should be informed by the likelihood of the detections being indicative of pest population occurrence as opposed to transient. In Table 11 we provide some guidelines for the interpretation of new molecular detections and suggest potential responses, which may assist in determining whether a pest population is truly present, and which escalate with increasing likelihood of the detections indicating an incursion. Where detections occur in few samples, re-testing the extracted DNA can provide further information on the pest DNA concentration in the sample. Confirmatory tests for assays could also be used for additional verification of initial results, but are not yet available for these species and would need to be developed and validated. In considering whether a result is likely to be transient material, possible sources (vectors) for the detection should be identified. If a potential vector was present in the vicinity at the time of sampling, it supports that the detections may be transient. We note that with ballast water regulations in place, the risk of pest transport in ballast water will be reduced, but detections may still occur of transient propagules, including from non-viable organisms, fouling, and organisms that are not deactivated by decontamination units or not cleared by ballast

exchange. In the absence of potential vectors an incursion should be considered more likely. Repeating molecular surveillance, especially within the optimum sampling season for the newly detected pest (where this is known), would assist in elucidating whether detections are transient. If a new incursion is present, this would be confirmed by further detections, which should be more numerous and likely at lower C_t values, while an absence of detection in the optimum season would indicate the original detections to be probably transient. Where there are >5 detections of a new pest, or even as few as 2 detections but with relative low C_t (= high DNA concentration), and especially where these are spatially distributed or occur outside the known optimal sampling season for a pest, it is likely that a pest population is present. In this case, tracing vectors to determine the source of the DNA would focus on identifying areas within the port that may harbor adult pest populations.

A possible disadvantage of the molecular method in comparison to traditional sampling is that molecular detections may occur some distance from adult populations. In the case of defining ballast water risk this may not be of concern because it is the presence of propagules around wharves that is of most interest, but molecular detections provide only a guide to pest population location and abundance and may not accurately reflect the specific location of the pest occurrence or range. An aim of this project was to obtain data to assess how molecular detections relate to the proximity and density of adult pest populations. The traditional sampling data obtained was, however, not suitable for this purpose. Although abundance data were recorded for most species and traditional sample types, the MDeT surveys are intended for detection not determination of density or distribution. The number and spatial arrangement of traditional samples was inadequate to characterise these aspects, and results were further compromised by inaccurate location data, sampling not being spatially well distributed, and difficulty in identifying individuals of some species in the field. Development of fine-scale hydrodynamic models for ports of interest could assist in indicating likely locations for source populations for molecular detections, and aid in interpretation of molecular results. Formally assessing the relationship between adult pest population location and densities and molecular detections would, however, require much more comprehensive (and expensive) surveys of adult pest populations than are required for detection. While hydrodynamic models would assist in identifying potential source locations for new molecular detections, confirming the specific location, range, and abundance of any new pest incursion would require follow-up traditional surveys to be conducted. Such follow up surveys could inform further management decisions, including indicating whether an incursion is likely to be eradicable, and would enable collection of taxonomic specimens for lodgment with relevant

museums and for confirmation of identification if necessary. If traditional surveys are infeasible, however, the precautionary principle suggests that ports with multiple molecular detections, especially at low C_t values, should be considered positive for that pest.

Table 12. Guidelines for interpretation and suggested responses to new marine pest environmental detections from validated assays. Re-test refers to re-testing the DNA extracted from the sample. Trace vectors refers to assessing potential sources of a pest detection (tracing backward), including transient sources. Re-survey (molecular) refers to re-sampling and testing using molecular analysis. Traditional survey refers to a survey to obtain specimens and delineate presence and abundance of the organism. Decisions need to be managed between State, Territory and Commonwealth Governments as part of response arrangements.

Number of detections	**Detection C_t**		
	>40	**35-39**	**<34**
1	Likely to be transient material • Re-test • Trace vectors	Likely to be transient material • Re-test • Trace vectors	Possible incursion • Re-test • Trace vectors • Re-survey (molecular)
2-5	Possible incursion • Re-test • Trace vectors • Re-survey (molecular)	Possible incursion • Re-test • Trace vectors • Re-survey (molecular)	Likely incursion • Trace vectors • Traditional survey, and/or • Consider port positive
6+	Possible incursion • Re-test • Trace vectors • Re-survey (molecular)	Likely incursion • Trace vectors • Traditional survey, and/or • Consider port positive	Likely incursion • Trace vectors • Traditional survey, and/or • Consider port positive

4.5. Summary

The molecular approach is fit-for-purpose for marine pest surveillance, can achieve higher survey sensitivity than traditional methods for lower cost and reduced time investment, can deliver results more quickly, and has lesser requirements for specific expertise and field equipment. Ballast water risk associated with propagules sourced from pest populations outside immediate wharf areas are better indicated by molecular methods than traditional surveillance. We have outlined that relatively few molecular samples are required to achieve a given survey sensitivity, especially where sampling takes place in the optimal season for a pest. The MDeT underestimates

sensitivity of the molecular method due to a range of assumptions and consideration should be made of improving approaches to survey design, including updating these assumptions. Seasonality should be considered in determining times to sample. The molecular method, however, is suitable for implementation for surveillance at Australian ports.

