Assessing natural resources in the Noosa Biosphere to restore a functional estuary: final report

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April 2021

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Report citation

McDougall C, Walker S, 2021. Assessing natural resources in the Noosa Biosphere to restore a functional estuary; final report. Australian Rivers Institute, Griffith University, Brisbane

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1. Executive Summary

Shellfish reefs are culturally, economically, and ecologically important but have almost entirely disappeared from Australia's coastlines. In the Noosa estuary, oysters were once abundant but have not recovered since overharvesting in the late 19th century. Oyster reef restoration projects have commenced but their efficacy to restore this lost habitat type is unknown. This project aims to 1) evaluate the distribution and diversity of oyster species within the estuary, 2) evaluate oyster settlement preferences, and 3) investigate environmental DNA techniques for detection and monitoring of restored reefs. This will directly inform the design of reef modules to maximise settlement of oyster species for ongoing restoration projects. The study finds that:

1. Minimally, there are four species of rock oyster present in the estuary. These include *Saccostrea glomerata* (the Sydney rock oyster), *Saccostrea lineage B*, *Saccostrea lineage G*, and *Ostrea equestris*.
2. The oysters (and their spat) cannot be reliably identified by morphology alone.
3. Only eDNA techniques were able to identify all four oyster species. *Saccostrea lineage B* was not found on settlement plates, and *Ostrea equestris* was not found via survey.
4. Each method provided a different picture of oyster distributions within the estuary. eDNA techniques are likely less reliable for this purpose, therefore oyster diversity appears to be higher near the river mouth than in the upper reaches, with *Saccostrea glomerata* the sole species present upstream (Lake Cooroibah mouth).
5. Several other native shellfish were identified in the estuary, including pearl oysters (*Pinctada albina/nigra* species complex), hairy mussel (*Trichomya hirsuta*) and leaf oyster (*Isognomon ephippium*, identified by morphology only) that are capable of clumping, consolidating rubble/shell substrate and contributing to restored shellfish reef habitats.
6. eDNA has been established as a useful technique for the initial detection of species within an estuary, however other methods are preferred for fine scale distribution assessments.

Each rock oyster species recorded, or a combination, could be targeted for shellfish reef restoration, however, considering the modern day dominance of *S. glomerata* it is an obvious choice. The current distribution of *S. glomerata* in the Noosa estuary appears to be largely limited to the intertidal zone, with only sparse individuals observed subtidally (likely a result of complex ecological interactions between environmental conditions, predation, settlement and recruitment and other disturbance). This study identified key shellfish species in a tropical/sub-tropical setting, which will inform the future design phase of restoration efforts, including habitat suitability models and other aspects of the early design of habitats. This is expected to result in significant cost savings by helping improve the efficiency and efficacy of current and future restoration efforts aimed at reviving these lost or threatened ecosystems.

This study also provided an excellent opportunity to trial innovative 'environmental DNA' (eDNA) techniques for the detection of oyster species throughout the estuary. eDNA (DNA shed by organisms into the environment) can be obtained from water (or soil) samples, and can allow detection of organisms without needing to survey large numbers of (potentially poorly accessible) sites. This project provided the first comparison of oyster biodiversity obtained from eDNA sampling compared with traditional physical surveys throughout the estuary. In doing so, we have developed an efficient means of evaluating oyster populations which can be applied in the Noosa River estuary and to other estuaries around the world.

2. Project Background

Shellfish reefs (reefs composed predominately of bivalve molluscs) are not as famous, or perhaps as beautiful, as coral reefs, but were once common along Australia's coastline (Gillies et al., 2018) (Figure 1). These reefs provide important ecosystem functions including coastline protection, habitat provision, carbon sequestration, food provision, and improvement of water quality (Gillies et al., 2018; Grabowski et al., 2012). The importance of reefs to estuaries has underpinned the recognition of shellfish reefs as a wetland habitat type as part of the Convention on Wetlands of International Importance (The Ramsar Convention – habitat type 'Ga') (Kasoar et al., 2015). Today oyster reefs are considered globally threatened, with an estimated 85 per cent of historical reef lost (including from Australia) (Beck et al., 2011; Gillies et al., 2018). The functional loss of oyster reef habitats has been linked to overall declines in ecosystem productivity, particularly of fish stocks, likely due to the loss of foraging and nursery habitats (Gilby et al., 2018). The loss of shellfish reefs has been attributed to several factors including overexploitation, dredging, increased sedimentation, disease, and flood events (Beck et al., 2011). The persistence of many of these stressors may mean that these reefs cannot be restored (Gilby et al., 2018). However, examples of successful shellfish reef restoration projects in the United States, and the confirmation of oyster survival and recruitment on various projects within Australia, including within the Noosa River estuary indicate that, with suitable reef restoration design, ongoing monitoring and maintenance, many of these ecosystems may be able to be recovered.

Figure 1. Intertidal remnant oyster reef, Turkey Beach, Queensland

In the USA, oyster restoration is leading the way in estuarine restoration through the creation of “green” jobs following the US economic downturn (Edwards et al., 2013). These restoration projects provide substantial local employment and economic opportunities through the creation of natural infrastructure that can enhance ecosystem services (including coastal protection) worth millions of dollars and support other industries including fishing (boosting commercial and recreational productivity) and tourism (Grabowski et al., 2012; Peterson et al., 2003). Despite evidence of substantial declines in the condition of estuaries in Australia and calls to restore them to maintain fisheries productivity (Gillies et al., 2018), marine restoration in Australia has typically lagged behind the rest of the world. However, shellfish reef restoration projects are recently gaining momentum within Australia, with several large-scale projects investing upwards of $20 million towards restoration efforts. There are currently several initiatives within Australia that aim to provide science-based, well-considered restoration of habitats that have been lost, including the restoration of oyster reefs (www.shellfishrestoration.org.au). Two projects are currently underway in Queensland; one in Pumicestone Passage (Moreton Bay), and another in the Noosa River estuary.

Research into the history of the Noosa River estuary has revealed extensive Indigenous use of oysters (evidenced by large shell middens and references from early settlers), and the existence of a large oyster fishery in the late 19th and early 20th centuries (Thurston, 2015). Leases were granted from 1881 onward, and oysters were harvested, including by dredge, for markets in Brisbane and beyond (Thurston, 2015). Even after large oysters had all but disappeared, harvest of young oysters also occurred by dredge, these were transported live and on-grown in Moreton Bay (Thurston, 2015). By 1930 commercial oyster harvesting in the Noosa estuary had largely ceased, yet populations have still not recovered (Thurston, 2015). It is thought that the dredging of oyster banks removed a large proportion of the substrate required for oyster settlement, and that the human modifications of the estuary (particularly the Noosa River mouth) further reduced the availability and suitability of habitat (Thurston, 2015).

