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Applying molecular techniques to surveys for introduced marine pests at ports in Spencer Gulf, South Australia

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

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SARDI Aquatics Sciences
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May 2017

Report for PIRSA Biosecurity SA

PREMIUM FOOD AND WINE FROM OUR CLEAN ENVIRONMENT

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EXECUTIVE SUMMARY

Shipping is a major vector for marine pests, and port areas are therefore at high risk of new introductions. Domestic ballast water regulations will be implemented in Australia from 2017, and require knowledge of the status of several pests of concern at Australian ports. The port of Adelaide was surveyed for introduced marine species (IMS) in 2001, 2007-8 and 2010-11, but other ports in South Australia have rarely been surveyed. Spencer Gulf contains three major regional ports: Whyalla, Wallaroo and Port Lincoln. IMS surveys were carried out in Port Lincoln in 1996 and at Port Lincoln, Whyalla, and other parts of Eyre Peninsula in 2010. Formal IMS surveys are lacking at Wallaroo, but there are some verified records of IMS there. Molecular techniques for marine pest surveillance offer cost and time savings over traditional IMS surveys. Molecular survey methods using plankton tows and quantitative polymerase chain reaction (qPCR) assays were successfully applied in the Port of Adelaide, and tested and refined during surveys of several ports around Australia. We applied these refined methods to molecular surveys of Whyalla, Wallaroo and Port Lincoln in parallel with diver visual surveys. The qPCR surveys detected the majority of target IMS which occur at each site: *Sabella spallanzanii* (European fanworm) at Whyalla and Port Lincoln, and *Ciona intestinalis* (vase tunicate) at all three ports. *Sabella spallanzanii* was not detected by qPCR in Wallaroo despite being confirmed present by the diver visual survey. *Crassostrea gigas* (Pacific oyster) is recorded at Port Lincoln and Whyalla, but was not detected by qPCR or visual surveys. *Corbula gibba* (European clam) was detected by qPCR in plankton samples from Port Lincoln and Whyalla, but has not been recorded previously at these sites and was not observed in the visual surveys, perhaps because it is a small species that lives buried in soft substrates. The assay for *C. gibba* showed cross-reaction with non-target species in other surveys, however, and has low specificity. Further research is needed to assess what species the assay detected and refine the *C. gibba* detection tool. This survey provides data from one season at these ports. Seasonality is important for molecular surveys, and sampling should be conducted throughout the year to maximize detections and obtain information about the seasonality of IMS detections using qPCR. These findings support Spencer Gulf and Gulf St Vincent being regarded as a ‘same-risk’ for domestic ballast water controls, since the pests of concern occurring in each are mostly the same species. Port Adelaide, however, is more heavily invaded than the Spencer Gulf ports and should be prioritised for domestic surveillance to understand the risk posed to ports which receive shipping and therefore ballast water from this region.

1. INTRODUCTION

1.1. Background

Introduced marine species (IMS) transported outside their native range by human activity can cause ecological and economic harm through loss of biodiversity, reduction in revenue from aquaculture and fisheries, damage to infrastructure, loss of amenity and, in some instances, impacts on human health (Hayes *et al.* 2005; Molnar *et al.* 2008). Not all species introduced to new locations establish or become pests, but increases in global trade and connectivity have increased the rate of new introductions, with a concomitant increase in the risk of pest establishment and impacts (Bax *et al.* 2003; Williams *et al.* 2013). Shipping is an important vector for marine species, through ballast water, fouling on hulls and equipment (e.g. fishing nets), and in recesses such as sea chests (Bax *et al.* 2003; Molnar *et al.* 2008).

Port areas are at high risk of new introductions. With the *International Convention for the Control and Management of Ships' Ballast Water and Sediments* (the Convention) becoming active in September 2017 (IMO 2017), Australia is developing a domestic ballast water management system, with oceanic ballast exchange used as a risk mitigation tool until all vessels are fitted with ballast water treatment systems. The ballast water management system will require knowledge of the status of several pests of concern (based on Hayes *et al.* 2005) at ports around Australia. Ports in close proximity and with a similar suite of pests can be regarded as 'same risk areas' under the Convention and not require oceanic ballast exchange, while journeys from ports where pests occur to those known to be free of pests will require oceanic ballast exchange (Saunders *et al.* 2016).