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APPENDIX 1. PROPOSED SAMPLE LOCATIONS

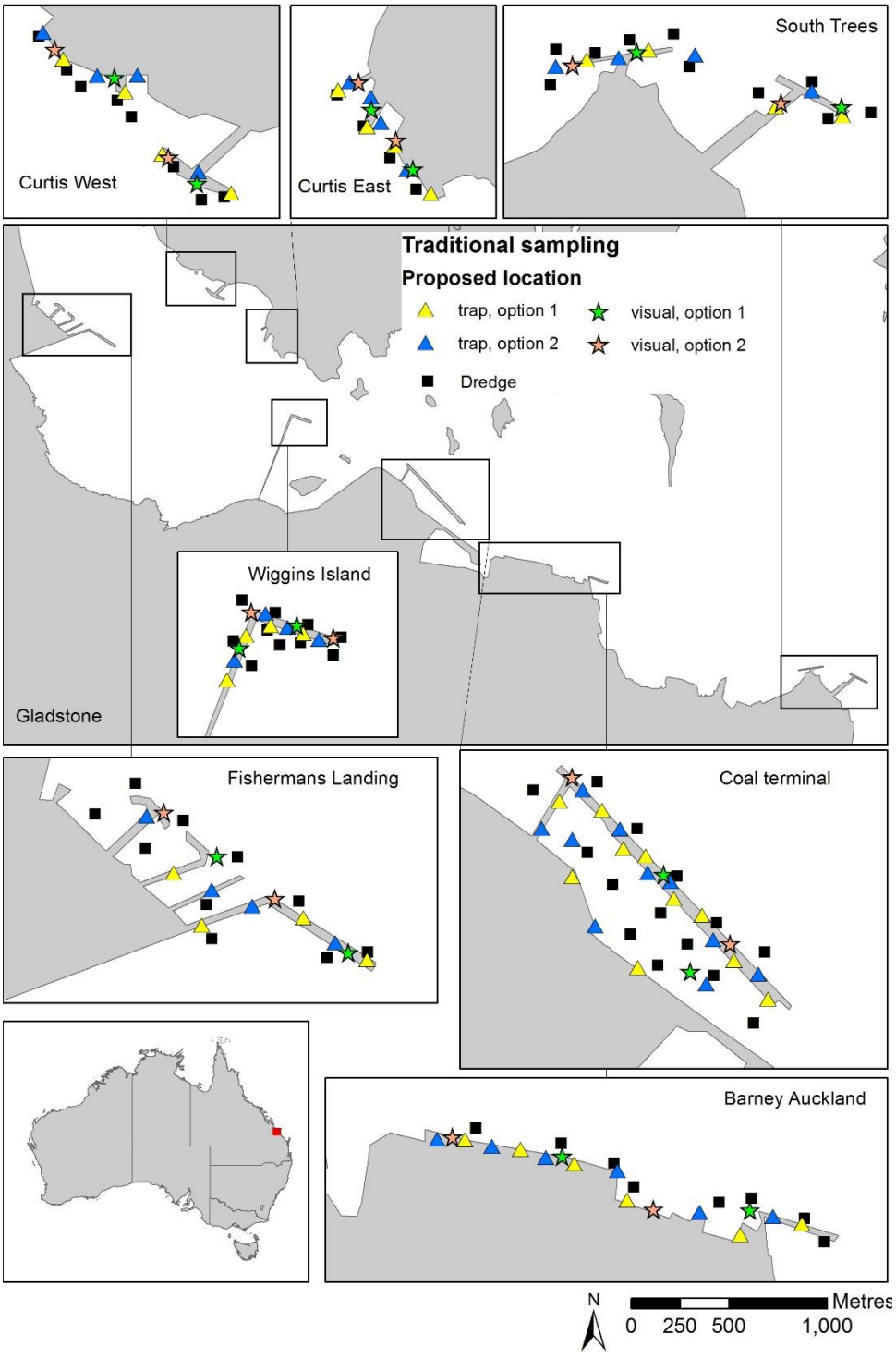

Figure 65. Map of proposed traditional survey sample locations for Gladstone.