It is generally assumed that the oyster species harvested from the Noosa estuary was the Sydney Rock oyster, however historical records point towards morphological differences between ‘dredge’ and ‘bank’ shellfish (Saville-Kent, 1891). A recent survey of oyster species around the Queensland coast has revealed higher than expected diversity, with a number of currently unrecognised species commonly found in similar habitats to Sydney Rock oysters (McDougall et al., 2020). It is therefore possible that dredge and bank oysters represent distinct species; this would have important implications for shellfish reef restoration projects and may explain why settlement has not been observed on submerged substrates in areas with healthy Sydney Rock oyster populations in some trials within the Noosa estuary (Gilby, 2018; The Nature Conservancy and Ecological Service Professionals, 2015).

In Noosa, an oyster restoration program is underway to restore rock oyster reefs that were thought to have consisted of Sydney rock oysters. Unexpectedly, early results have indicated that several types of oyster are settling – the identity of these ‘other’ oysters is unknown, and likely reflects the paucity of information regarding oyster biodiversity, rather than the recruitment of exotic species. The significant lack of knowledge of shellfish reef ecosystems

has been highlighted in the literature as a priority for research, and prior studies have often oversimplified shellfish reef ecosystems (Gillies et al., 2018); many more oyster species are capable of forming reefs, and reefs are often composed of multiple shellfish species (McDougall, unpublished data). Interspecific differences in biology and habitat preferences mean that some oyster species are likely to be more suitable for reef restoration projects than others, but this knowledge is critically lacking. Understanding which species are being targeted in reef restoration projects is essential for optimal design, which should take into account the habitat preferences of the species naturally (and/or historically) found in the area. Furthermore, increasing diversity of ecosystems can improve their capacity to respond to, and recover from, future disturbances.

Understanding of the ecology of target species is an essential component for successful restoration projects, however due to taxonomic complexities in this important shellfish group, it has become evident that a genetic screen is necessary to determine the wealth of shellfish diversity. This information would inform design of large-scale restoration initiatives, and addresses an important knowledge gap identified during the trial reef restoration phase in Noosa. Therefore, the aims of this project were to:

1. Evaluate the distribution and diversity of oyster species within the Noosa estuary.
2. Evaluate the settlement preferences of detected oyster species.
3. Investigate the utility of eDNA for detection and monitoring of oyster biodiversity in the estuary.

3. Methods

Ambient water quality

Water quality parameters were measured in surface waters (30 cm below the surface) at each site on several days during deployment of settlement plates and while collecting eDNA samples. Water quality was logged using a YSI ProDSS multi-parameter water quality sonde. The unit was calibrated prior to deployment manufacturer calibration methods and sampling completed in accordance with the Queensland *Monitoring and Sampling Manual 2018* (Department of Environment and Science, 2018) for the following parameters: water temperature; dissolved Oxygen; specific conductance; salinity; pH; and, turbidity.

Aim 1 - Evaluate the distribution and diversity of oyster species within the Noosa estuary.

Sampling of oysters in the Noosa Estuary was conducted by foot on the 18th of December 2019 and by boat on the 24th of August 2020. Eight collection sites were selected based on the availability of hard substrate and the presence of oysters (Figure 2). Water quality parameters were recorded at each site. Between three and twelve specimens were collected at each site depending on the observed morphological diversity apparent in the population. Oysters were stored in cooler boxes on ice and returned to the laboratory, where samples were stored in 70% ethanol until processed. No adult oysters were observed on mangrove roots at site SD1 although oysters did settle on experimental settlement plates.

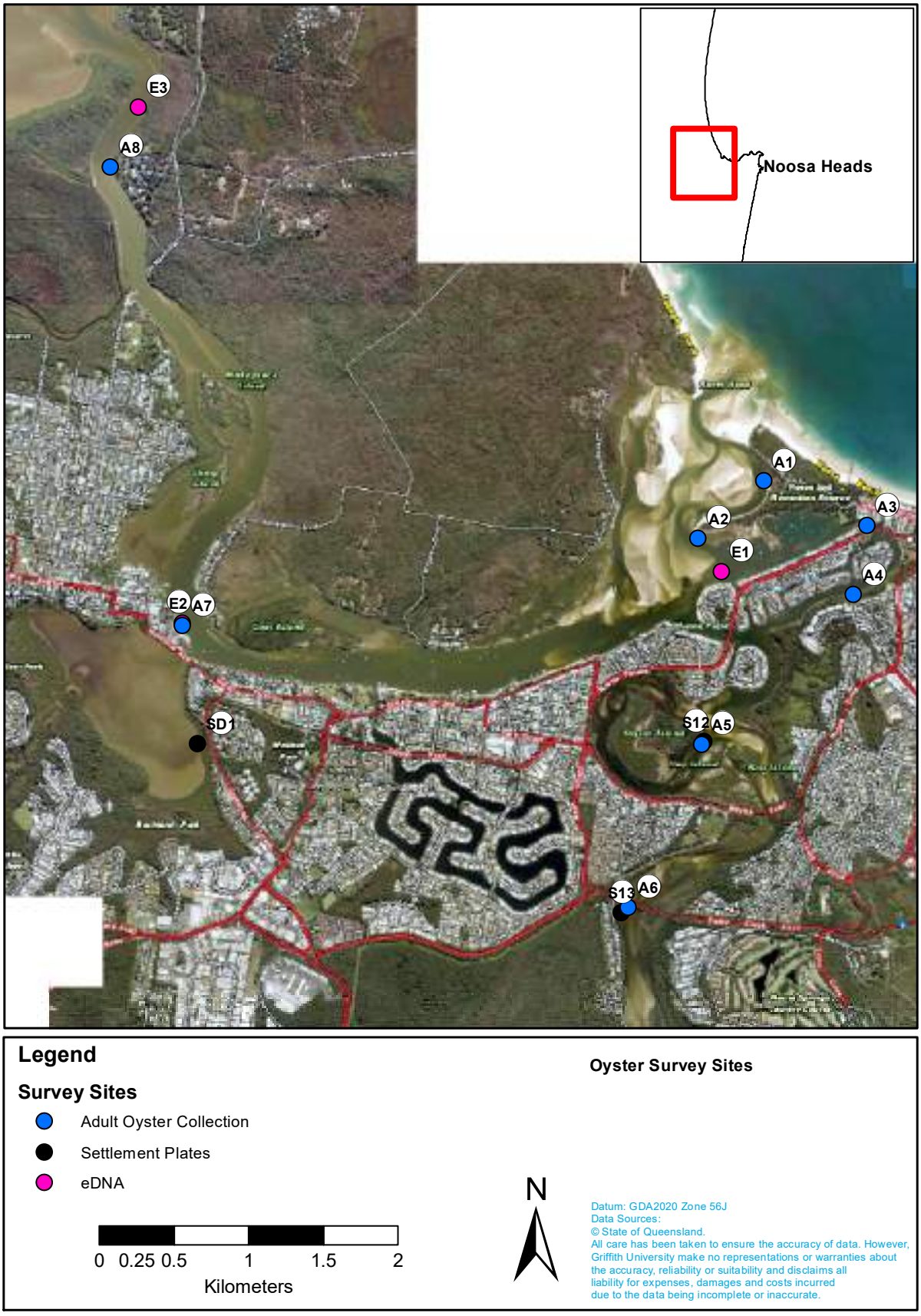

Figure 2. Sites in the lower Noosa Estuary. Blue dots: adult oyster collection site. Black dots: settlement rack deployment site. Pink dots: eDNA sample collection site.