IMS surveys have been carried out in Port Adelaide in 2001 (Cohen *et al.* 2002), 2007-8 (Rowling 2009) and 2010-11 (Wiltshire and Deveney 2011), but few surveys have been done in other port areas of South Australia (SA) (Wiltshire *et al.* 2010). Other major SA ports are located in Spencer Gulf, with Wallaroo, Whyalla and Port Lincoln receiving the greatest volume of shipping (Gillanders *et al*. 2016). Port Lincoln was surveyed for IMS in 1996 (Hewitt *et al.* 1997). In 2010, Port Lincoln and Whyalla were surveyed (Dittmann *et al.* 2010) as part of broader Eyre Peninsula surveys. Wallaroo has not been formally surveyed for IMS but there are records of IMS occurrences there (Wiltshire *et al.* 2010).

Traditional surveys, such as those of Port Adelaide, Port Lincoln and Whyalla, typically involve physical sampling using a range of techniques, including visual surveys, dredging, trawl sampling

and trapping, followed by sorting and identification. These processes are time consuming and require considerable expertise, which has motivated progress towards DNA-based methods for IMS detection (Bott *et al.* 2010b). Several molecular methods have been applied to IMS detection, with polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays being favoured due to their high sensitivity and specificity and relatively low cost (Bott *et al.* 2010b). SARDI developed qPCR assays for ten IMS of concern, (Table 1), including the seven macroscopic species of concern for domestic ballast water management (Hayes *et al.* 2005). These assays were applied to plankton samples collected during the 2010-11 IMS survey of Port Adelaide, where traditional survey techniques were also used (Wiltshire and Deveney 2011). The plankton sampling field and qPCR laboratory methods (Ophel-Keller *et al.* 2008; Wiltshire and Deveney 2011; Giblot-Ducray and Bott 2013) developed and applied for Port Adelaide were verified and refined during surveys of Darwin, Cairns, Sydney, Melbourne, Hobart, Perth and Adelaide over 2015-16 (Deveney *et al.* 2016).

The refined molecular methods were applied to surveys of three major ports in Spencer Gulf, SA: Whyallla, Port Lincoln and Wallaroo, along with simultaneous visual surveys to verify the occurrence of conspicuous IMS. Results were also compared to IMS records for these ports. These surveys provide baseline data for the IMS status of these ports and inform biosecurity management for ports in SA, including risk status for domestic ballast water management.

1.2. Objectives

This project aimed to provide improved understanding of marine pest distributions and status at key sites in South Australia and to further validate SARDI's molecular tools for detection of marine pests.

2. METHODS

The ports of Whyalla, Port Lincoln and Wallaroo were surveyed between 19 May and 7 June 2016. At each port, five visual surveys were carried out by SCUBA divers, and 30 plankton samples plus five controls were taken for molecular analysis. These were collected within ~2 km of the main wharf area in each case. Sampling locations are shown in Figure 1.

Plankton samples were collected using a 50 µm mesh conical plankton net (Sea-Gear® Melbourne, Florida, USA) fitted with a flow meter and towed for 100 m behind a vessel at a speed of ~1 kt. The length of tow was calculated based on GPS coordinates. Effective tow length was calculated based on flow meter readings and compared to GPS distance as a measure of sampling efficiency. Plankton was coarse filtered using 2 mm mesh and concentrated to ~40 mL by tilting water through the cod end mesh. At regular times throughout the field sampling, five samples of filtered seawater were also collected. These samples consisted of 40 mL seawater that was filtered by being passed through a 0.22 µm Millipore filter using a syringe. Plankton and filtered water samples were transferred immediately upon collection to 120 mL sample tubes containing 80 mL sulphate based preservative (based on recipe of De Wit *et al.* 2012), sealed, and placed on ice. All of the filtered water sample tubes and ten of the thirty plankton sample tubes also contained a dose of *Artemia salina* (hereafter *Artemia*) nauplii (Ocean Nutrition™ Newark California, USA *Instant Baby Brine Shrimp*) as a sampling quality assurance (SQA) control. The site name/code, GPS waypoint identifiers (start and finish), flow meter readings (start and finish) and any other relevant notes were recorded. Plankton nets and all sampling equipment exposed to seawater were cleaned in 60°C freshwater containing 200 mg/L active hypochlorite between field sites.