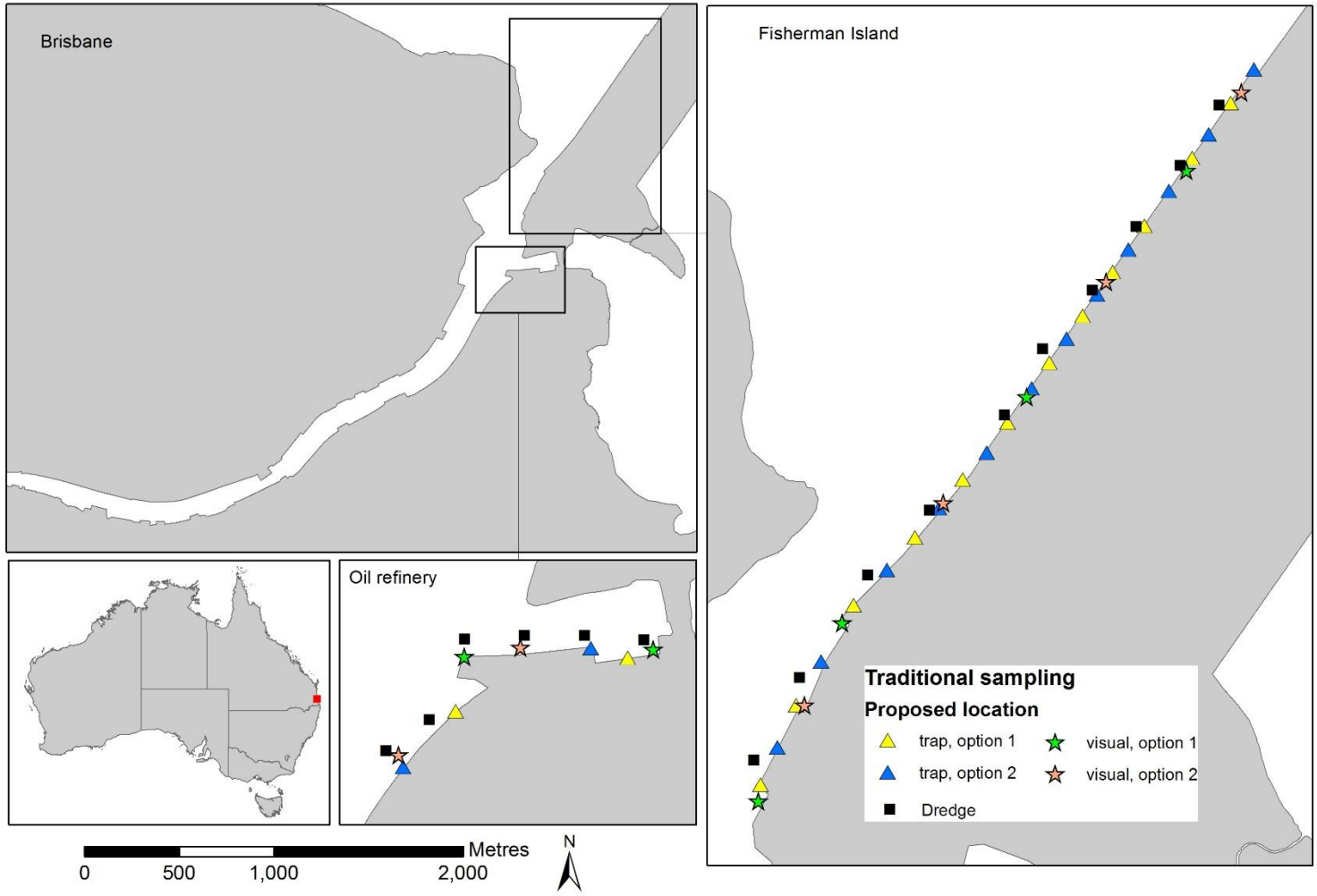

Figure 66. Map of proposed traditional survey sample locations for Fisherman Island and Oil Refinery sublocations in Brisbane.