Oysters were photographed, shucked, and a small sample of adductor muscle was dissected. Extractions were performed using the DNeasy Blood and Tissue DNA Kit (Qiagen) as per the manufacturer's instructions. A partial fragment of the 16S gene was amplified by PCR using the the primers 16S_Fwd (Banks et al., 1993) and 16Sbr-H (Palumbi et al., 1991) and the following thermoprofile: 95°C for 30 seconds, followed by 30 cycles of 95°C for 1 minute, 51°C for 1 minute and 68°C for 1 minute, and a final extension at 68°C for 5 minutes. PCR products were gel purified and sent to Macrogen (South Korea) for sequencing. Resulting traces were quality trimmed. Species identification was performed by aligning the resulting sequences against an in-house database of oyster 16S sequences.

Aim 2 - Evaluate the settlement preferences of detected oyster species.

Settlement racks were deployed at three sites on the 18th of December 2019 (Figure 2). Racks contained paired stacked plates (one nylon, one concrete), with four pairs placed horizontally on the top of the unit, and four positioned vertically at the end of the posts (Figure 3). Treated shells (both SRO and Pacific) were cable-tied on to each of the posts (4 of each species on each rack). Units were deployed at two existing restoration sites and one new site in Lake Doonella, and were positioned in order to be exposed during low tide. Water quality parameters were recorded at each site.

Figure 3. Settlement plate rack design. Top: construction of settlement racks. Bottom: settlement rack in situ at site SD1.

Settlement plates were collected on 8th of May 2020. The abundance of attached spat was recorded (based on morphology), individuals were photographed, and then each individual was carefully removed (avoiding cross contamination) and preserved separately in 70% ethanol for sequencing. DNA extraction and 16S PCR was performed as described above. Differences in the distribution and abundance of rock oysters were compared among the types of settlement plates (cement sheet and nylon), surface orientations (vertical and horizontal), and sites using a three factor PERMANOVA. Untransformed oyster abundance was converted to Euclidean distance matrix prior to analysis. Due to substantial losses of the oyster shell treatment at some sites, these were excluded from formal statistical analyses; however, where settlement onto oyster shell occurred, the results for comparison with the other treatments.

Aim 3 - Investigate the utility of eDNA for detection and monitoring of oyster biodiversity.

Water samples (2 L water sample, four replicates per site) were taken into sterile plastic containers at three sites visited on the 24th of August, 2020 (Figure 2), from site E1 at slack low tide, site E3 on an incoming tide, and site E2 at slack high tide. Samples were taken from the middle of the channel and approximately 10 cm below the surface. Water samples were kept on ice in the field and at 4°C upon return to the laboratory, and were filtered through sterile 250 mL 0.45 µm cellulose nitrate filter funnels (Nalgene 145-2045) using a vacuum manifold. Filtration was completed within 24 hours of sample collection. Three additional sterile plastic containers were filled with water from the laboratory sink (water the bottles had been rinsed in) and filtered to provide a 'blank' control. DNA extraction was performed using the DNeasy Blood and Tissue kit (Qiagen) protocol, with a modified lysis step as performed by Alexander et al., 2019.

As conventional universal DNA barcoding primers generally perform very poorly on bivalves, three new primer sets (incorporating Illumina adaptor sequences) were designed using the software PrimerDesign-M (Yoon and Leitner, 2015) and an alignment of 16S and COI sequences (standard DNA barcoding sequences) from all known Queensland oyster species (McDougall, unpublished data). 16S primers were designed *de novo*; COI forward primers were modified from those reported in Leray *et al*., 2013, and used with a reverse primer reported in Folmer *et al*., 1994. Each primer set was initially optimised on *Saccostrea glomerata* DNA, before being tested on DNA from a range of oyster and non-oyster species to determine specificity. Initial PCRs on extracted eDNA suggested the presence of PCR inhibitors in the samples, therefore DNA was further purified using a DNeasy Powerclean Pro Cleanup Kit (Qiagen). PCR reactions were performed in triplicate for all samples, for the blank control, and a mock community containing equal amounts of DNA (10ng) from a range of species to allow assessment of primer specificity (Table 1). One primer set (COI_sacc) was trialled at two different annealing temperatures to assess the effect on PCR specificity. The resulting PCR product was checked for concentration and quality, and triplicates were pooled. Products were then sent to AGRF for indexing and sequencing on a MiSeq (500 cycle kit, 250bp paired end). Bioinformatics (QC, chimera detection, clustering, OTU generation, mapping, and annotation via megaBLAST) was conducted by AGRF, and separate mapping of raw reads to sequences of known QLD oyster species was performed via HTSeq v 0.9.1 on the Galaxy Australia server.

Table 1. Species composition of 'Mock' DNA community for assessment of specificity

Species	Description
Saccostrea glomerata	Rock oyster
Saccostrea scyphophilla A/B	Rock oyster
Saccostrea scyphophilla C	Rock oyster
Saccostrea lineage B	Rock oyster
Saccostrea lineage F	Rock oyster
Saccostrea lineage G	Rock oyster
Saccostrea lineage I	Rock oyster
Saccostrea lineage J	Rock oyster
Magallana gigas	Rock oyster
Magallana bilineata	Rock oyster
Talonostrea sp. nov.	Rock oyster
Ostrea equestris	Rock oyster
Dendostrea crenulifera	Rock oyster
Ostrea/Dendostrea sp 1	Rock oyster
Ostrea/Dendostrea sp 2	Rock oyster
Hyotissa hyotis	Honeycomb oyster
Pinctada albina	Pearl oyster
Plicatula sp.	Kitten's paw oyster
Pipi_AL1.1	Pipi
Trichomya hirsuta	Mussel
Haliotis asinina	Abalone
Anentome sp. A	Freshwater snail
Cassiopea xamachana	Jellyfish
Nemertean_AL1.4	Worm

4. Results and Discussion

Characterisation of collection sites

Collection sites spanned from the Noosa River mouth upstream to the entrance to Lake Cooroibah, and included sites in Lake Doonella and Weyba Creek (Figure 2). The water quality parameters measured in situ at the various sites were typical of that in lower to mid-estuarine waters, with temerpature reflecting the ambient air temperature, good dissolved oxygen concentration and pH typical of seawater (Table 2). Turbidity ranged from 1 to 39 NTU, with the highest records occurring in Lake Doonella (site SD1). The exception was salinity (and specific conductance), which typically ranged from 23 to 42 PSU. The highest records occurred at sites in Weyba Creek (sites S12 & S13) in December 2019 (Table 2), following an extremely dry period with very low rainfall. These hypersaline conditions exceeded the normal growing conditions; however, salinity measured in March 2020 following rainfall had declined to expected salinity levels, and well within the optimal growth range for rock oysters (Table 3).