After collection, samples were kept cold (on ice or in a refrigerator at <4°C) until processing. In the laboratory, samples were filtered on paper discs using a manifold following Giblot-Ducray and Bott (2013). Filter papers were transferred to 50 mL centrifuge tubes and frozen at -20°C. Samples were freeze dried until completely dehydrated prior to DNA extraction. DNA was extracted from samples using the method developed by SARDI Molecular Diagnostics (Ophel-Keller *et al.* 2008). An aliquot of 20 mL of DNA extraction buffer containing an internal control (exogenous organism added to each sample in a standardised amount) was added to each sample before physical disruption (see Ophel-Keller et al. 2008). Final DNA elution was done in 160 µL elution buffer. Each DNA extract was then tested in singleplex quantitative polymerase chain reaction (qPCR) using the SARDI-developed IMS assays shown in Table 1. Assays are referred to hereafter by the genus name of the target. *Artemia* DNA yield was

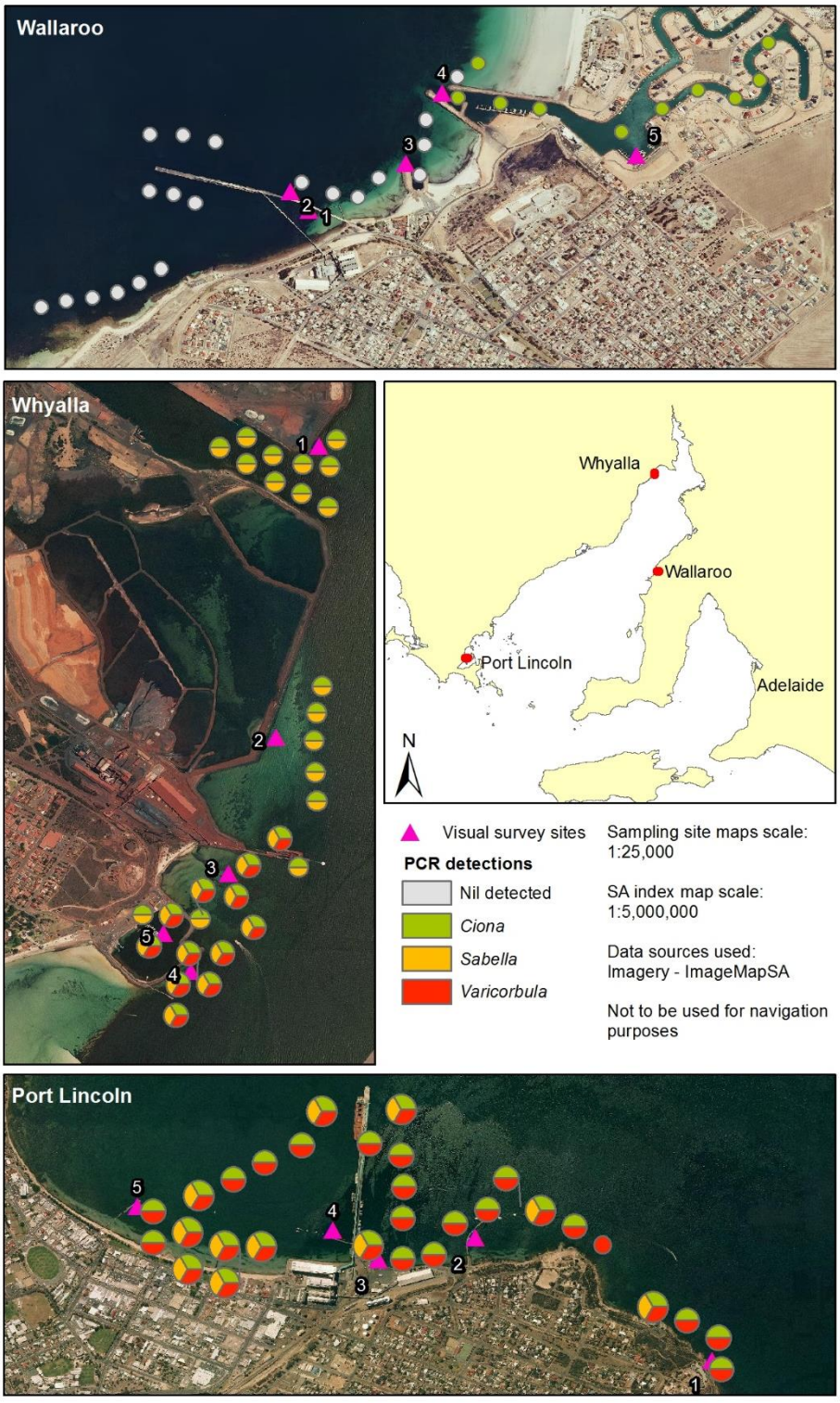
determined using an *Artemia* qPCR assay adapted from Mackie and Geller (2010) and compared between those plankton samples containing the SQA and filtered water samples. Each DNA extract was also tested using a qPCR assay specific to the internal control (Ophel-Keller *et al.* 2008). A sample of 2 µg mL^{-1} of a pure culture of the control organism in MilliQ™ (Merck Millipore, Billerica, MA, USA) water with a filter paper added was also extracted and tested by qPCR to assess DNA yield from a pure sample, and used as a reference to determine if PCR inhibition occurred during analysis. A scaling factor was calculated for each sample by comparing the amount of internal control DNA detected in each sample to that detected in the reference sample. Scaling >1.6 indicates inhibition of the PCR reaction, which may be problematic for detection.