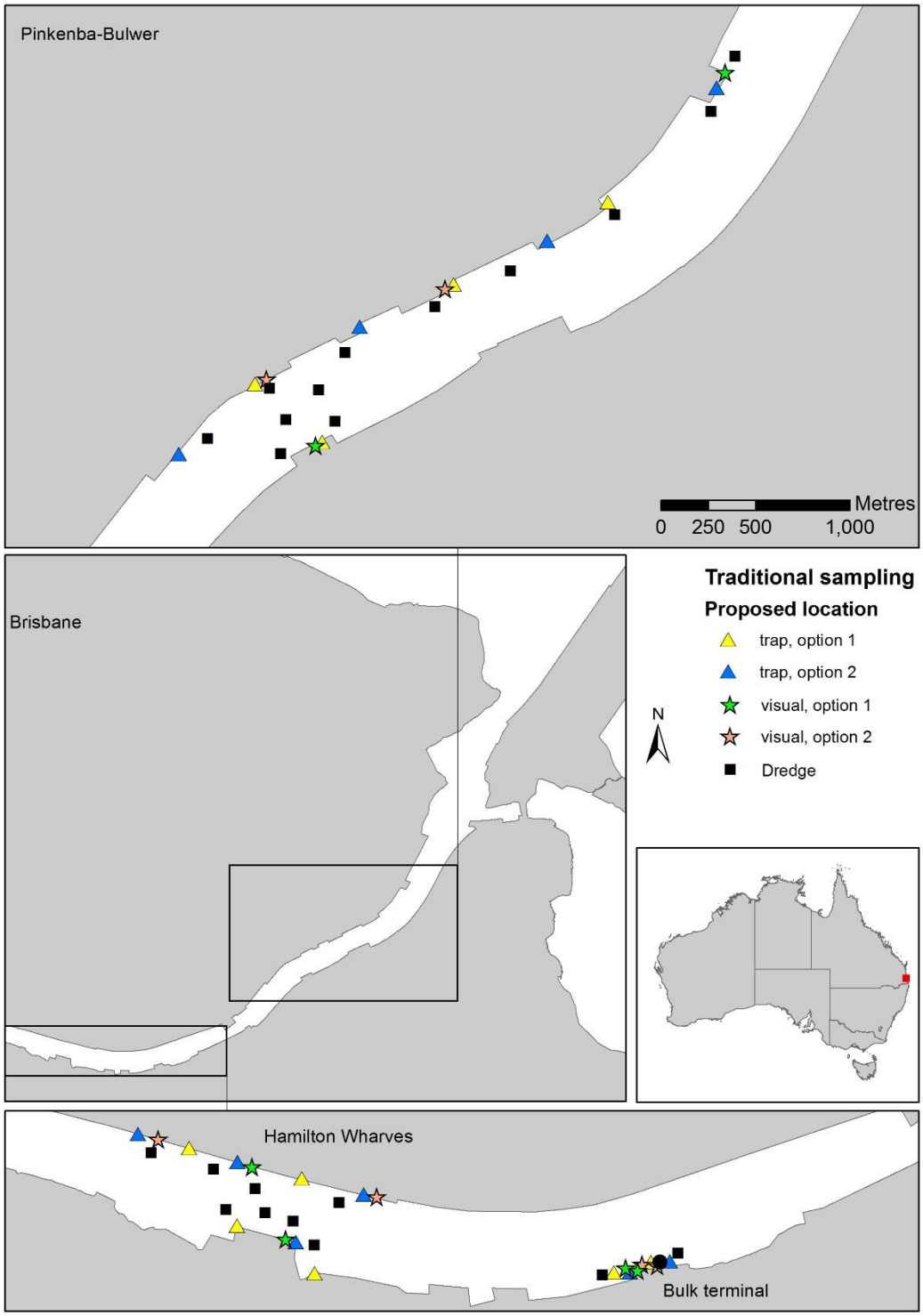

Figure 67. Map of proposed traditional survey sample locations for Pinkenba-Bulwer, Bulk Terminal and Hamilton Wharves sublocations in Brisbane.