Table 2. Water quality parameters measured in situ at each site

Site	Date	Time	Water Temperature (°C)	Dissolved Oxygen (mg/L)	Specific Conductance (µS/cm)	Salinity (psu)	pH	Turbidty (NTU)
SD1	18/12/19	12:36	26.89	6.54	53572	35.33	7.98	9.49
	30/4/20	15:15	29.22	8.37	36746	23.14	8.11	39.03
	8/5/20	11:00	22.07	6.57	52437	34.59	7.92	12.09
A5/S12	18/12/19	11:29	25.53	7.44	61798	41.56	7.87	3.09
	30/4/20	No data	No data	No data	No data	No data	No data	No data
	8/5/20	15:21	23.17	7.49	45396	29.42	8.03	5.64
A6/S13	18/12/19	9:45	23.85	7.26	62662	42.26	7.81	8.24
	30/4/20	13:00	25.20	6.91	41210	26.38	8.05	4.89
	8/5/20	12:35	22.51	7.15	48062	31.36	8.00	3.31
A4	24/8/20	9:20	17.83	7.57	49615	32.52	8.22	2.81
E1	24/8/20	8:16	18.00	7.22	50393	33.09	8.16	0.99
E2	18/12/19	13:00	25.90	6.25	54201	35.82	7.97	6.67
	24/8/20	14:06	18.90	7.58	51590	33.98	8.20	1.48
A8/E3	24/8/20	12:28	16.61	7.39	46727	30.41	8.05	5.22
A1	24/8/20	10:41	19.40	7.50	53330	35.27	8.32	9.64
A2	24/8/20	10:04	18.17	7.37	51475	33.89	8.24	1.21
A3	24/8/20	8:30	17.50	7.22	49667	32.56	8.18	1.08

The distribution and diversity of oyster species within the Noosa estuary.

16S fragments were successfully amplified and sequenced for 49 oysters collected throughout the Noosa Estuary. Sequence analysis revealed that the oysters belonged to three different species; *Saccostrea glomerata* (Sydney rock oyster), *Saccostrea lineage B* and *Saccostrea lineage G*. The use of *Saccostrea* 'lineages' as opposed to species is established in the literature because the difficulty in morphologically identifying oyster species has prevented the confident assignment of species names (Lam and Morton, 2006; Sekino and Yamashita, 2016). Indeed, in this study it was impossible to distinguish *Saccostrea glomerata* and *Saccostrea lineage G* morphologically (Figure 4). A 16S fragment was also collected for a 'hairy mussel' collected from site A2. The sequence confirms that this species is *Trichomya hirsuta.*

The finding of *Saccostrea glomerata* in the Noosa Estuary was expected, and is congruent with the known natural distribution of the species (from the Town of Seventeen Seventy in Queensland to the Bass Strait, and New Zealand). *Saccostrea lineage B* was previously reported from Yepoon and Cairns (Lam and Morton, 2006), and both *Saccostrea lineages B* and *G* were found to be broadly distributed in Queenland in a recent survey (McDougall et al., 2020). These two species have broad Indo-Pacific distributions, occurring as far north as Japan (Sekino and Yamashita, 2016).

Saccostrea glomerata was found at all sites surveyed, other than site SD1 at which no adult oysters were found. *Saccostrea lineage G* was found at sites close to the river mouth and at Tewantin boat ramp (sites A1, A2, A3 and A7), but not in Weyba Creek, Lake Doonella, or at the mouth of lake Cooroibah. *Saccostrea lineage B* was found near the river mouth only (site A1, however juveniles of this species were observed at site A2 also). These results indicate that the three species have different habitat requirements; this may be related to different salinity tolerances and/or ability to withstand sedimentation.