Table 1. SARDI-developed qPCR assays for IMS. Species marked (*) are regarded as pests of concern for domestic ballast water management

Species	**Common Name**	**Assay reference(s)**
*Asterias amurensis** (Echinodermata:Asteroidea)	Northern Pacific seastar	Bott *et al.* (2010a); Bott and Giblot-Ducray (2011a)
*Carcinus maenas** (Arthropoda: Malacostraca)	European shore crab	
*Undaria pinnatifida** (Ochrophyta: Phaeophyceae)	Japanese seaweed, wakame	
Ciona intestinalis (Chordata: Ascidiacea)	Vase tunicate	
Arcuatula (= *Musculista*) *senhousia** (Mollusca:Bivalvia)	Asian date mussel, bag mussel	Ophel-Keller *et al.* 2007; Bott and Giblot-Ducray 2011b
*Corbula gibba** (Mollusca:Bivalvia)	European clam	
Perna canaliculus (Mollusca:Bivalvia)	New Zealand green-lipped mussel	
*Sabella spallanzanii** (Annelida: Polychaeta)	European fanworm	Ophel-Keller *et al.* 2007
*Crassostrea gigas** (Mollusca:Bivalvia)	Pacific oyster	Bott and Giblot-Ducray 2012
Mytilopsis sallei (Mollusca:Bivalvia)	Black-striped mussel	Bott *et al.* 2012

Each visual survey consisted of searches of at least 10 minutes duration (typically 15-20 minutes) and minimum survey length of 200 m. Searches targeted suitable substrates for the target IMS. Most target IMS are associated with hard substrate, often being found on jetty pilings, pontoons and other artificial substrates (Dafforn *et al.* 2009; Ruiz *et al.* 2009). *Corbula*, however, occurs in soft substrates; *Carcinus* occurs in intertidal and shallow areas on a variety of substrates, and *Arcuatula* may occur on hard or soft substrate. Searches focused on pilings, pontoons and rock walls, but also encompassed surrounding soft substrates, shallow rocky areas and seagrass.

Records of target IMS for each location were obtained from the review of Wiltshire *et al.* (2010). Identifications of several IMS from the Eyre Peninsula survey (Dittmann *et al.* 2010), which were uncertain when that review was published, were confirmed by Thierry Laperousaz of the SA Museum. Voucher specimens of these records are held by the SA Museum (SAM).



Visual survey site names:

Wallaroo

1. Main wharf
2. Spur jetty
3. Ferry dock
4. Marina entrance
5. Marina inside

Whyalla

1. Steelworks entrance
2. Pellet plant wall
3. Old jetty
4. Marina outside to town jetty
5. Marina inside

Port Lincoln

1. Kirton Point
2. Fuel wharf
3. Main wharf
4. Recreational jetty
5. Town jetty

Figure 1. Map of sampling locations and target pest qPCR detections. Symbols are shown scaled to the number of detections. To avoid symbol overlap, some samples are shown offset with a leader line to their actual position. Position shown is the midpoint of each tow.