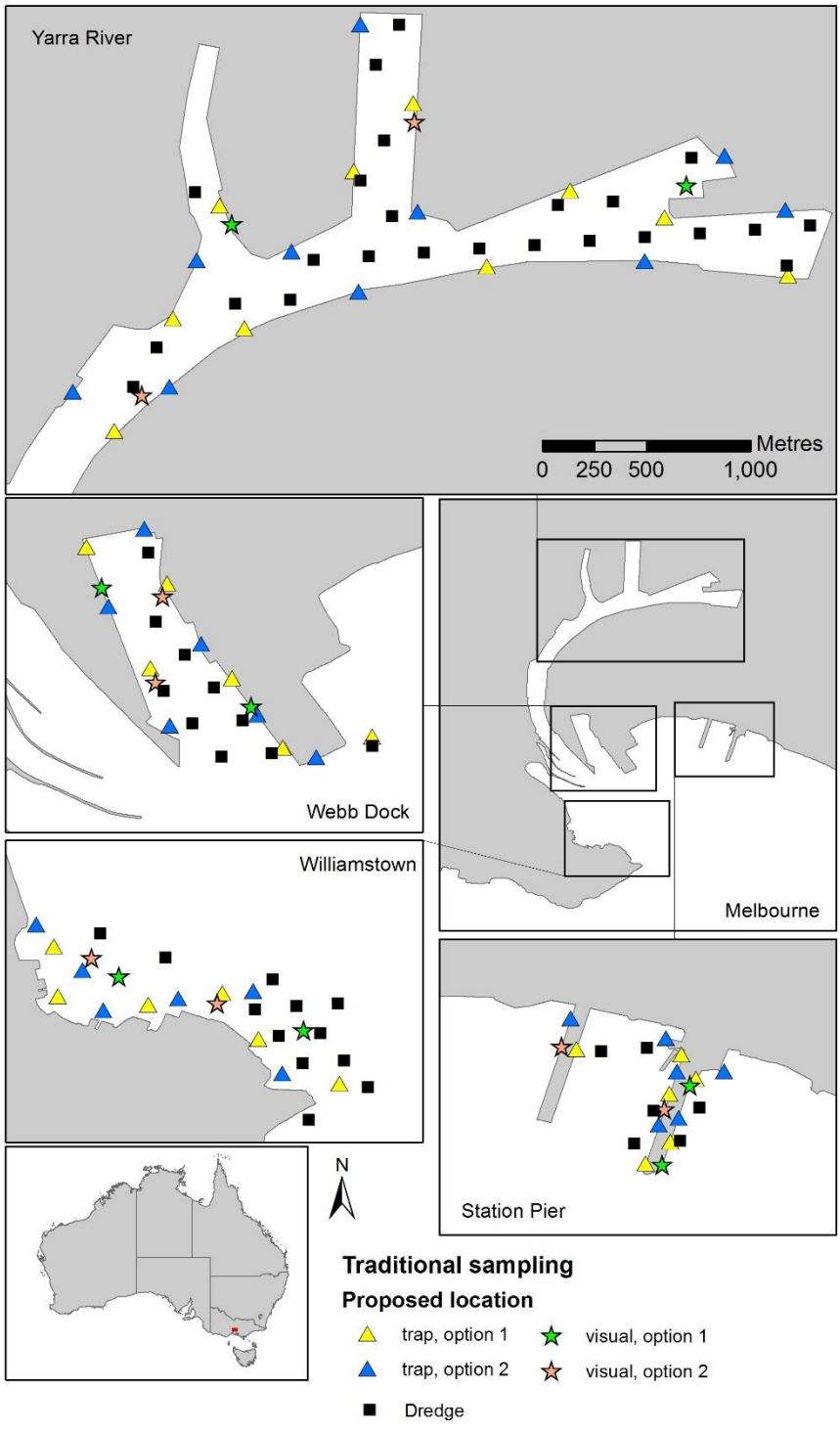

Figure 68. Map of proposed traditional survey sample locations for Melbourne.

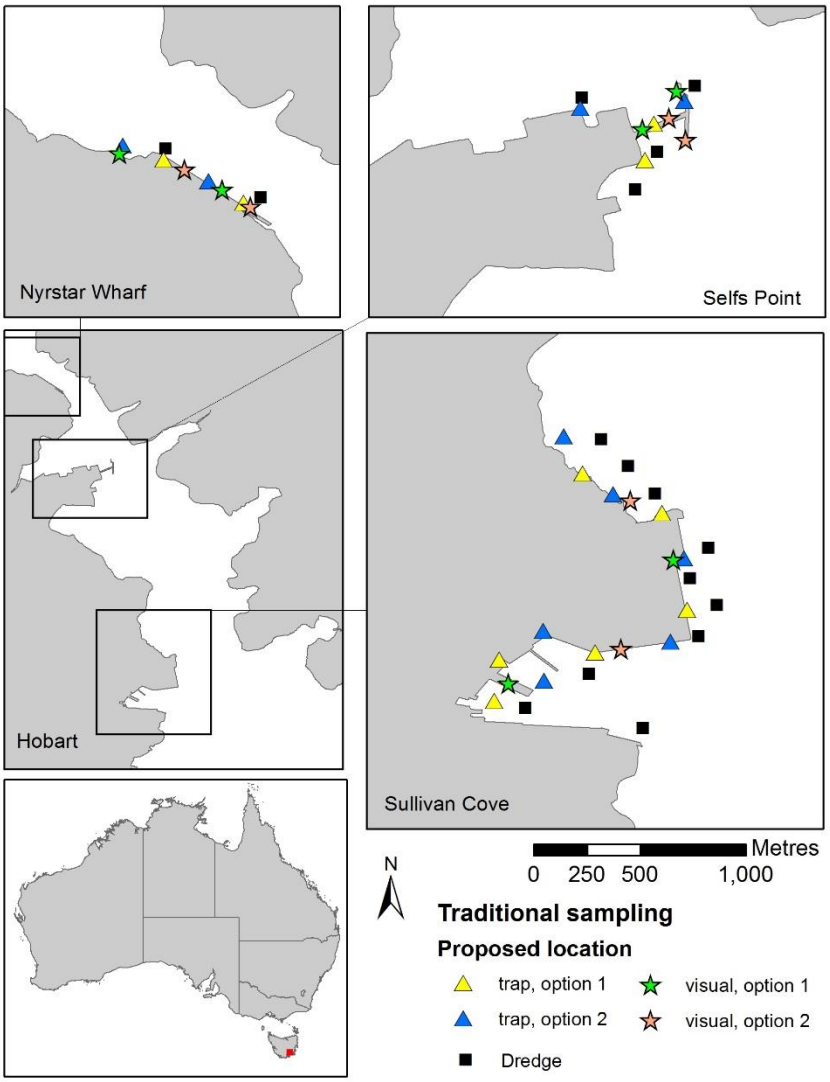

Figure 69. Map of proposed traditional survey sample locations for Hobart.