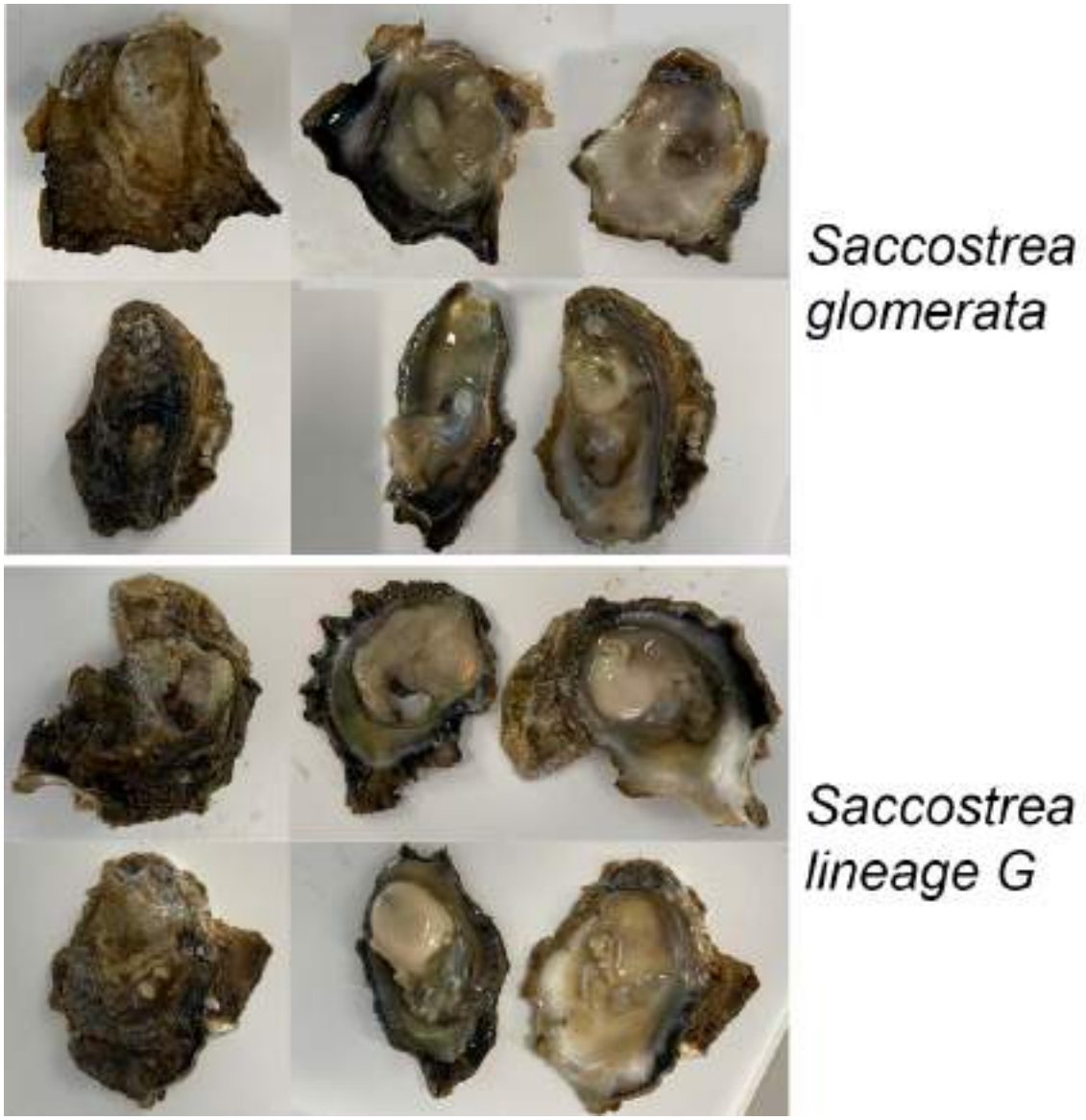

Figure 4. Morphology of adult Saccostrea glomerata and Saccostrea lineage G.

The accommodation space represents the area in an estuary with environmental conditions suitable for growth of oysters (Volety, 2013). Oysters grow from the intertidal to depths of at least 20 metres and this is where much of the hand gathering or dredging of oysters has historically occurred (Nell, 2001). However, it is thought that due to changes in ambient conditions and a variety of external factors, rock oysters in Southern Queensland are typically found only in the intertidal zone and have been functionally lost from subtidal habitats (Diggles, 2013). Conditions suitable for optimal *Saccostrea glomerata* growth are summarised below from (Holliday, 1995; Nell and Holliday, 1988; Rubio, 2007). Adult rock oysters are able to close their valve for several days if salinity is less than 15 ppt, although prolonged periods of low salinity can cause death (Holliday, 1995; Nell and Holliday, 1988). Based on ambient conditions, it would be possible for oysters to grow at any of the sites surveyed, where suitable subtsrata is available for settlement (Figure 5 & 6). Optimal conditions for the other bivalve species identified within this study are not presently known.

Table 3. Temperature and salinity for optimum growth and tolerance limit for Saccostrea glomerata

Parameter	Units	Life History Stage		
		Larvae	Spat	Adult
Temperature optimum growth	°C	24-26	14-28	18-26
Temperature tolerance limit	°C	unknown	11-30	11-30
Salinity optimum growth	ppt	23-39	20-40	25-35
Salinity tolerance limit	ppt	15-39	0-40	0-50

Figure 5. Subtidal rock rubble and oyster bank at Tewantin

Figure 6. Low relief aggregation of subtidal leaf oyster bed over sand and mud in Weyba Creek

Settlement preferences of oyster species in the Noosa estuary

DNA fragments were sequence from a total of 47 oyster spat collected on settlement racks at three sites throughout the estuary. Three species were detected; *Saccostrea glomerata*, *Saccostrea lineage G,* and *Ostrea equestris*. *Ostrea equestris* has a wide distribution in both Atlantic and Pacific oceans, including Argentina, U.S.A., Japan, China and New Zealand (Hu et al., 2019). The presence of the species in Australia has, until now, not been confirmed with genetic evidence (although specimens of the same species were previously discovered on settlement plates in the Pumicestone Passage, McDougall unpublished data). It is a subtidal species (which explains why it was not found in the adult oyster surveys, which predominately explored the intertidal zone), and is reported to survive in high salinity environments (Galtsoff and Merrill, 1962). Two small pearl oyster specimens were also sequenced; they both belong to the *Pinctada albina/nigra* species complex.

The external morphology of oyster spat was recorded in photographs taken prior to dissection. Once sequencing results had been returned photographs were examined to determine whether species could be reliably identified by morphology. Extensive overlapping variation was observed across the three species (Figure) indicating that morphological identification is not feasible.

All three species settled onto plates at sites S12 and S13 (Weyba Creek), however only *Saccostrea glomerata* spat were detected at site SD1 (Lake Doonella). This is in contrast with adult oyster distributions, with only *Saccostrea glomerata* adults detected at sites S12 and S13. The lack or reduced abundance of other species (specifically, *Saccostrea lineage G*) at these sites is not due to lack of larval recruitment, but most likely due to unfavourable environmental conditions that cause mortality of settled individuals enabling the numerical dominance of *S. glomerata*. Spat of *Saccostrea lineage B* were not detected on any of the plates; this could have several explanations, for example, lack of larval supply (adults of the species were only detected at the river mouth), lack of suitability of plates for settlement, early mortality, or the lack of a spawning event. It is notable that the 2019/2020 summer was particularly dry, possibly affecting spawning behaviour. Adults were generally in low densities where recorded at the mouth of the river.

There were differences in the density (individuals per 0.01 m^2) of oysters on plates around the estuary as well as on different surface orientations and types of settlement surface (Table : PERMANOVA site x orientation interaction pseudo-$F_{2,36}$ = 5.55, p = 0.002, Figure 8 & 9). There was also a general increase in the density of oysters settling onto nylon than concrete sheet (Table : PERMANOVA substrate type pseudo-$F_{1,36}$ = 4.56, p = 0.029, Figure 8). The average density was greatest on horizontal surfaces, particularly on the undersides of plates. A layer of sediment had accumulated on the upper surface of each plate (Figure , up to 5 mm thick on some plates), which prevented successful attachment. The greatest density of oysters was recorded at site S12, which is closest to the mouth of Weyba Creek. On vertical surfaces, the density of recently settled oyster spat onto oyster shells was typically similar to that found on vertical nylon sheets at site S12, but much greater on oyster shell than on the plates at site S13 (Figure).