3. RESULTS AND DISCUSSION

The target IMS, their expected occurrence from previous records and detections by qPCR and visual surveys at each port are summarised in Table 2. Visual survey site numbers correspond to survey locations shown in Figure 1. The location of plankton samples and qPCR results are also shown in Figure 1.

Ciona and *Sabella* have been previously recorded at each of the three surveyed ports, while *Crassostrea* is recorded from Whyalla and Port Lincoln (Dittmann *et al.* 2010; Wiltshire *et al.* 2010; SAM specimens). The continued presence of *Ciona* and *Sabella* at each of these ports was confirmed by the visual surveys, but *Crassostrea* and other target species were not observed (Table 2). *Sabella* was abundant on pontoons within the marinas at Wallaroo (visual site 5) and Whyalla (visual site 5) and on the recreational jetty pilings in Port Lincoln (visual site 4). *Ciona* was most abundant inside the Whyalla marina (Figure 1, Table 2). *Ciona*, *Sabella* and *Corbula* were detected by qPCR in plankton samples from Whyalla, but only *Ciona* was detected in samples from Wallaroo (Figure 1, Table 2). Sampling efficiency, calculated from the ratio of flowmeter to GPS distance, was very low (<5%) at all sites (Figure 2), indicating severe net clogging.

There was no indication of sample degradation from *Artemia* results in the current study samples. Scaling factors for qPCR analysis were >1 for 26 samples from Whyalla, 16 samples from Port Lincoln and 14 from Wallaroo, indicating some inhibition. One sample from Wallaroo had a scale factor >1.6 (2.54), which is regarded as high scaling, i.e. above which likelihood of detection may be compromised. Scale factors up to 5, however, did not significantly impact likelihood of IMS detection (binomial GLM $p=0.486$) in recent testing of plankton samples from ports around Australia, when samples were processed using the method applied in the current survey (Deveney *et al.* 2016).

Table 2. Summary of survey results. Target species are shown as + previously recorded and detected by visual surveys, previously recorded but not observed in visual surveys, - not expected to be present, and qPCR detection results are the number of samples with the target present (n=30 samples). Consistent detections are highlighted green. Lack of PCR detection for a species verified to be present by the simultaneous visual survey is highlighted orange, and qPCR detection of species not expected to be present in purple. Lack of detection is not shown for those species not expected to occur, and not highlighted for those not observed in visual surveys.

Target / Port	*Arcuatula*	*Asterias*	*Carcinus*	*Ciona*	*Crassostrea*	*Mytilopsis*	*Perna*	*Sabella*	*Undaria*	*Corbula*
Wallaroo										
Expected	-	-	-	+	-	-	-	+	-	-
Detected - qPCR				30				0		
Visual 1				N				N		
Visual 2				N				Y		
Visual 3				N				N		
Visual 4				N				N		
Visual 5				Y				Y		
Whyalla										
Expected	-	-	-	+	?	-	-	+	-	-
Detected - qPCR				30	0			30		12
Visual 1				N	N			N		N
Visual 2				N	N			N		N
Visual 3				N	N			N		N
Visual 4				N	N			Y		N
Visual 5				Y	N			Y		N
Pt Lincoln										
Expected	-	-	-	+	?	-	-	+	-	-
Detected - qPCR				29	0			11		30
Visual 1				N	N			N		N
Visual 2				Y	N			Y		N
Visual 3				N	N			Y		N
Visual 4				N	N			Y		N
Visual 5				Y	N			Y		N