APPENDIX 2. ADDITIONAL RESULTS AND JAGS CODE

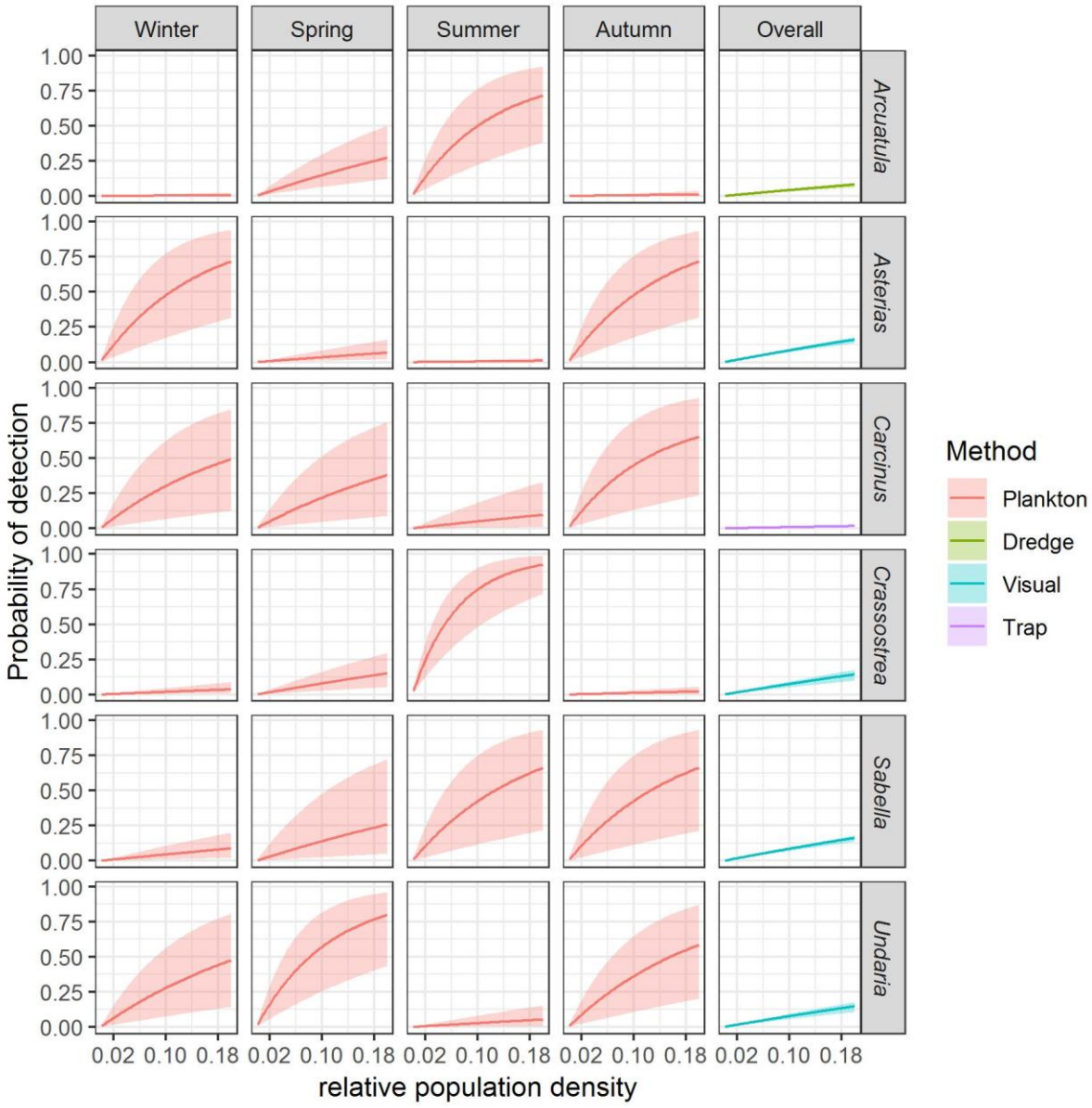

Figure 70. Posterior predictions of per sample likelihood of detection range of relative pest density values. Line shows mean predicted value with shading indicating 95% credible intervals.