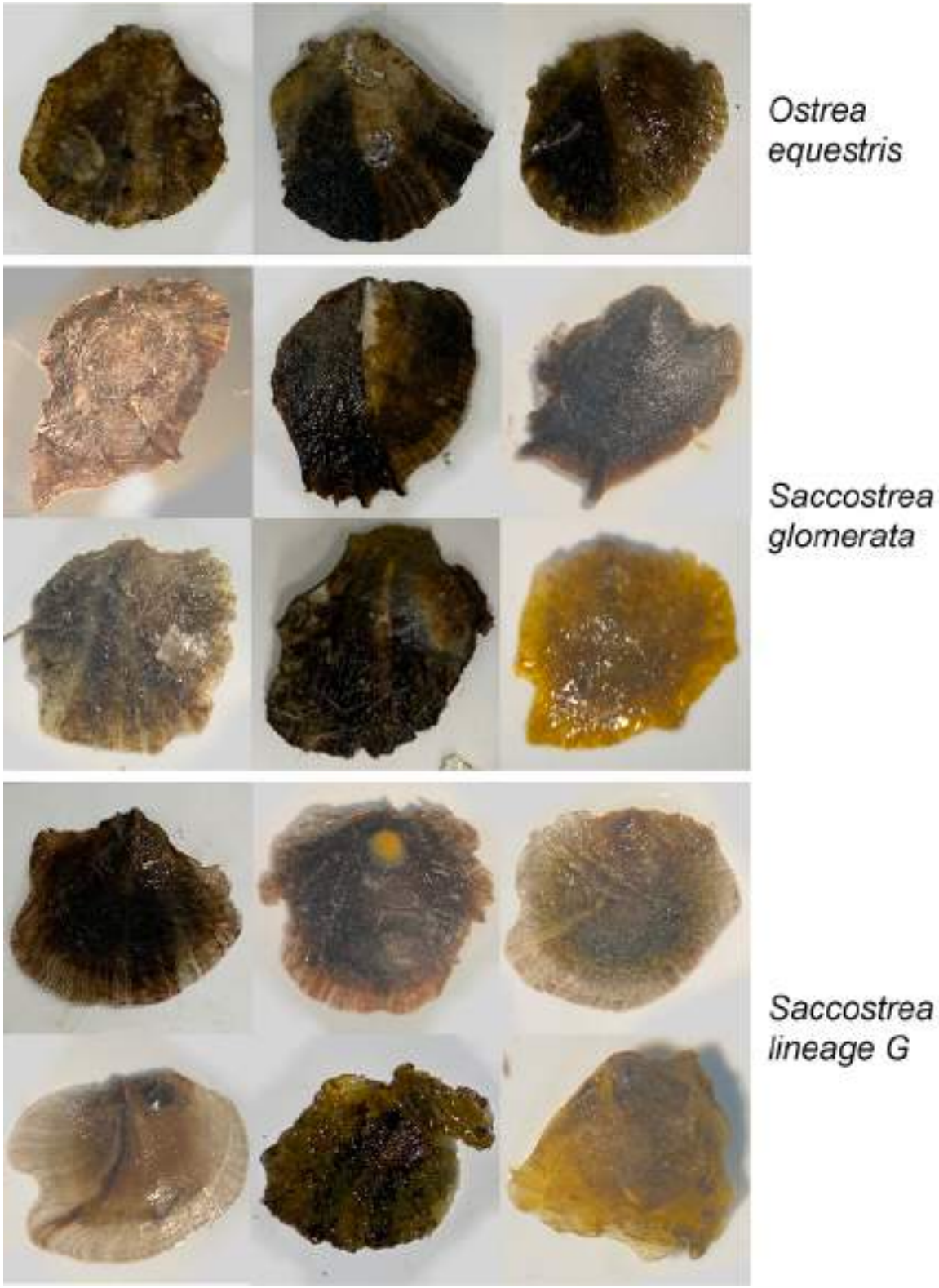

Figure 7. Morphology of spat of each detected species.

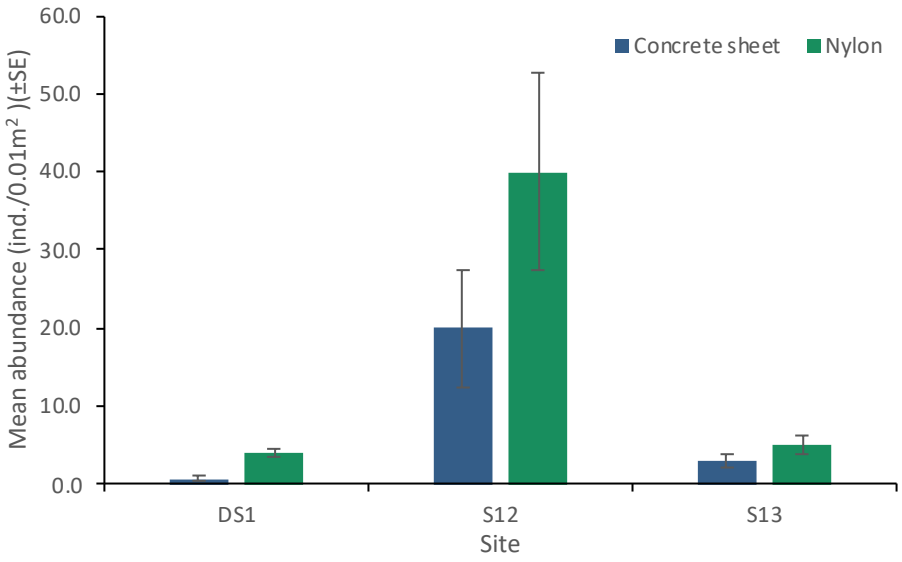

Figure 8. Average abundance (±SE) of rock oysters on horizontal settlement plates among sites

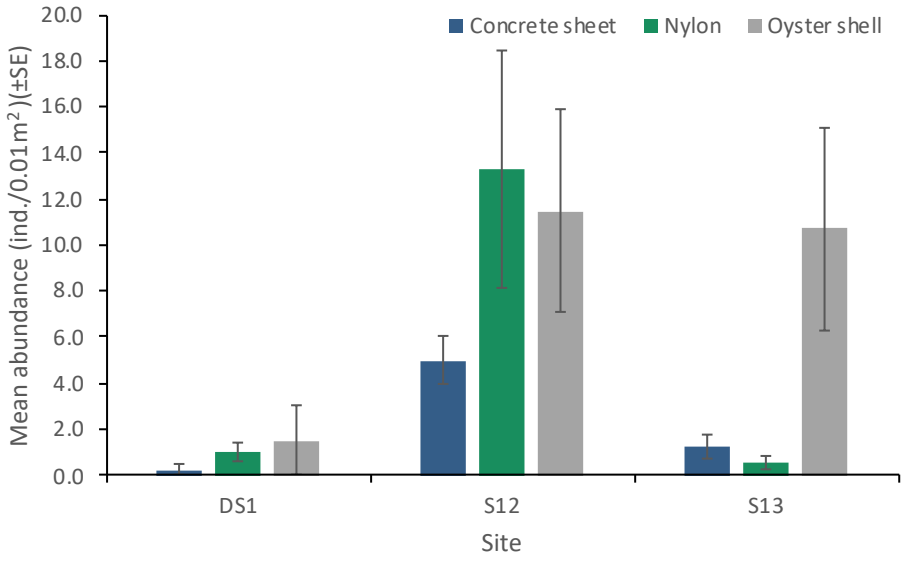

Figure 9. Average abundance (±SE) of rock oysters on vertical settlement plates among sites

Table 4. PERMANOVA results for differences in oyster density among site, substrate types and surface orientations.