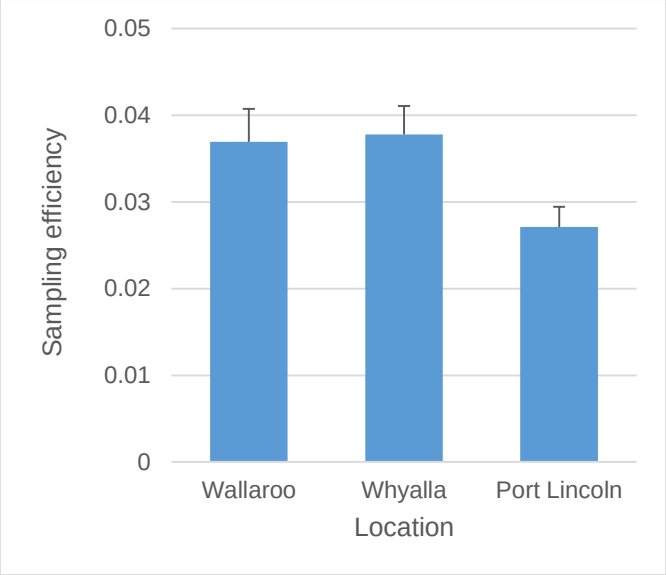


Figure 2. Sampling efficiency (flowmeter distance/GPS distance) for each port. Error bars show standard error (n=30).

Detections by qPCR were largely consistent with previous records and visual survey results of IMS at each site, aside from the failure to detect *Sabella* at Wallaroo, especially within the marina where a large population was observed in visual surveys. In previous sampling in Port Adelaide, *Sabella* was sporadically detected with qPCR in four of seven sets of samples collected between January 2015 and March 2016, with no clear seasonal pattern (Deveney *et al.* 2016). Where this species was detected, it was found in <50% of samples each time, and in as few as 2 of 36 samples (Deveney *et al.* 2016) despite the presence of a large established population of *Sabella* at Port Adelaide (Wiltshire *et al.* 2010; Wiltshire and Deveney 2011). This suggests that sampling should be repeated in multiple seasons to maximise probability of *Sabella* detection. It is more surprising that this species was detected in all molecular samples from Whyalla, since our visual surveys only observed it in the marina area. We were not able to access the Whyalla main wharf or ore-loading facilities, however, and additional populations may be present in those areas. Sampling may also have coincided with a spawning event in this area. There are conflicting data on seasonality of spawning in *Sabella*, with Currie *et al.* (2000) reporting spawning in autumn/winter in Port Phillip Bay, Victoria, while Lee (2013) reported spawning *Sabella* in late summer in Wirrina Cove, Gulf St Vincent, South Australia. The spawning period for *Sabella* in the Mediterranean varies temporally, occurring in late summer in 1993 and 2001-2005, but in late

spring in 2008 (Giangrande *et al.* 2010). Repeat sampling would provide information needed to optimise detection of *Sabella* in South Australia.

The lack of detection of *Crassostrea* in Whyalla and Port Lincoln is not surprising, as this species may not have established or persisted at these locations. The record at Port Lincoln was of empty shells from the Port Lincoln marina, with no live animals found (Dittmann *et al.* 2010). The marina is 3 km+ away from the nearest location sampled for plankton in this study. The single record of *Crassostrea* at Whyalla was as “rope fouling” at the Whyalla marina (SAM specimen) and it is unclear if that occurrence was on a marina structure or vessel. *Crassostrea* most likely does not occur at the sampled sites, however its presence cannot be completely excluded because of the limited coverage of the visual surveys, and collection of plankton samples outside the main spawning season for this species. Elsewhere in Australia, qPCR detected *Crassostrea* in plankton samples from Sydney and Hobart, where large populations exist, with detections in all 22 samples from each site collected during summer (January/February). In Sydney there were no detections in 30 samples collected in spring (October), while *Crassostrea* was detected in Hobart in only 1 of 30 samples collected in autumn (May) (Deveney *et al.* 2016). Plankton sampling in summer is necessary to maximise the probability of detecting this species by qPCR.

Corbula is not recorded from Whyalla or Port Lincoln, and was not observed in our visual surveys. It is a small species that lives buried in soft substrates and we are unlikely to have observed it using this method even if it was present. Grab or dredge sampling, rather than visual surveys, are traditional methods recommended to target this species (National System for the Prevention and Management of Marine Pest Incursions 2010). The assay for *Corbula* was designed from limited material (Bott and Giblot-Ducray 2011b). Sampling in other locations showed qPCR detections at sites outside the thermal range of this species (Deveney *et al.* 2016). It is likely that the *Corbula* assay cross-reacts with DNA from another species, probably a native corbulid. Further investigation is required to determine if the detections at Whyalla and Port Lincoln are *C. gibba* or the result of a detection of a related native species. Refinement of the *Corbula* detection tool is planned for 2017-18.