JAGS code for probabilistic model

```
model {
for(i in 1:N){
 # Y - number of detections for method/location/time
 # n - number of samples for method/location/time
 # pi - probability of detection
 # ppres - probability of presence in a sample unit
 # conc - adult pest concentration in sublocation where present
 # z - indicator:pest present/absent in sublocation
 # Nprop - multiplier between adult and planktonic target concentration
 # meff - method efficiency

 Y[i] ~ dbin(pi[i], n[i])
 pi[i] <- Meff[sp[i],m[i]] * ppres[sp[i],loc[i], t[i], m[i]]

 }
 #Priors
 for(s in 1:Nspp){
  for(l in 1:Nloc){
    for(t in 1:Ntimes){
      # Method 1 = plankton (molecular)
      ppres[s,l,t,1] <- 1 - exp(-1*conc[s,l]*Nprop[s,t]*svol[1])
    }
    #Traditional methods
      for(m in 2:Nmethod){
          #detections only at times 1,3 as traditional methods not applied other times
        ppres[s,l,1,m] <- 1 - exp(-1*conc[s,l]*svol[m])
        ppres[s,l,2,m] <- 0
        ppres[s,l,3,m] <- 1 - exp(-1*conc[s,l]*svol[m])
        ppres[s,l,4,m] <- 0
      }
    z[s,l] ~ dbern(tau[s,l])
    tau[s,l] ~ dbeta(0.1,0.1)
    conc[s,l] <- z[s,l]*tconc[s,l]
    log(tconc[s,l]) <- lc[s,l]
    lc[s,l] ~ dnorm(mean.c,prec.c)
```

```
    }
    for(t in 1:Ntimes){
     Nprop[s,t] ~ dnorm(0,0.001)T(0,)
    }
   for(m in 1:Nmethod){
     Meff[s,m] ~ dbeta(alpha[s,m],beta[s,m])
   }
}
mean.c ~ dnorm(0,0.01)
prec.c <- pow(sigma.c,-2)
sigma.c ~ dunif(0,10)
}
```

Figure 71. Predicted survey sensitivity for each detected target species in Melbourne and Hobart based on MDeT calculated sample numbers to achieve 0.6 sensitivity for each method. Bars show mean predicted sensitivity, with error bars showing 95% credible intervals.