Factor	**df**	**MS**	**Pseudo-F**	**P(perm)**
Site	2	1654	20.16	**0.001**
Substrate Type	1	374	4.56	**0.029**
Orientation	1	884	10.77	**0.002**
Site x Substrate Type	2	221	2.69	0.081
Site x Orientation	2	455	5.55	**0.002**
Substrate Type x Orientation	1	96	1.17	0.302
Site x Substrate Type x Orientation	2	28	0.34	0.743
Error	36	82		
Pariwise Comparisons				
(b) Horizontal vs Vertical				
	t-value	P(perm)		
S12	2.68	0.010		
S13	4.11	0.002		
DS1	4.43	0.001		
	(c) Horizontal		(d) Vertical	
Differences between sites	t-value	P(perm)	t-value	P(perm)
S12 vs S13	3.53	0.005	3.12	0.004
S12 vs DS1	3.76	0.003	3.22	0.003
S13 vs DS1	2.10	0.061	0.68	0.586

Figure 10. Settlement plates with high coverage of sediment on upper surface in May 2020

eDNA analysis of oyster biodiversity

Details of the three new primer sets (one designed to 16S, the other two designed to the same COI region, but with slightly different sequences) can be found in Table 5. Each set was tested for species specificity in a series of single-target PCRs, and were found to preferentially amplify ostreid (oyster) DNA (data not shown). PCR product was successfully amplified for each eDNA sample and the mock community, however products were also observed in the 'blank' (tap water) control for some reactions, indicating contamination. No products were observed in the PCR no-template control. Sequencing was successful for all samples, and generated between 118,206 and 371,256 paired-end reads (50,288-192,794 reads for 'blank' samples).

Table 5. Details of primers designed and used in the study.

Primer name	Primer sequence	Product	Tm	Reference
16S Ostreid_16S_Fwd Ostreid_16S_Rev	AACGGCCGCCCTAGCGTGAGG AGCCTGTTATCCCCGGCGTAAC	317bp	72˚C	This study
COI_oys mlCOIintF_oys HC02198	GGWGCWGGWTGAACWATWTAYCCYCC TAAACTTCAGGGTGACCAAAAAATCA	356bp	56.6˚C	Modifed from Leray *et al*., 2013 Folmer *et al.,*1994
COI_sacc mlCOIintF_sacc HC02198	GGNGCYGGYTGRACTATYTAYCCBCC TAAACTTCAGGGTGACCAAAAAATCA	356bp	52˚C (low) 63˚C (high)	Modifed from Leray *et al*., 2013 Folmer *et al.,*1994

Analysis of the mock community sequencing results (Table 6) revealed that the 16S primer set only amplified ostreid COI sequences. Single-target PCRs had indicated that these primers could amplify products in other taxa (non-oyster bivalves and the cnidarian *Cassiopea*); results from the mock community indicate that the primers bind preferentially to oyster targets in mixed community samples. It is also clear that some oyster species amplify more efficiently than others; *Saccostrea scyphophilla A/B* recorded the highest number of reads (45,085), and *Ostrea equestris* the least (750 reads). Data should therefore not be used as a proxy for species abundance. COI primers performed less well, with several oyster species poorly represented in the sequencing results (e.g., *Magallana bilineata* and *Talonostrea sp. nov*.), and amplification of non-oyster sequences (particularly the freshwater snail *Anentome sp. A*).

Table 6. Sequencing results (number of raw reads) for each primer set on the mock community sample.

Species	16S	COI_oy	COI_Sacc_Hi	COI_Sacc_Lo
Saccostrea glomerata	4688	37689	1957	23682
Saccostrea scyphophilla A/B	45085	398	13	2542
Saccostrea scyphophilla C	12744	400	32	1837
Saccostrea lineage B	7960	126160	299109	61402
Saccostrea lineage F	13462	38265	6711	22117
Saccostrea lineage G	22446	20	4	18
Saccostrea lineage I	11515	221	1	736
Saccostrea lineage J	1045	42	83	28
Magallana gigas	1732	19265	1	1283
Magallana bilineata	1466	0	0	2
Talonostrea sp. nov.	10686	0	0	161
Ostrea equestris	750	4	2	3
Dendostrea crenulifera	1817	n/a	n/a	n/a
Ostrea/Dendostrea sp 1	4168	n/a	n/a	n/a

Ostrea/Dendostrea sp 2	n/a	941	9	2153
Hyotissa hyotis	0	0	0	0
Pinctada albina	0	0	0	0
Plicatula sp.	n/a	n/a	n/a	n/a
Pipi_AL1.4	n/a	1	0	1
Trichomya hirsuta	0	0	0	115
Haliotis asinina	0	0	0	0
Anentome sp. A	0	182577	293247	182577
Cassiopea xamachana	0	0	0	0
Nemertean_AL1.1	n/a	2	0	109

* Sequences are not available for all species; indicated by n/a.

Sequencing results for each primer set are summarised in Figure 11. The 16S primers were highly specific for bivalves, whereas the COI primers amplified a range of taxa. COI_oy and COI_sacc (high annealing temperature) primers preferentially amplified red algae sequences (up to 86% of reads), whereas the COI_sacc primers at the lower annealing temperature preferentially amplified fish sequences (up to 86% of reads). Given that none of the COI primers produced high coverage of bivalve sequences they are not recommended for eDNA profiling of oyster species.