There is substantial domestic vessel traffic to Spencer Gulf ports including from Port Adelaide (Gillanders *et al*. 2016). Australia is developing a domestic ballast water management system for implementation by 8 September 2017 to comply with the Convention. Oceanic ballast exchange or ballast water treatment will be used as risk mitigation tools, but most vessels are not yet fitted with treatment systems and oceanic ballast exchange between the Port Adelaide and Spencer

Gulf ports is unlikely to be safe or feasible. Domestic ballast water risk ratings will be based on presence of seven listed pests of concern that are established in Australia (Hayes *et al.* 2005; highlighted in Table 1).

Port Adelaide contains a greater range of IMS than the Spencer Gulf ports, with high abundance for some. Of the seven pests of concern for ballast water, four are recorded from Port Adelaide, but not all are established (Deveney *et al.* 2016; Wiltshire *et al.* 2010). *Arcuatula* was recorded in Port Adelaide, but has not been observed since 2001 despite targeted surveys (Wiltshire *et al.* 2010). *Corbula* shells, but no live animals, were found during the 2007-8 survey of Port Adelaide, and were identified at the time as a native, *C. stolata* (Rowling 2009). Subsequently, these shells were re-examined and found to be *C. gibba* (SAM specimens), but there have been no more recent records. *Crassostrea* has recently established in Port Adelaide and was detected by qPCR (Deveney *et al.* 2016) and incidental observations (SARDI unpublished data). *Carcinus* is present in Port Adelaide but its population density varies widely; it was common from the 1980s to early 2000s then was undetected from 2004-2009 (Wiltshire *et al.* 2010). Its abundance has since increased again, with recent records from several sites around Adelaide, including Port Adelaide (Dittmann *et al.* 2016; SARDI unpublished data) and qPCR detections in plankton from Port Adelaide collected in 2015-2016 (Deveney *et al.* 2016). There is a large established population of *Sabella* in Port Adelaide (Wiltshire *et al.* 2010; Wiltshire and Deveney 2011). *Ciona* is not one of the current ballast water pests of concern, but it is also established and abundant in Port Adelaide (Wiltshire *et al.* 2010).

Our survey supports a ‘same-risk’ area classification (Saunders *et al.* 2016) being applied to Spencer Gulf and Gulf St Vincent. The pests of concern occurring in Port Adelaide and Spencer Gulf are largely the same species, although Port Adelaide may pose a threat as a donor port to the Spencer Gulf ports for *Carcinus*. *Crassostrea* was not detected at the surveyed Spencer Gulf Ports in this study, but is farmed at several sites within Spencer Gulf (Wiltshire *et al.,* 2010), so transport of this species to Spencer Gulf by ballast water from Port Adelaide poses a negligible risk. Within the ‘same-risk’ area, Port Adelaide should be prioritised for domestic surveillance to understand the risk that vessels leaving this area pose to other ports in Australia, because it is the most heavily invaded port in the area.

4. CONCLUSIONS

The continued presence of *Sabella* and *Ciona* at Wallaroo, Whyalla and Port Lincoln was confirmed by visual surveys, with qPCR also detecting both species at Whyalla and Port Lincoln, and *Ciona* at Wallaroo. Repeated plankton sampling may be required to reliably detect *Sabella* using qPCR, but the best season to target this species is unclear and may vary between locations and years. The current status of *Crassostrea* at Whyalla and Port Lincoln is uncertain; none were observed in visual surveys or detected by qPCR. Conducting plankton tows in summer for qPCR testing would maximise the probability of detecting *Crassostrea* by this method, although it is possible that records of this species at these sites were isolated occurrences and that it is not established at either location. *Corbula* was detected in plankton from Whyalla and Port Lincoln by qPCR, but the assay for this species has low specificity. It is likely that these detections are the result of cross-reaction with DNA of a related native species. The *C. gibba* assay requires further testing against samples from other corbulids, and further investigation is required to determine if *C. gibba* occurs at either site. Results of these surveys support Spencer Gulf and Port Adelaide being regarded as ‘same-risk’ for ballast water management, given that the pests of concern occurring in each are largely the same.

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