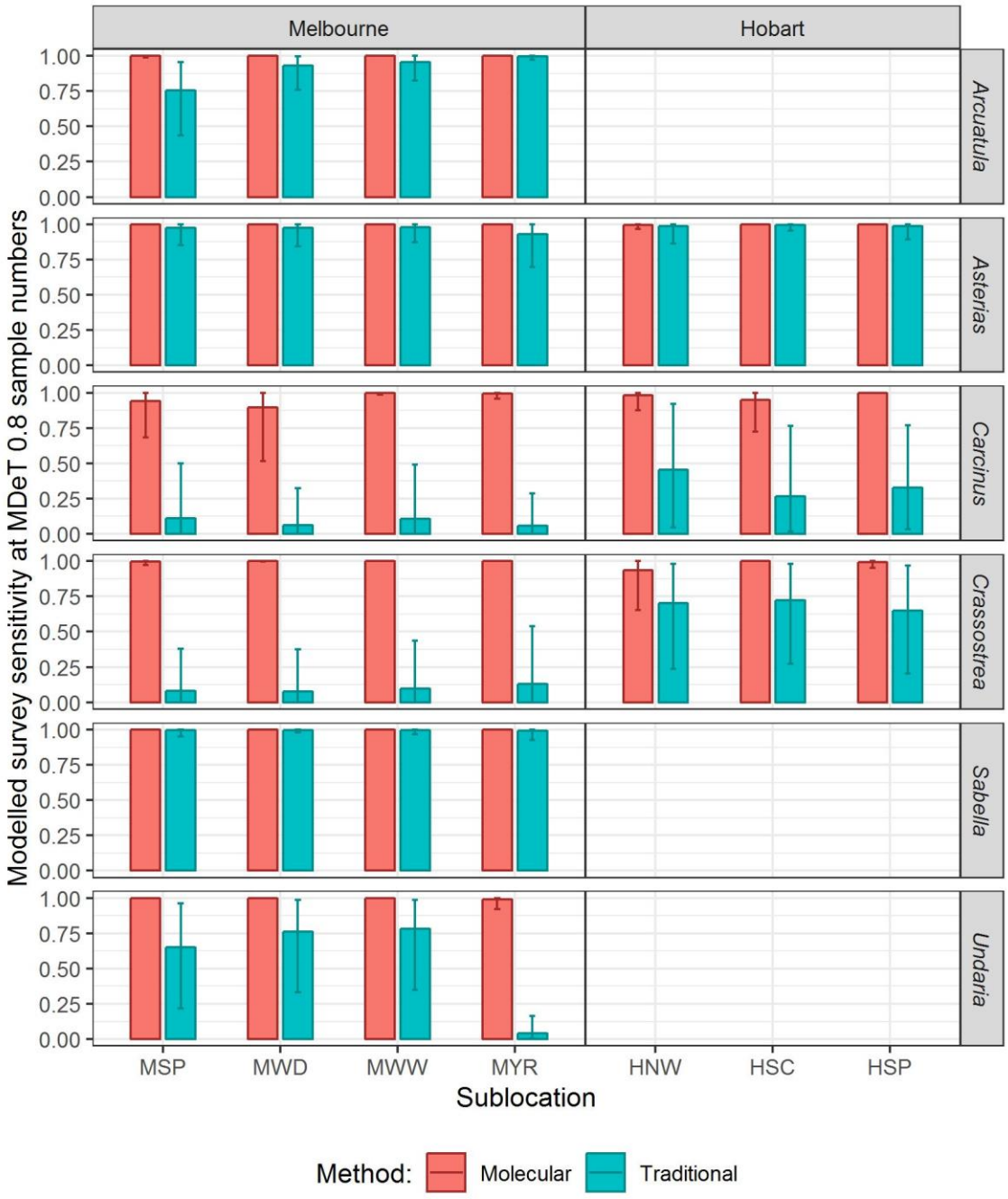

Figure 72. Predicted survey sensitivity for each detected target species in Melbourne and Hobart based on MDeT calculated sample numbers to achieve 0.8 sensitivity for each method. Bars show mean predicted sensitivity, with error bars showing 95% credible intervals.

Deviance information criterion results for logistic models

Table 13. Deviance Information Criterion (DIC) difference between logistic models of the effect of sample type (molecular/traditional) and sublocation on likelihood of detection including and excluding the interaction term sample type x sublocation. A DIC difference value of > –2 indicates that the simpler model (no interaction) is preferred.

Port	Species	DIC difference
Melbourne	*Arcuatula*	3.53
	Asterias	−0.55
	Carcinus	−0.05
	Crassostrea	0.44
	Sabella	0.21
	Undaria	2.91
Hobart	*Asterias*	0.21
	Carcinus	0.70
	Crassostrea	2.57

JAGS code for logistic model

```
model{
 # Priors
 # for binomial component, scaled t, 7df, scale 10 for intercept, 2.5 for coefficients
 beta[1]~ dt(0,pow(10,-2),7)
 for (i in 2:K) {beta[i] ~ dt(0,pow(2.5,-2),7)}

 # Likelihood
 for (i in 1:N) {
   # logistic component, p is the probability of detection
   # Y is count of detections, n is number of samples
   # X is model matrix for included predictors
   Y[i] ~ dbern(p[i])
   logit(p[i]) <- inprod(beta[],X[i,])
 }
}
```

JAGS code for zero-altered lognormal model

```
model{
 # Priors
 # for binomial component, scaled t, 7df, scale 10 for intercept, 2.5 for coefficients
 betaB[1]~ dt(0,pow(10,-2),7)
 for (i in 2:Kb) {betaB[i] ~ dt(0,pow(2.5,-2),7)}
 # for continuous (lognormal) component
 for(i in 1:Kc) {betaC[i] ~ dnorm(0, 0.0001)}
 # Prior for sigmaLN (lognormal variance)
 sigmaLN ~ dunif(0,10)
 # Prior for scale factor multiplier
 betaS ~ dnorm(1,4)

 # For the zeros trick
 C <- 10000
 # Likelihood
 for (i in 1:N) {
   # logistic component, w is the probability of detection
   # Xb is model matrix for included predictors in binary component
   logit(w[i]) <- etaB[i]
   etaB[i] <- inprod(betaB[],Xb[i,])
   # continuous relDNA component, lognormal likelihood
   #inclFM set to 0 for sample DNA model, 1 for field DNA model
   # Xc is model matrix for included predictors in continuous component
   mu[i] <- inprod(betaC[],Xc[i,]) - betaS*logSF[i] + inclFM*logFM[i]
   # Use the zeros trick
   Zeros[i] ~ dpois(-ll[i] + C)
   ln1[i] <- -(log(Y[i]) + log(sigmaLN) + log(sqrt(2*sigmaLN)))
   ln2[i] <- -0.5*pow((log(Y[i]) - mu[i]),2)/(sigmaLN*sigmaLN)
   LN[i] <- ln1[i] +ln2[i]
   z[i] <- step(Y[i] - 0.001) #Test if Y[i] is <0.0001, i.e. effectively zero
   l1[i] <- (1 - z[i]) * log(1 - w[i]) # log-likelihood where no detection
   l2[i] <- z[i] * (log(w[i]) +LN[i]) # log-likelihood where detected
   ll[i] <- l1[i] + l2[i]
 }
}
```