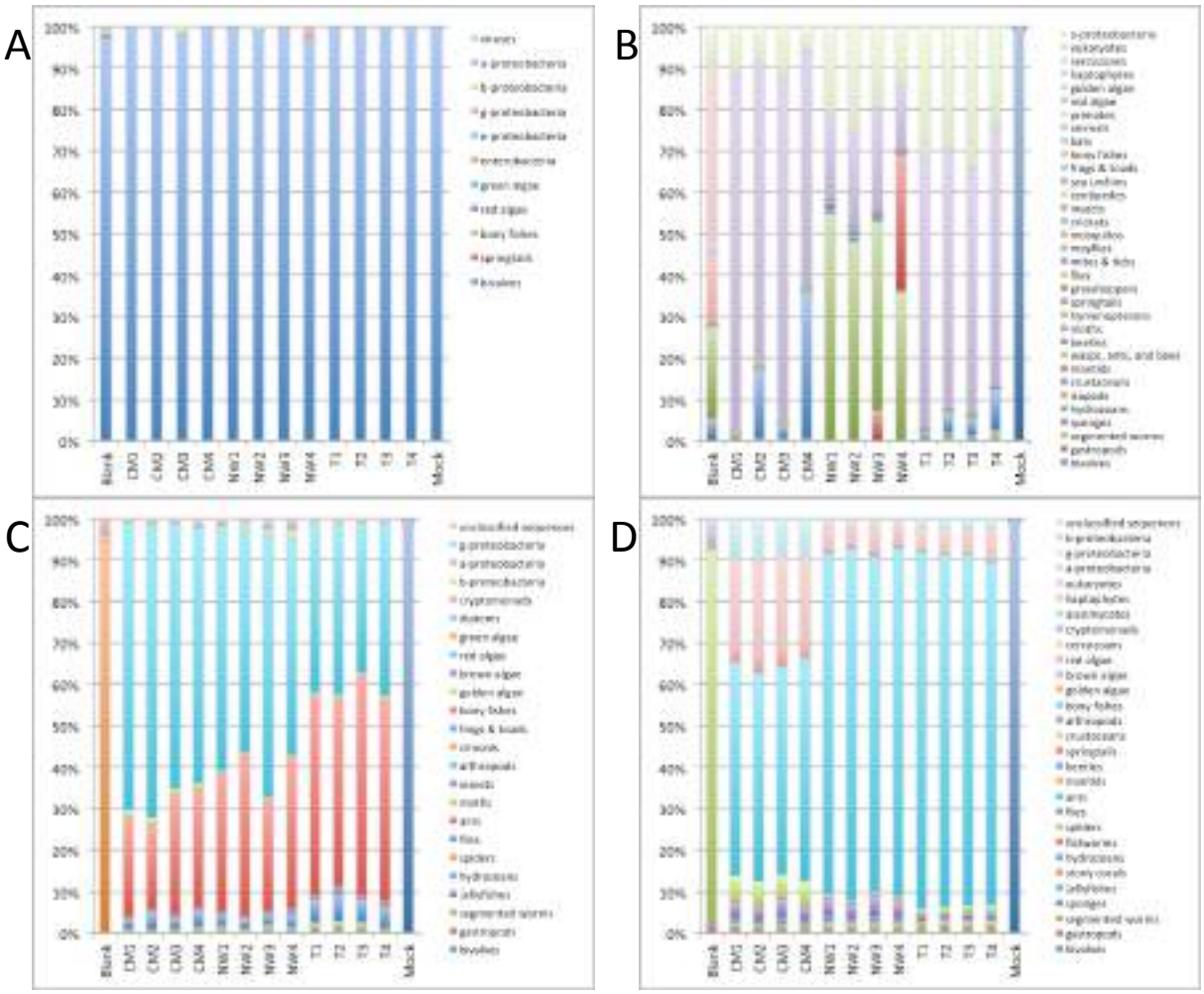

Figure 11. Comparison of eDNA sequencing from different primer sets. A. 16S primers. B. COI_oys primers. C. COI_sacc primers at high annealing temperature. D. COI_sacc primers at low annealing temperature. CM, Cooroibah mouth; NW, Noosa Woods; T, Tewantin.

Within the 16S sequencing results, only four species were represented within the 'bivalve' reads. These include *Saccostrea glomerata*, *Saccostrea lineage G, Saccostrea lineage B,* and *Ostrea equestris* (Table 7), all of the species that had previously been identified in the Noosa estuary by the other methods. No additional species were detected. Given that this primer set was able to amplify other oyster sequences within the mock community, and that *Ostrea equestris* was among the oyster species for which these primers appeared to work less optimally, it is likely that these are the only oyster species that inhabit the Noosa estuary in significant numbers. Unfortunately a large number of *Saccostrea glomerata* reads were obtained from the 'blank' (tap water) control. It is unclear where this contamination originated, however it is possible that our equipment sterilisation technique (bleach, rinsing in tap water, then autoclaving) is not sufficient to remove trace DNA. Given this, there is some uncertaintly about the level of detection of *Saccostrea glomerata* DNA in the eDNA samples.

Table 7. Raw read counts for oyster species within the 16S sequencing results.

		Noosa Woods				Tewantin				Lake Cooroibah Mouth			
	Blank	1	2	3	4	1	2	3	4	1	2	3	4
Saccostrea glomerata	100530	190322	270472	173321	30125	244699	69152	358857	0	146164	185787	17564	129826
Saccostrea lineage G	0	0	0	0	19241	66969	133292	0	316647	121713	42063	61563	116046
Saccostrea lineage B	0	0	0	0	0	0	0	0	0	0	0	130415	0
Ostrea equestris	0	0	9554	0	121398	0	15105	0	0	0	0	0	0

Comparison of methods for species detection

Neither adult oyster surveys or settlement plate analysis gave a comprehensive picture of the species present within the Noosa estuary. Oyster surveys would possibly have been more complete had subtidal collection techniques been utilised, and additional species may have been collected on settlement plates if they had been deployed at additional sites. Each of these modification presents additional logistical challenges; use of divers for collection is expensive (and dredges hit-and-miss), and settlement plates deployed at locations with higher human traffic (such as around the river mouth) are more likely to be interfered with. While the eDNA methodology developed in this study needs some refinement, it is extremely promising and was able to detect all four species. Given the expense of eDNA techniques (approximately $100/sample), consideration does need to be given to the required comprehensiveness for the study when evaluating which methodology is desirable (e.g., physical oyster collection and barcoding or eDNA analysis).

Historical significance and implications for shellfish design

The historical presence of oysters in the Noosa estuary is well recorded, both within middens and in the literature (Thurston, 2015). While no mention is made of the species of oyster present, 'bank' and 'dredge' oysters are distinguished. Saville-Kent (1891) believed that these represented two forms of *Saccostrea glomerata*, and noted that the difference in morphology was likely an environmental effect, with subtidal 'dredge' oysters having 'a smoother and more ponderous form, with often an abnormally elongated contour, and a more or less complete absence of the conspicuous colouration characteristic of the shells exposed to light

and air'. Despite this, he also noted that the two 'forms' of oysters occurred within the same clump, leaving open the possibility that they are distinct species. In addition to these reports, a newspaper article from 1915 authored by Harry C. Perry states that 'Mud oysters are plentiful in Lake Weyba' (Perry, 1915). While 'mud oysters' in southern states refer to *Ostrea angasi*, the species does not occur in Queensland. Other articles indicate that the term 'mud oyster' was used for any oyster occurring below the low water mark (1934). Given the difficulty in distinguishing oyster species by morphology, determining what species were historically present in the Noosa estuary will be challenging, particularly if living specimens have become locally extinct and archeological evidence from middens needs to be relied upon. *Saccostrea glomerata, Saccostrea lineage G* and *Saccostrea lineage B* are morphologically very similar as adults, and were likely considered the one species in historical accounts. Whether accurate determinations can be made from midden specimens remains to be established.

This study has shown that there are at least four species of rock oyster present in the Noosa estuary, presumably each of these, or a combination, could be targeted for shellfish reef restoration. Given that each species likely has unique environmental requirements, and that modelling for appropriate reef restoration sites is generally based on parameters for *Saccostrea glomerata*, consideration of alternative species may significantly increase the number of possible sites. The detection of a subtidal species, *Ostrea equestris*, suggests that submerged reefs may be possible; however, reports indicate that the species does not naturally form reefs (Galtsoff and Merrill, 1962). Given the results presented here, we recommend trials to explore the attributes of each species (resilience, performance, provision of ecosystem services) under different environmental conditions to provide essential baseline information to integrate with shellfish reef restoration designs.

5. Acknowledgements

The authors would like to acknowledge the project funders, Griffith University and the Noosa Biosphere Reserve Foundation, Nikolina Nenadic for laboratory assistance, and Bryan Walsh from the Noosa Parks Association for field assistance.

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